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COVER NOTE

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Subject:	Annex to COMMISSION REGULATION (EU) .../... of XXX amending, for the purpose of its adaptation to technical progress, the Annex to Regulation (EC) No 440/2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH)

Delegations will find attached document Annex to D060575/02.

Encl.: Annex to D060575/02

EN

ANNEX

The Annex to Regulation (EC) No 440/2008 is amended as follows:

(1) In part B, Chapter B.4 is replaced by the following:

"B.4 ACUTE DERMAL IRRITATION/CORROSION**INTRODUCTION**

1. This test method is equivalent to OECD test guideline (TG) 404 (2015). OECD guidelines for testing of Chemicals are periodically reviewed to ensure that they reflect the best available science. In the review of OECD TG 404, special attention was given to possible improvements in relation to animal welfare concerns and to the evaluation of all existing information on the test chemical in order to avoid unnecessary testing in laboratory animals. The updated version of OECD TG 404 (originally adopted in 1981, revised in 1992, 2002 and 2015) includes reference to the Guidance Document on Integrated Approaches to Testing and Assessment (IATA) for Skin Irritation/Corrosion (1), proposing a modular approach for skin irritation and skin corrosion testing. The IATA describes several modules which group information sources and analysis tools, and (i) provides guidance on how to integrate and use existing testing and non-testing data for the assessment of the skin irritation and skin corrosion potentials of chemicals and (ii) proposes an approach when further testing is needed (1). In addition, where needed, the successive, instead of simultaneous, application of the three test patches to the animal in the initial *in vivo* test is recommended in that Guideline.
2. Definitions of dermal irritation and corrosion are set out in the Appendix to this test method.

INITIAL CONSIDERATIONS

3. In the interest of both sound science and animal welfare, *in vivo* testing should not be undertaken until all available data relevant to the potential dermal corrosivity/irritation of the test chemical have been evaluated in a weight-of-the-evidence (WoE) analysis as presented in the Guidance Document on Integrated Approaches to Testing and Assessment for Skin Corrosion and Irritation, i.e. over the three Parts of this guidance and their corresponding modules (1). Briefly, under Part 1 existing data is addressed over seven modules covering human data, *in vivo* data, *in vitro* data, physico-chemical properties data

(e.g. pH, in particular strong acidity or alkalinity) and non-testing methods. Under Part 2, WoE analysis is performed. If this WoE is still inconclusive, Part 3 should be conducted with additional testing, starting with *in vitro* methods, and *in vivo* testing is used as last resort. This analysis should therefore decrease the need for *in vivo* testing for dermal corrosivity/irritation of test chemicals for which sufficient evidence already exists from other studies as to those two endpoints.

PRINCIPLE OF THE *IN VIVO* TEST

4. The test chemical to be tested is applied in a single dose to the skin of an experimental animal; untreated skin areas of the test animal serve as the control. The degree of irritation/corrosion is read and scored at specified intervals and is further described in order to provide a complete evaluation of the effects. The duration of the study should be sufficient to evaluate the reversibility or irreversibility of the effects observed.
5. Animals showing continuing signs of severe distress and/or pain at any stage of the test should be humanely killed, and the test chemical assessed accordingly. Criteria for making the decision to humanely kill moribund and severely suffering animals are the subject of a separate Guidance Document (2).

PREPARATIONS FOR THE *IN VIVO* TEST

Selection of animal species

6. The albino rabbit is the preferable laboratory animal, and healthy young adult rabbits are used. A rationale for using other species should be provided.

Preparation of the animals

7. Approximately 24 hours before the test, fur should be removed by closely clipping the dorsal area of the trunk of the animals. Care should be taken to avoid abrading the skin, and only animals with healthy, intact skin should be used.
8. Some strains of rabbit have dense patches of hair that are more prominent at certain times of the year. Such areas of dense hair growth should not be used as test sites.

Housing and feeding conditions

9. Animals should be individually housed. The temperature of the experimental animal room should be 20°C ($\pm$ 3°C) for rabbits. Although the relative humidity should be at least 30% and preferably not exceed 70%, other than during room cleaning, the aim should be 50-60%. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unrestricted supply of drinking water

TEST PROCEDURE

Application of the test chemical

10. The test chemical should be applied to a small area (approximately 6 cm²) of skin and covered with a gauze patch, which is held in place with non-irritating tape. In cases in which direct application is not possible (e.g. liquids or some pastes), the test chemical should first be applied to the gauze patch, which is then applied to the skin. The patch should be loosely held in contact with the skin by means of a suitable semi-occlusive dressing for the duration of the exposure period. If the test chemical is applied to the patch, it should be attached to the skin in such a manner that there is good contact and uniform distribution of the test chemical on the skin. Access by the animal to the patch and ingestion or inhalation of the test chemical should be prevented.
11. Liquid test chemicals are generally used undiluted. When testing solids (which may be pulverised, if considered necessary), the test chemical should be moistened with the smallest amount of water (or, where necessary, of another suitable vehicle) sufficient to ensure good skin contact. When vehicles other than water are used, the potential influence of the vehicle on irritation of the skin by the test chemical should be minimal, if any.
12. At the end of the exposure period, which is normally 4 hours, residual test chemical should be removed, where practicable, using water or an appropriate solvent without altering the existing response or the integrity of the epidermis.

Dose level

13. A dose of 0.5 ml of liquid or 0.5 g of solid or paste is applied to the test site.

Initial test (*In vivo* dermal irritation/corrosion test using one animal)

14. When a test chemical has been judged to be corrosive, irritant or non-classified on the basis of a weight of evidence analyses or of previous *in vitro* testing, further *in vivo* testing is normally not necessary. However, in the cases where additional data are felt warranted, the *in vivo* test is performed initially using one animal and applying the following approach. Up to three test patches are applied sequentially to the animal. The first patch is removed after three minutes. If no serious skin reaction is observed, a second patch is applied at a different site and removed after one hour. If the observations at this stage indicate that exposure can humanely be allowed to extend to four hours, a third patch is applied and removed after four hours, and the response is graded.
15. If a corrosive effect is observed after any of the three sequential exposures, the test is immediately terminated. If a corrosive effect is not observed after the last patch is removed, the animal is observed for 14 days, unless corrosion develops at an earlier time point.

16. In those cases in which the test chemical is not expected to produce corrosion but may be irritating, a single patch should be applied to one animal for four hours.

Confirmatory test (*In vivo* dermal irritation test with additional animals)

17. If a corrosive effect is not observed in the initial test, the irritant or negative response should be confirmed using up to two additional animals, each with one patch, for an exposure period of four hours. If an irritant effect is observed in the initial test, the confirmatory test may be conducted in a sequential manner, or by exposing two additional animals simultaneously. In the exceptional case, in which the initial test is not conducted, two or three animals may be treated with a single patch, which is removed after four hours. When two animals are used, if both exhibit the same response, no further testing is needed. Otherwise, the third animal is also tested. Equivocal responses may need to be evaluated using additional animals.

Observation period

18. The duration of the observation period should be sufficient to evaluate fully the reversibility of the effects observed. However, the experiment should be terminated at any time that the animal shows continuing signs of severe pain or distress. To determine the reversibility of effects, the animals should be observed up to 14 days after removal of the patches. If reversibility is seen before 14 days, the experiment should be terminated at that time.

Clinical observations and grading of skin reactions

19. All animals should be examined for signs of erythema and oedema, and the responses scored at 60 minutes, and then at 24, 48 and 72 hours after patch removal. For the initial test in one animal, the test site is also examined immediately after the patch has been removed. Dermal reactions are graded and recorded according to the grades in the Table below. If there is damage to skin which cannot be identified as irritation or corrosion at 72 hours, observations may be needed until day 14 to determine the reversibility of the effects. In addition to the observation of irritation, all local toxic effects, such as defatting of the skin, and any systemic adverse effects (e.g. effects on clinical signs of toxicity and body weight), should be fully described and recorded. Histopathological examination should be considered to clarify equivocal responses.
20. The grading of skin responses is necessarily subjective. To promote harmonisation in grading of skin response and to assist testing laboratories and those involved in making and interpreting the observations, the personnel performing the observations need to be adequately trained in the scoring system used (see Table below). An illustrated guide for grading skin irritation and other lesions could be helpful (3).

DATA AND REPORTING

21. Study results should be summarised in tabular form in the final test report and should cover all items listed in paragraph 24.

Evaluation of results

22. The dermal irritation scores should be evaluated in conjunction with the nature and severity of lesions, and their reversibility or lack of reversibility. The individual scores do not represent an absolute standard for the irritant properties of a material, as other effects of the test material are also evaluated. Instead, individual scores should be viewed as reference values, which need to be evaluated in combination with all other observations from the study.
23. Reversibility of dermal lesions should be considered in evaluating irritant responses. When responses such as alopecia (limited area), hyperkeratosis, hyperplasia and scaling, persist to the end of the 14-day observation period, the test chemical should be considered an irritant.

Test report

24. The test report must include the following information:

Rationale for in vivo testing:

- Weight-of-evidence analysis of pre-existing test data, including results from sequential testing strategy;
- Description of relevant data available from prior testing;
- Data derived at each stage of testing strategy;
- Description of *in vitro* tests performed, including details of procedures, results obtained with test/reference substances;
- Weight-of-the-evidence analysis for performing *in vivo* study.

Test chemical:

- Mono-constituent substance: chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc;
- Multi-constituent substance, mixture and substances of unknown or variable composition, complex reaction products or biological materials (UVCB): characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physico-chemical properties of the constituents;
- Physical appearance, water solubility, and additional relevant physico-chemical properties;
- Source, lot number if available;

- Treatment of the test chemical/control substance prior to testing, if applicable (e.g. warming, grinding);
- Stability of the test chemical, limit date for use, or date for re-analysis if known;
- Storage conditions.

Vehicle:

- Identification, concentration (where appropriate), volume used;
- Justification for choice of vehicle.

Test animal(s):

- Species/strain used, rationale for using animal(s) other than albino rabbit;
- Number of animal(s) of each sex;
- Individual animal weight(s) at start and conclusion of test;
- Age at start of study;
- Source of animal(s), housing conditions, diet, etc.

Test conditions:

- Technique of patch site preparation;
- Details of patch materials used and patching technique;
- Details of test chemical preparation, application, and removal.

Results:

- Tabulation of irritation/corrosion response scores for each animal at all time points measured;
- Descriptions of all lesions observed;
- Narrative description of nature and degree of irritation or corrosion observed, and any histopathological findings;
- Description of other adverse local (e.g. defatting of skin) and systemic effects in addition to dermal irritation or corrosion.

Discussion of results

Conclusions

LITERATURE

- (1) OECD (2014). Guidance document on Integrated Approaches to Testing and Assessment for Skin Irritation/Corrosion. Environmental Health and Safety Publications, Series on Testing and Assessment, (No 203), Organisation for Economic Cooperation and Development, Paris.
- (2) OECD (1998) Harmonized Integrated Hazard Classification System for Human Health and Environmental Effects of Chemical Substances, as endorsed by the 28th Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, November 1998 .
- (3) OECD (2000). Guidance Document on the Recognition, Assessment and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation. Environmental Health and Safety Publications, Series on Testing and Assessment (No 19), Organisation for Economic Cooperation and Development, Paris.

TABLE: GRADING OF SKIN REACTIONS**Erythema and Eschar Formation**

No erythema.....	0
Very slight erythema (barely perceptible).....	1
Well defined erythema.....	2
Moderate to severe erythema.....	3
Severe erythema (beef redness) to eschar formation preventing grading of erythema...	4
Maximum possible: 4	

Oedema Formation

No oedema.....	0
Very slight oedema (barely perceptible).....	1
Slight oedema (edges of area well defined by definite raising).....	2
Moderate oedema (raised approximately 1 mm).....	3
Severe oedema (raised more than 1 mm and extending beyond area of exposure)....	4
Maximum possible: 4	

Histopathological examination may be carried out to clarify equivocal responses.

Appendix

DEFINITIONS

Chemical is a substance or a mixture.

Dermal irritation is the production of reversible damage of the skin following the application of a test chemical for up to 4 hours.

Dermal corrosion is the production of irreversible damage of the skin; namely, visible necrosis through the epidermis and into the dermis, following the application of a test chemical for up to four hours. Corrosive reactions are typified by ulcers, bleeding, bloody scabs, and, by the end of observation at 14 days, by discolouration due to blanching of the skin, complete areas of alopecia, and scars. Histopathology should be considered to evaluate questionable lesions.

Test chemical is any substance or mixture tested using this test method"

(2) In Part B, Chapter B.17 is replaced by the following:

"B.17 *IN VITRO* MAMMALIAN CELL GENE MUTATION TESTS USING THE HPRT AND XPRT GENES

INTRODUCTION

1. This test method (TM) is equivalent to the OECD test guideline 476 (2016). Test methods are periodically reviewed in the light of scientific progress, changing regulatory needs and animal welfare. This current revised version of TM B.17 reflects nearly thirty years of experience with this test and also results from the development of a separate new method dedicated to *in vitro* mammalian cell gene mutation tests using the thymidine kinase gene. TM B.17 is part of a series of test methods on genetic toxicology. A document that provides succinct information on genetic toxicology testing and an overview of the recent changes that were made to genetic toxicity OECD test guidelines has been developed by OECD (1).
2. The purpose of the *in vitro* mammalian cell gene mutation test is to detect gene mutations induced by chemicals. The cell lines used in these tests measure forward mutations in reporter genes, specifically the endogenous hypoxanthine-guanine phosphoribosyl transferase gene (Hprt in rodent cells, HPRT in human cells; collectively referred to as the Hprt gene and HPRT test in this test method), and the xanthine-guanine phosphoribosyl transferase transgene (gpt) (referred to as the XPRT test). The HPRT and XPRT mutation tests detect different spectra of genetic events. In addition to the mutational events detected by the HPRT test (e.g. base pair substitutions, frameshifts, small deletions and insertions) the autosomal location of the gpt transgene may allow the detection of mutations resulting from large deletions and possibly mitotic recombination not detected by the HPRT test because the Hprt gene is located on the X-chromosome (2) (3) (4) (5) (6) (7). The XPRT is currently less widely used than the HPRT test for regulatory purposes.
3. Definitions used are provided in Appendix 1.

INITIAL CONSIDERATIONS AND LIMITATIONS

4. Tests conducted *in vitro* generally require the use of an exogenous source of metabolic activation. The exogenous metabolic activation system does not entirely mimic *in vivo* conditions.
5. Care should be taken to avoid conditions that would lead to artefactual positive results, (i.e. possible interaction with the test system), not caused by direct interaction between the test chemicals and the genetic material of the cell; such conditions include changes in pH or osmolality (8) (9) (10), interaction with the medium components (11) (12), or excessive

levels of cytotoxicity (13). Cytotoxicity exceeding the recommended top cytotoxicity levels as defined in paragraph 19 is considered excessive for the HPRT test.

6. Before use of the test method on a mixture for generating data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed when there is a regulatory requirement for testing of the mixture.

PRINCIPLE OF THE TEST

7. Mutant cells deficient in Hprt enzyme activity in the HPRT test or xpRT enzyme activity in the XPRT test are resistant to the cytostatic effects of the purine analogue 6-thioguanine (TG). The Hprt (in the HPRT test) or gpt (in XPRT test) proficient cells are sensitive to TG, which causes the inhibition of cellular metabolism and halts further cell division. Thus, mutant cells are able to proliferate in the presence of TG, whereas normal cells, which contain the Hprt (in the HPRT test) or gpt (in XPRT test) enzyme, are not.
8. Cells in suspension or monolayer cultures are exposed to the test chemical, both with and without an exogenous source of metabolic activation (see paragraph 14), for a suitable period of time (3-6 hours), and then sub-cultured to determine cytotoxicity and to allow phenotypic expression prior to mutant selection (14) (15) (16) (17). Cytotoxicity is determined by relative survival (RS), i.e. cloning efficiency measured immediately after treatment and adjusted for any cell loss during treatment as compared to the negative control (paragraph 18 and Appendix 2). The treated cultures are maintained in growth medium for a sufficient period of time, characteristic of each cell type, to allow near-optimal phenotypic expression of induced mutations (typically a minimum of 7-9 days). Following phenotypic expression, mutant frequency is determined by seeding known numbers of cells in medium containing the selective agent to detect mutant colonies, and in medium without selective agent to determine the cloning efficiency (viability). After a suitable incubation time, colonies are counted. Mutant frequency is calculated based on the number of mutant colonies corrected by the cloning efficiency at the time of mutant selection.

DESCRIPTION OF THE METHOD

Preparations

Cells

9. The cell types used for the HPRT and XPRT tests should have a demonstrated sensitivity to chemical mutagens, a high cloning efficiency, a stable karyotype, and a stable spontaneous mutant frequency. The most commonly used cells for the HPRT test include the CHO, CHL and V79 lines of Chinese hamster cells, L5178Y mouse lymphoma cells,

and TK6 human lymphoblastoid cells (18) (19). CHO-derived AS52 cells containing the gpt transgene (and having the Hp^rt gene deleted) are used for the XPRT test (20) (21); the HPRT test cannot be performed in AS52 cells because the hp^rt gene has been deleted. The use of other cell lines should be justified and validated.

10. Cell lines should be checked routinely for the stability of the modal chromosome number and the absence of Mycoplasma contamination (22) (23), and cells should not be used if contaminated or if the modal chromosome number has changed. The normal cell cycle time used in the testing laboratory should be established and should be consistent with the published cell characteristics. The spontaneous mutant frequency in the master cell stock should also be checked, and the stock should not be used if the mutant frequency is not acceptable.
11. Prior to use in this test, the cultures may need to be cleansed of pre-existing mutant cells, e.g. by culturing in HAT medium for HPRT test and MPA for XPRT test (5) (24) (See Appendix 1). The cleansed cells can be cryopreserved and then thawed to use as working stocks. The newly thawed working stock can be used for the test after normal doubling times are attained. When conducting the XPRT test, routine culture of AS52 cells should use conditions that assure the maintenance of the gpt transgene (20).

Media and culture conditions

12. Appropriate culture medium and incubation conditions (culture vessels, humidified atmosphere of 5% CO₂, and incubation temperature of 37°C) should be used for maintaining cultures. Cell cultures should always be maintained under conditions that ensure that they are growing in log phase. It is particularly important that media and culture conditions be chosen to ensure optimal growth of cells during the expression period and optimal cloning efficiency for both mutant and non-mutant cells.

Preparation of cultures

13. Cell lines are propagated from stock cultures, seeded in culture medium at a density such that the cells in suspensions or in monolayers will continue to grow exponentially through the treatment and expression periods (e.g. confluence should be avoided for cells growing in monolayers).

Metabolic activation

14. Exogenous metabolising systems should be used when employing cells which have inadequate endogenous metabolic capacity. The most commonly used system, that is recommended by default, unless otherwise justified, is a co-factor-supplemented post-mitochondrial fraction (S9) prepared from the livers of rodents (generally rats) treated with enzyme-inducing agents such as Aroclor 1254 (25) (26) (27) (28) or a combination of phenobarbital and β -naphthoflavone (29) (30) (31) (32). The latter combination does not

conflict with the Stockholm Convention on Persistent Organic Pollutants (33) and has been shown to be as effective as Aroclor 1254 for inducing mixed-function oxidases (29) (31). The S9 fraction typically is used at concentrations ranging from 1 to 2% (v/v) but may be increased to 10% (v/v) in the final test medium. The choice of the type and concentration of exogenous metabolic activation system or metabolic inducer employed may be influenced by the class of substances being tested (34) (35) (36).

Test chemical preparation

15. Solid test chemicals should be prepared in appropriate solvents and diluted, if appropriate, prior to treatment of the cells (see paragraph 16). Liquid test chemicals may be added directly to the test system and/or diluted prior to treatment of the test system. Gaseous or volatile test chemicals should be tested by appropriate modifications to the standard protocols, such as treatment in sealed culture vessels (37) (38). Preparations of the test chemical should be made just prior to treatment unless stability data demonstrate the acceptability of storage.

TEST CONDITIONS

Solvents

16. The solvent should be chosen to optimise the solubility of the test chemicals without adversely impacting the conduct of the test e.g. changing cell growth, affecting the integrity of the test chemical, reacting with culture vessels, impairing the metabolic activation system. It is recommended that, wherever possible, the use of an aqueous solvent (or culture medium) should be considered first. Well established solvents are for example, water and dimethyl sulfoxide. Generally, organic solvents should not exceed 1% (v/v) and aqueous solvents (saline or water) should not exceed 10% (v/v) in the final treatment medium. If the solvents used are not well-established (e.g. ethanol or acetone), their use should be supported by data indicating their compatibility with the test chemicals and the test system, and their lack of genetic toxicity at the concentration used. In the absence of that supporting data, it is important to add untreated controls (see Appendix 1) to demonstrate that no deleterious or mutagenic effects are induced by the chosen solvent.

Measuring cytotoxicity and choosing exposure concentrations

17. When determining the highest test chemical concentration, concentrations that have the capability of producing artefactual positive responses, such as those producing excessive cytotoxicity (see paragraph 20), precipitation in the culture medium (see paragraph 21), or marked changes in pH or osmolality (see paragraph 5) should be avoided. If the test chemical causes a marked change in the pH of the medium at the time of addition, the pH might be adjusted by buffering the final treatment medium so as to avoid artefactual positive results and to maintain appropriate culture conditions.

18. Concentration selection is based on cytotoxicity and other considerations (see paragraphs 20-22). While the evaluation of cytotoxicity in an initial test may be useful to better define the concentrations to be used in the main experiment, an initial test is not required. Even if an initial cytotoxicity evaluation is performed, the measurement of cytotoxicity for each culture is still required in the main experiment. Cytotoxicity should be evaluated using RS, i.e. cloning efficiency (CE) of cells plated immediately after treatment, adjusted by any loss of cells during treatment, based on cell count, as compared with adjusted cloning efficiency in negative controls (assigned a survival of 100%) (see Appendix 2 for the formula).
19. At least four test concentrations (not including the solvent and positive controls) that meet the acceptability criteria (appropriate cytotoxicity, number of cells, etc.) should be evaluated. While the use of duplicate cultures is advisable, either replicate or single treated cultures may be used at each concentration tested. The results obtained in the independent replicate cultures at a given concentration should be reported separately but can be pooled for the data analysis (17). For test chemicals demonstrating little or no cytotoxicity, concentration intervals of approximately 2 to 3 fold will usually be appropriate. Where cytotoxicity occurs, the test concentrations selected should cover a range from that producing cytotoxicity to concentrations at which there is moderate and little or no cytotoxicity. Many test chemicals exhibit steep concentration response curves and in order to cover the whole range of cytotoxicity or to study the concentration response relationship in detail, it may be necessary to use more closely spaced concentrations and more than four concentrations, in particular in situations where a repeat experiment is required (see paragraph 43). The use of more than 4 concentrations may be particularly important when using single cultures.
20. If the maximum concentration is based on cytotoxicity, the highest concentration should aim to achieve between 20 and 10% RS. Care should be taken when interpreting positive results only found at 10% RS or below (paragraph 43).
21. For poorly soluble test chemicals that are not cytotoxic at concentrations below the lowest insoluble concentration, the highest concentration analysed should produce turbidity or a precipitate visible by eye or with the aid of an inverted microscope at the end of the treatment with the test chemical. Even if cytotoxicity occurs above the lowest insoluble concentration, it is advisable to test at only one concentration producing turbidity or with a visible precipitate because artefactual effects may result from the precipitate. At the concentration producing a precipitate, care should be taken to assure that the precipitate does not interfere with the conduct of the test. The determination of solubility in the culture medium prior to the experiment may be useful.
22. If no precipitate or limiting cytotoxicity is observed, the highest test concentration should correspond to 10 mM, 2 mg/ml or 2 µl/ml, whichever is the lowest (39) (40). When the test

chemical is not of defined composition, e.g. substance of unknown or variable composition, complex reaction products or biological materials (i.e. Chemical Substances of Unknown or Variable Composition (UVCBs)) (41), environmental extracts, etc., the top concentration may need to be higher (e.g. 5 mg/mL), in the absence of sufficient cytotoxicity, to increase the concentration of each of the components. It should be noted however that these requirements may differ for human pharmaceuticals (42).

Controls

23. Concurrent negative controls (see paragraph 16), consisting of solvent alone in the treatment medium and handled in the same way as the treatment cultures, should be included for every experimental condition.
24. Concurrent positive controls are needed to demonstrate the ability of the laboratory to identify mutagens under the conditions of the test protocol used and the effectiveness of the exogenous metabolic activation system, when applicable. Examples of positive controls are given in Table 1 below. Alternative positive control substances can be used, if justified. Because *in vitro* mammalian cell tests for genetic toxicity are sufficiently standardised, tests using treatments with and without exogenous metabolic activation may be conducted using only a positive control requiring metabolic activation. In this case, this single positive control response will demonstrate both the activity of the metabolic activation system and the responsiveness of the test system. Each positive control should be used at one or more concentrations expected to give reproducible and detectable increases over background in order to demonstrate the sensitivity of the test system, and the response should not be compromised by cytotoxicity exceeding the limits specified in this test method (see paragraph 20).

Table 1: Reference substances recommended for assessing laboratory proficiency and for selection of positive controls.

Metabolic condition	Activation	Locus	Substance and CAS No
Absence of exogenous metabolic activation		<i>Hprt</i>	Ethylmethanesulfonate [CAS no. 62-50-0] Ethylnitrosourea [CAS no. 759-73-9] 4-Nitroquinoline 1-oxide [CAS no. 56-57-5]
		<i>xprt</i>	Streptonigrin [CAS no. 3930-19-6] Mitomycin C [CAS no. 50-07-7]
Presence of exogenous metabolic activation		<i>Hprt</i>	3-Methylcholanthrene [CAS no. 56-49-5] 7,12-Dimethylbenzanthracene [CAS no. 57-97-6] Benzo[a]pyrene [CAS no. 50-32-8]
		<i>xprt</i>	Benzo[a]pyrene [CAS no. 50-32-8]

PROCEDURE

Treatment with test chemical

25. Proliferating cells are treated with the test chemical in the presence and absence of a metabolic activation system. Exposure should be for a suitable period of time (usually 3 to 6 hours is adequate).
26. The minimum number of cells used for each test (control and treated) culture at each stage in the test should be based on the spontaneous mutant frequency. A general guide is to treat and passage sufficient cells as to maintain 10 spontaneous mutants in every culture in all phases of the test (17). The spontaneous mutant frequency is generally between 5 and 20×10^{-6} . For a spontaneous mutant frequency of 5×10^{-6} and to maintain a sufficient number of spontaneous mutants (10 or more) even for the cultures treated at concentrations that cause 90% cytotoxicity during treatment (10% RS), it would be necessary to treat at least 20×10^6 cells. In addition a sufficient number of cells (but never less than 2 million) must be cultured during the expression period and plated for mutant selection (17).

Phenotypic expression time and measuring mutant frequency

27. After the treatment period, cells are cultured to allow expression of the mutant phenotype. A minimum of 7 to 9 days generally is sufficient to allow near optimal phenotypic expression of newly induced Hprt and xprt mutants (43) (44). During this period, cells are regularly sub-cultured to maintain them in exponential growth. After phenotypic expression, cells are re-plated in medium with and without selective agent (6-thioguanine) for the determination of the number of mutants and cloning efficiency at the time of selection, respectively. This plating can be accomplished using dishes for monolayer cultures or microwell plates for cells in suspension. For mutant selection, cells should be plated at a density to assure optimum mutant recovery (i.e. avoid metabolic cooperation) (17). Plates are incubated for an appropriate length of time for optimum colony growth (e.g. 7-12 days) and colonies counted. Mutant frequency is calculated based on the number of mutant colonies corrected by the cloning efficiency at the time of mutant selection (see Appendix 2 for formulas).

Proficiency of the laboratory

28. In order to establish sufficient experience with the test prior to using it for routine testing, the laboratory should have performed a series of experiments with reference positive substances acting via different mechanisms (at least one active with and one active without metabolic activation selected from the substances listed in Table 1) and various negative controls (using various solvents/vehicles). These positive and negative control responses should be consistent with the literature. This is not applicable to laboratories that have experience, i.e. that have an historical data base available as defined in paragraphs 30 to 33.

29. A selection of positive control substances (see Table 1 in paragraph 25) should be investigated in the absence and in the presence of metabolic activation, in order to demonstrate proficiency to detect mutagenic chemicals, to determine the effectiveness of the metabolic activation system and to demonstrate the appropriateness of the cell growth conditions during treatment, phenotypic expression and mutant selection and of the scoring procedures. A range of concentrations of the selected substances should be chosen so as to give reproducible and concentration-related increases above the background in order to demonstrate the sensitivity and dynamic range of the test system.

Historical control data

30. The laboratory should establish:

- A historical positive control range and distribution,
- A historical negative (untreated, solvent) control range and distribution.

31. When first acquiring data for an historical negative control distribution, concurrent negative controls should be consistent with published control data (22). As more experimental data are added to the control distribution, concurrent negative controls should ideally be within the 95% control limits of that distribution (17) (45) (46).

32. The laboratory's historical negative control database should initially be built with a minimum of 10 experiments but would preferably consist of at least 20 experiments conducted under comparable experimental conditions. Laboratories should use quality control methods, such as control charts (e.g. C-charts or X-bar charts (47)), to identify how variable their positive and negative control data are, and to show that the methodology is 'under control' in their laboratory (46). Further recommendations on how to build and use the historical data (i.e. criteria for inclusion and exclusion of data in historical data and the acceptability criteria for a given experiment) can be found in the literature (45).

33. Negative control data should consist of mutant frequencies from single or preferably replicate cultures as described in paragraph 23. Concurrent negative controls should ideally be within the 95% control limits of the distribution of the laboratory's historical negative control database (17) (45) (46). Where concurrent negative control data fall outside the 95% control limit they may be acceptable for inclusion in the historical control distribution as long as these data are not extreme outliers and there is evidence that the test system is 'under control' (see above) and there is evidence of no technical or human failure.

34. Any changes to the experimental protocol should be considered in terms of their consistency with the laboratory's existing historical control databases. Any major inconsistencies should result in the establishment of a new historical control database.

DATA AND REPORTING

Presentation of the results

35. The presentation of results should include all of the data needed to calculate cytotoxicity (expressed as RS). The data, for both treated and control cultures, should include the number of cells at the end of treatment, the number of cells plated immediately following treatment, and the colony counts (or number of wells without colonies for the microwell method). RS for each culture should be expressed as a percentage relative to the concurrent solvent control (refer to Appendix 1 for definitions).
36. The presentation of results should also include all of the data needed to calculate the mutant frequency. Data for both treated and control cultures, should include: (1) the number of cells plated with and without selective agent (at the time the cells are plated for mutant selection), and (2) the number of colonies counted (or the number of wells without colonies for the microwell method) from the plates with and without selective agent. Mutant frequency is calculated based on the number of mutant colonies (in the plates with selective agent) corrected by the cloning efficiency (from the plates without selective agent). The mutant frequency should be expressed as the number of mutant cells per million viable cells (refer to Appendix 1 for definitions).
37. Individual culture data should be provided. Additionally, all data should be summarised in tabular form.

Acceptability Criteria

38. Acceptance of a test is based on the following criteria:
 - The concurrent negative control is considered acceptable for addition to the laboratory historical negative control database as described in paragraph 33.
 - Concurrent positive controls (see paragraph 24) should induce responses that are compatible with those generated in the historical positive control data base and produce a statistically significant increase compared with the concurrent negative control.
 - Two experimental conditions (i.e. with and without metabolic activation) were tested unless one resulted in positive results (see paragraph 25).
 - Adequate number of cells and concentrations are analysable (paragraphs 25, 26 and 19).
 - The criteria for the selection of top concentration are consistent with those described in paragraphs 20, 21 and 22.

Evaluation and interpretation of results

39. Providing that all acceptability criteria are fulfilled, a test chemical is considered to be clearly positive if, in any of the experimental conditions examined:

- at least one of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control,
- the increase is concentration-related when evaluated with an appropriate trend test,
- any of the results are outside the distribution of the historical negative control data (e.g. Poisson-based 95% control limit; see paragraph 33).

When all of these criteria are met, the test chemical is then considered able to induce gene mutations in cultured mammalian cells in this test system. Recommendations for the most appropriate statistical methods can be found in the literature (46) (48).

40. Providing that all acceptability criteria are fulfilled, a test chemical is considered clearly negative if, in all experimental conditions examined:

- none of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control,
- there is no concentration-related increase when evaluated with an appropriate trend test,
- all results are inside the distribution of the historical negative control data (e.g. Poisson-based 95% control limit; see paragraph 33).

The test chemical is then considered unable to induce gene mutations in cultured mammalian cells in this test system.

41. There is no requirement for verification of a clearly positive or negative response.

42. In cases when the response is neither clearly negative nor clearly positive as described above, or in order to assist in establishing the biological relevance of a result, the data should be evaluated by expert judgement and/or further investigations. Performing a repeat experiment possibly using modified experimental conditions (e.g. concentration spacing, other metabolic activation conditions [i.e. S9 concentration or S9 origin]) could be useful.

43. In rare cases, even after further investigations, the data set will preclude making a conclusion of positive or negative results. Therefore the test chemical response should be concluded to be equivocal (interpreted as equally likely to be positive or negative).

Test report

44. The test report should include the following information:

Test chemical:

- source, lot number, limit date for use, if available;
- stability of the test chemical itself, if known;
- solubility and stability of the test chemical in solvent, if known;

- measurement of pH, osmolality and precipitate in the culture medium to which the test chemical was added, as appropriate.

Mono-constituent substance:

- physical appearance, water solubility, and additional relevant physicochemical properties;
- chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc.

Multi-constituent substance, UVCBs and mixtures:

- characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents.

Solvent:

- justification for choice of solvent;
- percentage of solvent in the final culture medium.

Cells:

For Laboratory master cultures:

- type, source of cell lines;
- number of passages, if available, and history in the laboratory;
- karyotype features and/or modal number of chromosomes;
- methods for maintenance of cell cultures;
- absence of mycoplasma;
- cell doubling times.

Test conditions:

- rationale for selection of concentrations and number of cultures including, e.g. cytotoxicity data and solubility limitations;
- composition of media, CO₂ concentration, humidity level;
- concentration of test chemical expressed as final concentration in the culture medium (e.g. µg or mg/ml or mM of culture medium);
- concentration (and/or volume) of solvent and test chemical added in the culture medium;
- incubation temperature;
- incubation time;

- duration of treatment;
- cell density during treatment;
- type and composition of metabolic activation system (source of S9, method of preparation of the S9 mix, the concentration or volume of S9 mix and S9 in the final culture medium, quality controls of S9);
- positive and negative control substances, final concentrations for each condition of treatment;
- length of expression period (including number of cells seeded, and subcultures and feeding schedules, if appropriate);
- identity of the selective agent and its concentration;
- criteria for acceptability of tests;
- methods used to enumerate numbers of viable and mutant cells;
- methods used for the measurements of cytotoxicity;
- any supplementary information relevant to cytotoxicity and method used;
- duration of incubation times after plating;
- criteria for considering studies as positive, negative or equivocal;
- methods used to determine pH, osmolality and precipitation.

Results:

- number of cells treated and number of cells sub-cultured for each culture;
- cytotoxicity measurements and other observations if any;
- signs of precipitation and time of the determination;
- number of cells plated in selective and non-selective medium;
- number of colonies in non-selective medium and number of resistant colonies in selective medium, and related mutant frequencies;
- concentration-response relationship, where possible;
- concurrent negative (solvent) and positive control data (concentrations and solvents);
- historical negative (solvent) and positive control data, with ranges, means and standard deviations and confidence interval (e.g. 95%) as well as the number of data;
- statistical analyses (for individual cultures and pooled replicates if appropriate), and p-values if any.

Discussion of the results.

Conclusion

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Appendix 1

DEFINITIONS

Base pair substitution mutagens: chemicals that cause substitution of base pairs in the DNA.

Chemical: A substance or a mixture.

Cloning efficiency: The percentage of cells plated at a low density that are able to grow into a colony that can be counted.

Concentrations: refer to final concentrations of the test chemical in culture medium

Cytotoxicity: For the assays covered in this test method, cytotoxicity is identified as a reduction in relative survival of the treated cells as compared to the negative control (see specific paragraph).

Forward mutation: a gene mutation from the parental type to the mutant form which gives rise to an alteration or a loss of the enzymatic activity or the function of the encoded protein.

Frameshift mutagens: chemicals which cause the addition or deletion of single or multiple base pairs in the DNA molecule.

Genotoxic: a general term encompassing all types of DNA or chromosomal damage, including DNA breaks, adducts, rearrangements, mutations, chromosome aberrations, and aneuploidy. Not all types of genotoxic effects result in mutations or stable chromosomal damage

HAT medium: medium containing Hypoxanthine, Aminopterin and Thymidine, used for cleansing of Hprt mutants.

Mitotic recombination: during mitosis, recombination between homologous chromatids possibly resulting in the induction of DNA double strand breaks or in a loss of heterozygosity.

MPA medium: medium containing Xanthine, Adenine, Thymidine, Aminopterin and Mycophenolic acid, used for cleansing of Xprt mutants.

Mutagenic: produces a heritable change of DNA base-pair sequences(s) in genes or of the structure of chromosomes (chromosome aberrations).

Mutant frequency (MF): the number of mutant colonies observed divided by the number of cells plated in selective medium, corrected for cloning efficiency (or viability) at the time of selection.

Phenotypic expression time: The time after treatment during which the genetic alteration is fixed within the genome and any preexisting gene products are depleted to the point that the

phenotypic trait is altered.

Relative survival (RS): RS is used as the measure of treatment-related cytotoxicity. RS is cloning efficiency (CE) of cells plated immediately after treatment adjusted by any loss of cells during treatment as compared with cloning efficiency in negative controls (assigned a survival of 100%).

S9 liver fractions: supernatant of liver homogenate after 9000g centrifugation, i.e. raw liver extract

S9 mix: mix of the liver S9 fraction and cofactors necessary for metabolic enzyme activity.

Solvent control: General term to define the control cultures receiving the solvent alone used to dissolve the test chemical.

Test chemical: Any substance or mixture tested using this test method.

Untreated control: cultures that receive no treatment (i.e. neither test chemical nor solvent) but are processed concurrently and in the same way as the cultures receiving the test chemical

UVCB: Chemical Substances of Unknown or Variable Composition, Complex Reaction Products and Biological Materials

Appendix 2

FORMULAS FOR ASSESSMENT OF CYTOTOXICITY AND MUTANT FREQUENCY

Cytotoxicity is evaluated by relative survival, i. e., cloning efficiency (CE) of cells plated immediately after treatment adjusted by any loss of cells during treatment as compared with adjusted cloning efficiency in negative controls (assigned a survival of 100%) (see RS formula below).

Adjusted CE for a culture treated by a test chemical is calculated as:

$$\text{Adjusted CE} = \frac{\text{Number of cells at the end of treatment}}{\text{Number of cells at the beginning of treatment}}$$

RS for a culture treated by a test chemical is calculated as:

$$\text{RS} = \frac{\text{Adjusted CE in treated culture}}{\text{Adjusted CE in the solvent control}} \times 100$$

Mutant frequency is the cloning efficiency of mutant colonies in selective medium divided by the cloning efficiency in non-selective medium measured for the same culture at the time of selection.

$$\text{Mutant frequency} = \frac{\text{Cloning efficiency of mutant colonies in selective medium}}{\text{Cloning efficiency in non – selective medium}}$$

When plates are used for cloning efficiency:

$$\text{CE} = \text{Number of colonies} / \text{Number of cells plated.}$$

When micro-well plates are used for cloning efficiency:

The number of colonies per well on micro-wells plates follows a Poisson distribution.

$$\text{Cloning Efficiency} = -\text{LnP}(0) / \text{Number of cells plated per well}$$

Where $-\text{LnP}(0)$ is the probable number of empty wells out of the seeded wells and is described by the following formula

$$\text{LnP}(0) = -\text{Ln} (\text{number of empty wells} / \text{number of plated wells})"$$

(3) In Part B, Chapter B.22 is replaced by the following:

"B.22 RODENT DOMINANT LETHAL TEST

INTRODUCTION

1. This test method (TM) is equivalent to the OECD test guideline (TG) 478 (2016). Test methods are periodically reviewed in the light of scientific progress, changing regulatory needs, and animal welfare considerations. This modified version of the test method reflects more than thirty years of experience with this test and the potential for integrating or combining this test with other toxicity tests such as developmental, reproductive toxicity, or genotoxicity studies; however due to its limitations and the use of a large number of animals this assay is not intended for use as a primary method, but rather as a supplemental test method which can only be used when there is no alternative for regulatory requirements. Combining toxicity testing has the potential to spare large numbers of animals from use in toxicity tests. A document that provides succinct information on genetic toxicology testing and an overview of the recent changes that were made to genetic toxicity OECD test guidelines has been developed by OECD (1).
2. The purpose of the Dominant lethal (DL) test is to investigate whether chemicals produce mutations resulting from chromosomal aberrations in germ cells. In addition, the dominant lethal test is relevant to assessing genotoxicity because, although they may vary among species, factors of *in vivo* metabolism, pharmacokinetics and DNA-repair processes are active and contribute to the response. Induction of a DL mutation after exposure to a test chemical indicates that the chemical has affected germinal tissue of the test animal.
3. DL mutations cause embryonic or foetal death. Induction of DL mutation after exposure to a test chemical indicates that the chemical has affected the germ cells of the test animal.
4. A DL assay is useful for confirmation of positive results of tests using somatic *in vivo* endpoints, and is a relevant endpoint for the prediction of human hazard and risk of genetic diseases transmitted through the germline. However, this assay requires a large number of animals and is labour-intensive; as a result, it is very expensive and time-consuming to conduct. Because the spontaneous frequency of dominant lethal mutations is quite high, the sensitivity of the assay for detection of small increases in the frequency of mutations is generally limited.
5. Definitions of key terms are set out in Appendix 1.

INITIAL CONSIDERATIONS

6. The test is most often conducted in mice (2) (3) (4) but other species, such as rats (5) (6) (7) (8), may in some cases be appropriate if scientifically justified. DLs generally are the result of gross chromosomal aberrations (structural and numerical abnormalities) (9) (10) (11), but gene mutations cannot be excluded. A DL mutation is a mutation occurring in a germ cell per se, or is fixed post fertilisation in the early embryo, that does not cause dysfunction of the gamete, but is lethal to the fertilised egg or developing embryo.
7. Individual males are mated sequentially to virgin females at appropriate intervals. The number of matings following treatment is dependent on the ultimate purpose of the DL study (Paragraph 23) and should ensure that all phases of male germ cell maturation are evaluated for DLs (12).
8. If there is evidence that the test chemical, or its metabolite(s), will not reach the testis, it is not appropriate to use this test.

PRINCIPLE OF THE TEST

9. Generally, male animals are exposed to a test chemical by an appropriate route of exposure and mated to untreated virgin females. Different germ cell types can be tested by the use of sequential mating intervals. Following mating, the females are euthanised after an appropriate period of time, and their uteri are examined to determine the numbers of implants and live and dead embryos. The dominant lethality of a test chemical is determined by comparing the live implants per female in the treated group with the live implants per female in the vehicle/solvent control group. The increase of dead implants per female in the treated group over the dead implants per female in the control group reflects the test-chemical-induced post-implantation loss. The post-implantation loss is calculated by determining the ratio of dead to total implants in the treated group compared to the ratio of dead to total implants in the control group. Pre-implantation loss can be estimated by comparing corpora lutea counts minus total implants or the total implants per female in treated and control groups.

VERIFICATION OF LABORATORY PROFICIENCY

10. Competence in this assay should be established by demonstrating the ability to reproduce dominant lethal frequencies from published data (e.g. (13) (14) (15) (16) (17) (18)) with positive control substances (including weak responses) such as those listed in Table 1, and vehicle controls and obtaining negative control frequencies that are consistent acceptable range of data (see references above) or with the laboratory's historical control distribution, if available.

DESCRIPTION OF THE METHOD

Preparations

Selection of animal species

11. Commonly used laboratory strains of healthy sexually mature animals should be employed. Mice are commonly used but rats may also be appropriate. Any other appropriate mammalian species may be used, if scientific justification is provided in the report.

Animal housing and feeding conditions

12. For rodents, the temperature in the animal room should be 22°C ($\pm 3^\circ\text{C}$). Although the relative humidity ideally should be 50-60%, it should be at least 40% and preferably not exceed 70%, other than during room cleaning. Lighting should be artificial, the sequence being 12 hours light, followed by 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. The choice of diet may be influenced by the need to ensure a suitable admixture of a test chemical when administered by this route. Prior to treatment or mating, rodents should be housed in small groups (no more than five) of the same sex if no aggressive behaviour is expected or observed, preferably in solid cages with appropriate environmental enrichment. Animals may be housed individually if scientifically justified.

Preparation of the animals

13. Healthy and sexually mature male and female adult animals are randomly assigned to the control and treatment groups. The individual animals are identified uniquely using a humane, minimally invasive method (e.g. by ringing, tagging, micro-chipping, or biometric identification, but not toe and ear clipping) and acclimated to the laboratory conditions for at least five days. Cages should be arranged in such a way that possible effects due to cage placement are minimised. Cross contamination by the positive control and the test chemical should be avoided. At the commencement of the study, the weight variation of animals should be minimal and not exceed $\pm 20\%$ of the mean weight of each sex.

Preparation of doses

14. Solid test chemicals should be dissolved or suspended in appropriate solvents or vehicles or admixed in diet or drinking water prior to dosing of the animals. Liquid test chemicals may be dosed directly or diluted prior to dosing. For inhalation exposures, test chemicals can be administered as gas, vapour, or a solid/liquid aerosol, depending on their physicochemical properties. Fresh preparations of the test chemical should be employed unless stability data demonstrate the acceptability of storage and define the appropriate storage conditions.

Test Conditions

Solvent/vehicle

15. The solvent/vehicle should not produce toxic effects at the dose volumes used, and should not be suspected of chemical reaction with the test chemical. If other than well-known solvents/vehicles are used, their inclusion should be supported with reference data indicating their compatibility. It is recommended that wherever possible, the use of an aqueous solvent/vehicle should be considered first. Examples of commonly used compatible solvents/vehicles include water, physiological saline, methylcellulose solution, carboxymethyl cellulose sodium salt solution, olive oil and corn oil.

Positive controls

16. Concurrent positive control animals should always be used unless the laboratory has demonstrated proficiency in the conduct of the test and has used the test routinely in the recent past (e.g. within the last 5 years). However, it is not necessary to treat positive control animals by the same route as animals receiving the test chemical, or sample all the mating intervals. The positive control substances should be known to produce DLs under the conditions used for the test. Except for the treatment, animals in the control groups should be handled in an identical manner to animals in the treated groups.
17. The doses of the positive control substances should be selected so as to produce weak or moderate effects that critically assess the performance and sensitivity of the assay, but which consistently produce positive dominant lethal effects. Examples of positive control substances, and appropriate doses, are included in Table 1.

Table 1: Examples of Positive Control Substances.

Substance [CAS no.] (reference no.)	Effective Dose range (mg/kg) (rodent species)	Administration Time (days)
Triethylenemelamine [51-18-3] (15)	0.25 (mice)	1
Cyclophosphamide [50-18-0] (19)	50-150 (mice)	5
Cyclophosphamide [50-18-0] (5)	25-100 (rats)	1
Ethyl methanesulphonate [62-50-0] (13)	100-300 (mice)	5
Monomeric Acrylamide [79-06-1] (17)	50 (mice)	5
Chlorambucil [305-03-3] (14)	25 (mice)	1

Negative controls

18. Negative control animals, treated with solvent or vehicle alone, and otherwise treated in the same way as the treatment groups, should be included for every sampling time (20). In

the absence of historical or published control data showing that no DLs or other deleterious effects are induced by the chosen solvent/vehicle, untreated control animals should also be included for every sampling time in order to establish acceptability of the vehicle control.

PROCEDURE

Number of Animals

19. Individual males are mated sequentially at appropriate predetermined intervals (e.g. weekly intervals, Paragraphs 21 & 23) preferably to one virgin female. The number of males per group should be predetermined to be sufficient (in combination with the number of mated females at each mating interval) to provide the statistical power necessary to detect at least a doubling in DL frequency (Paragraph 44).
20. The number of females per mating interval should also be predetermined by statistical power calculations to permit the detection of at least a doubling in the DL frequency (i.e. sufficient pregnant females to provide at least 400 total implants) (20) (21) (22) (23) and that at least one dead implant per analysis unit (i.e. mating group per dose) is expected (24).

Administration Period and Mating Intervals

21. The number of mating intervals following treatment is governed by the treatment schedule and should ensure that all phases of male germ cell maturation are evaluated for DL induction (12) (25). For a single treatment up to five daily dose administrations, there should be 8 (mouse) or 10 (rat) matings conducted at weekly intervals following the last treatment. For multiple dose administrations, the number of mating intervals may be reduced in proportion to the increased time of the administration period, but maintaining the goal of evaluating all phases of spermatogenesis (e.g. after a 28-day exposure, only 4 weekly matings are sufficient to evaluate all phases of spermatogenesis in the mouse). All treatment and mating schedules should be scientifically justified.
22. Females should remain with the males for at least the duration of one oestrus cycle (e.g. one week covers one oestrus cycle in both mice and rats). Females that did not mate during a one-week interval can be used for a subsequent mating interval. Alternatively, until mating has occurred, as determined by the presence of sperm in the vagina or by the presence of a vaginal plug.
23. The exposure and mating regimen used is dependent on the ultimate purpose of the DL study. If the goal is to determine whether a given chemical induces DL mutations *per se*, then the accepted method would be to expose an entire round of spermatogenesis (e.g. 7 weeks in the mouse, 5-7 treatments per week) and mate once at the end. However, if the goal is to identify the sensitive germ cell type for DL induction, then a single or 5 day exposure followed by weekly mating is preferred.

Dose Levels

24. If a preliminary range-finding study is performed because there are no suitable data already available to aid in dose selection, it should be performed in the same laboratory, using the same species, strain, sex, and treatment regimen to be used in the main study (26). The study should aim to identify the maximum tolerated dose (MTD), defined as the highest dose that will be tolerated without evidence of study-limiting toxicity, relative to the duration of the study period (for example, abnormal behaviour or reactions, minor body weight depression or hematopoietic system cytotoxicity), but not death or evidence of pain, suffering or distress necessitating humane euthanasia (27).
25. The MTD must also not adversely affect mating success (21).
26. Test chemicals with specific biological activities at low non-toxic doses (such as hormones and mitogens), and chemicals which exhibit saturation of toxicokinetic properties may be exceptions to the dose-setting criteria and should be evaluated on a case-by-case basis.
27. In order to obtain dose response information, a complete study should include a negative control group and a minimum of three dose levels generally separated by a factor of 2, but not greater than 4. If the test chemical does not produce toxicity in a range-finding study, or based on existing data, the highest dose for a single administration should be 2000 mg/kg body weight. However, if the test chemical does cause toxicity, the MTD should be the highest dose administered and the dose levels used should preferably cover a range from the maximum to a dose producing little or no toxicity. For not-toxic chemicals, the limit dose for an administration period of 14 days or more is 1000 mg/kg body weight/day, and for administration periods of less than 14 days the limit dose is 2000 mg/kg body weight/day.

Administration of Doses

28. The anticipated route of human exposure should be considered when designing an assay. Therefore, routes of exposures such as dietary, drinking water, subcutaneous, intravenous, topical, inhalation, oral (by gavage), or implantation may be chosen as justified. In any case, the route should be chosen to ensure adequate exposure of the target tissue(s). Intraperitoneal injection is not normally recommended since it is not an intended route of human exposure, and should only be used with specific scientific justification. If the test chemical is admixed in diet or drinking water, especially in case of single dosing, care should be taken that the delay between food and water consumption and mating should be sufficient to allow detection of the effects (paragraph 31). The maximum volume of liquid that can be administered by gavage or injection at one time depends on the size of the test animal. The volume should not normally exceed 1 ml/100g body weight except in the case of aqueous solutions where a maximum of 2 ml/100g may be used. The use of volumes greater than this (if permitted by animal welfare legislation) should be justified. Variability

in test volume should be minimised by adjusting the concentration to ensure a constant volume in relation to body weight at all dose levels.

Observations

29. General clinical observations of the test animals should be made and clinical signs recorded at least once a day, preferably at the same time(s) each day and considering the peak period of anticipated effects after dosing. At least twice daily during the dosing period, all animals should be observed for morbidity and mortality. All animals should be weighed at the beginning of the study and at least once a week during repeated dose studies, and at the time of euthanasia. Measurements of food consumption should be made at least weekly. If the test chemical is administered via the drinking water, water consumption should be measured at each change of water and at least weekly. Animals exhibiting non-lethal indicators of excess toxicity should be euthanised prior to completion of the test period (27).

Tissue Collection and Processing

30. Females are euthanised in the second half of pregnancy at gestation day (GD) 13 for mice and GD 14-15 for rats. Uteri are examined for dominant lethal effects to determine the number of implants, live and dead embryos, and corpora lutea.
31. The uterine horns and ovaries are exposed for counting of corpora lutea, and fetuses are removed, counted, and weighted. Care should be taken to examine the uteri for resorptions obscured by live fetuses and to ensure that all resorptions are enumerated. Fetal mortality is recorded. The number of successfully impregnated females and the number of total implantations, pre-implantation losses, and post-implantation mortality (included early and late resorptions) also are recorded. In addition, the visible fetuses may be preserved in Bouin's fixative for at least 2 weeks followed by examination for major external malformations (28) to provide additional information on the reproductive and developmental effects of the test agent.

DATA AND REPORTING

Treatment of Results

32. Data should be tabulated to show the number of males mated, the number of pregnant females, and the number of non-pregnant females. Results of each mating, including the identity of each male and female, should be reported individually. The mating interval, dose level for treated males, and the numbers of live implants and dead implants should be enumerated for each female.

33. The post-implantation loss is calculated by determining the ratio of dead to total implants from the treated group compared to the ratio of dead to total implants from the vehicle/solvent control group.
34. Pre-implantation loss is calculated as the difference between the number of corpora lutea and the number of implants, or as a reduction in the average number of implants per female in comparison with control matings. Where pre-implantation loss is estimated, it should be reported.
35. The Dominant Lethal factor is estimated as: $(\text{post-implantation deaths} / \text{total implantations per female}) \times 100$.
36. Data on toxicity and clinical signs (as per Paragraph 29) should be reported.

Acceptability Criteria

37. The following criteria determine the acceptability of a test.
- Concurrent negative control is consistent with published norms for historical negative control data, and the laboratory's historical control data if available (see Paragraphs 10 and 18).
 - Concurrent positive controls induce responses that are consistent with published norms for historic positive control data, or the laboratory's historical positive control database, if available, and produce a statistically significant increase compared with the negative control (see Paragraphs 17 and 18).
 - Adequate number total implants and doses have been analysed (Paragraph 20).
 - The criteria for the selection of top dose are consistent with those described in Paragraphs 24 and 27.

Evaluation and Interpretation of Results

38. At least three treated dose groups should be analysed in order to provide sufficient data for dose-response analysis.
39. Providing that all acceptability criteria are fulfilled, a test chemical is considered a clear positive if:
- at least one of the test doses exhibits a statistically significant increase compared with the concurrent negative control;
 - the increase is dose-related in at least one experimental condition (e.g. a weekly mating interval) when evaluated with an appropriate test; and,

- any of the results are outside of the acceptable range of negative control data, or the distribution of the laboratory's historical negative control data (e.g. Poisson-based 95% control limit) if available.

The test chemical is then considered able to induce dominant lethal mutations in germ cells of the test animals. Recommendations for the most appropriate statistical methods are described in Paragraph 44; other recommend statistical approaches can also be found in the literature (20) (21) (22) (24) (29). Statistical tests used should consider the animal as the experimental unit.

40. Providing that all acceptability criteria are fulfilled, a test chemical is considered a clear negative if:

- none of the test doses exhibits a statistically significant increase compared with the concurrent negative control;
- there is no dose-related increase in any experimental condition; and
- all results are within acceptable range of negative control data, or the laboratory's historical negative control data (e.g. Poisson-based 95% control limit), if available.

The test chemical is then considered unable to induce dominant lethal mutations in germ cells of the test animals.

41. There is no requirement for verification of a clear positive or a clear negative response.

42. If the response is not clearly negative or positive, and in order to assist in establishing the biological relevance of a result (e.g. a weak or borderline increase), the data should be evaluated by expert judgment and/or further investigations using the existing experimental data, such as consideration whether the positive result is outside the acceptable range of negative control data, or the laboratory's historical, negative control data (30).

43. In rare cases, even after further investigations, the data set will preclude making a conclusion of positive or negative results, and will therefore be concluded as equivocal.

44. Statistical tests used should consider the male animal as the experimental unit. While it is possible that count data (e.g. number of implants per female) may be Poisson distributed and/or proportions (e.g. proportion of dead implants) may be binomially distributed, it is often the case that such data are overdispersed (31). Accordingly, statistical analysis should first employ a test for over- underdispersion using variance tests such as Cochran's binomial variance test (32) or Tarone's $C(\alpha)$ test for binomial overdispersion (31) (33). If no departure from binomial dispersion is detected, trends in proportions across dose levels may be tested using the Cochran-Armitage trend test (34) and pairwise comparisons with the control group may be tested using Fisher's exact test (35). Likewise, if no departure from Poisson dispersion is detected, trends in counts may be tested using Poisson

regression (36) and pairwise comparisons with the control group may be tested within the context of the Poisson model, using pairwise contrasts (36). If significant overdispersion or underdispersion is detected, nonparametric methods are recommended (23) (31). These include rank-based tests, such as the Jonckheere-Terpstra test for trend (37) and Mann-Whitney tests (38) for pairwise comparisons with the vehicle/solvent control group, as well as permutation, resampling, or bootstrap tests for trend and pairwise comparisons with the control group (31) (39).

45. A positive DL assay provides evidence for the genotoxicity of the test chemical in the germ cells of the treated male of the test species.
46. Consideration of whether the observed values are within or outside of the historical control range can provide guidance when evaluating the biological significance of the response (40).

Test Report

47. The test report should include the following information.

Summary.

Test chemical:

- source, lot number, limit date for use, if available;
- stability of the test chemical itself, if known;
- solubility and stability of the test chemical in solvent, if known;
- measurement of pH, osmolality, and precipitate in the culture medium to which the test chemical was added, as appropriate.

Mono-constituent substance:

- physical appearance, water solubility, and additional relevant physicochemical properties;
- chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc.

Multi-constituent substance, UVCBs and mixtures:

- characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents.

Test chemical preparation:

- justification for choice of vehicle;
- solubility and stability of the test chemical in the solvent/vehicle, if known;

- preparation of dietary, drinking water or inhalation formulations;
- analytical determinations on formulations (e.g. stability, homogeneity, nominal concentrations) when conducted.

Test animals:

- species/strain used and justification for the choice;
- number, age and sex of animals;
- source, housing conditions, diet, etc.;
- method of uniquely identifying the animals;
- for short-term studies: individual body weight of the male animals at the start and end of the test; for studies longer than one week: individual body weights during the study and food consumption. Body weight range, mean and standard deviation for each group should be included.

Test conditions:

- positive and negative (vehicle/solvent) control data;
- data from the range-finding study;
- rationale for dose level selection;
- details of test chemical preparation;
- details of the administration of the test chemical;
- rationale for route of administration;
- methods for measurement of animal toxicity, including, where available, histopathological or hematological analyses and the frequency with which animal observations and body weights were taken;
- methods for verifying that the test chemical reached the target tissue, or general circulation, if negative results are obtained;
- actual dose (mg/kg body weight/day) calculated from diet/drinking water test chemical concentration (ppm) and consumption, if applicable;
- details of food and water quality;
- details on cage environment enrichment;
- detailed description of treatment and sampling schedules and justifications for the choices;
- method of analgesia
- method of euthanasia;
- procedures for isolating and preserving tissues;

- source and lot numbers of all kits and reagents (where applicable);
- methods for enumeration of DLs;
- mating schedule;
- methods used to determine that mating has occurred;
- time of euthanasia;
- criteria for scoring DL effects, including, corpora lutea, implantations, resorptions and pre-implantation losses, live implants, dead implants.

Results:

- animal condition prior to and throughout the test period, including signs of toxicity;
- male body weight during the treatment and mating periods;
- number of mated females;
- dose-response relationship, where possible;
- concurrent and historical negative control data with ranges, means and standard deviations;
- concurrent positive control data;
- tabulated data for each dam including: number of corpora lutea per dam; number of implantations per dam; number of resorptions and pre-implantation losses per dam; number of live implants per dam; number of dead implants per dam; fetus weights;
- the above data summarised for each mating period and dose, with Dominant Lethal frequencies;
- statistical analyses and methods applied.

Discussion of the results.

Conclusion.

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Appendix 1

DEFINITIONS

Chemical: A substance or a mixture

Corpora luteum (lutea): the hormonal secreting structure formed on the ovary at the site of a follicle that has released the egg. The number of corpora lutea in the ovaries corresponds to the number of eggs that were ovulated.

Dominant Lethal Mutation: a mutation occurring in a germ cell, or is fixed after fertilisation, that causes embryonic or foetal death.

Fertility rate: the number of mated pregnant female over the number of mated females.

Mating interval: the time between the end of exposure and mating of treated males. By controlling this interval, chemical effects on different germ cell types can be assessed. In the mouse mating during the 1, 2, 3, 4, 5, 6, 7 and 8 week after the end of exposure measures effects in sperm, condensed spermatids, round spermatids, pachytene spermatocytes, early spermatocytes, differentiated spermatogonia, differentiating spermatogonia and stem cell spermatogonia.

Preimplantation loss: the difference between the number of implants and the number of corpora lutea. It can also be estimated by comparing the total implants per female in treated and control groups.

Postimplantation loss: the ratio of dead implant in the treated group compared to the ratio of dead to total implants in the control group.

Test chemical: Any substance or mixture tested using this test method.

UVCB: Chemical Substance of Unknown or Variable Composition, Complex Reaction Products and Biological Materials

Appendix 2

TIMING OF SPERMATOGENESIS IN MAMMALS

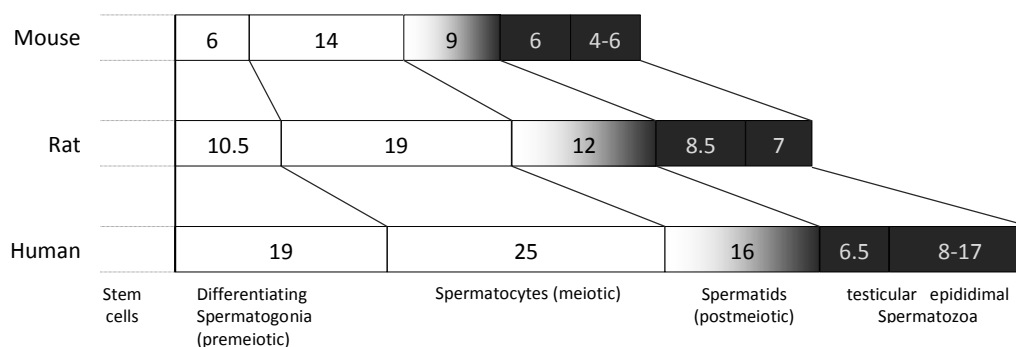


Fig.1: Comparison of the duration (days) of male germ cell development in mice, rats and humans. DNA repair does not occur during the periods indicated by shading.

A schematic of spermatogenesis in the mouse, rat and human is shown above (taken from Adler, 1996). Undifferentiated spermatogonia include: A-single; A-paired; and A-aligned spermatogonia (Hess and de Franca, 2008). A-single is considered the true stem cells; therefore, to assess effects on stem cells at least 49 days (in the mouse) must pass between the last injection of the test chemical and mating.

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(4) In Part B, Chapter B.23 is replaced by the following:

"B.23 MAMMALIAN SPERMATOGONIAL CHROMOSOMAL ABERRATION TEST

INTRODUCTION

1. This test method (TM) is equivalent to the OECD test guideline 483 (2016). Test methods are periodically reviewed in the light of scientific progress, changing regulatory needs, and animal welfare considerations. This modified version of the test method reflects many years of experience with this assay and the potential for integrating or combining this test with other toxicity or genotoxicity studies. Combining toxicity studies has the potential to reduce the numbers of animals used in toxicity testing. This test method is part of a series of test methods on genetic toxicology. A document that provides succinct information on genetic toxicology testing and an overview of the recent changes that were made to genetic toxicity OECD test guidelines has been developed by OECD (1).
2. The purpose of the *in vivo* mammalian spermatogonial chromosomal aberration test is to identify those chemicals that cause structural chromosomal aberrations in mammalian spermatogonial cells (2) (3) (4). In addition, this test is relevant to assessing genotoxicity because, although they may vary among species, factors of *in vivo* metabolism, pharmacokinetics and DNA-repair processes are active and contribute to the response. This test method is not designed to measure numerical abnormalities; the assay is not routinely used for this purpose.
3. This test measures structural chromosomal aberrations (both chromosome- and chromatid-type) in dividing spermatogonial germ cells and is, therefore, expected to be predictive of induction of heritable mutations in these germ cells.
4. Definitions of key terms are set out in the Appendix.

INITIAL CONSIDERATIONS

5. Rodents are routinely used in this test but other species may in some cases be appropriate if scientifically justified. Standard cytogenetic preparations of rodent testes generate mitotic (spermatogonia) and meiotic (spermatocyte) metaphases. Mitotic and meiotic metaphases are identified based on the morphology of the chromosomes (4). This *in vivo* cytogenetic test detects structural chromosomal aberrations in spermatogonial mitoses. Other target cells are not the subject of this test method.
6. To detect chromatid-type aberrations in spermatogonial cells, the first mitotic cell division following treatment should be examined before these aberrations are converted into chromosome-type-aberrations in subsequent cell divisions. Additional information from treated

spermatocytes can be obtained by meiotic chromosome analysis for chromosomal structural aberrations at diakinesis-metaphase I and metaphase II.

7. A number of generations of spermatogonia are present in the testis (5), and these different germ cell types may have a spectrum of sensitivity to chemical treatment. Thus, the aberrations detected represent an aggregate response of treated spermatogonial cell populations. The majority of mitotic cells in testis preparations are B spermatogonia, which have a cell cycle of approximately 26 hr (3).
8. If there is evidence that the test chemical, or its metabolite(s), will not reach the testis it is not appropriate to use this test.

PRINCIPLE OF THE TEST METHOD

9. Generally, animals are exposed to the test chemical by an appropriate route of exposure and are euthanised at appropriate times after treatment. Prior to euthanasia, animals are treated with a metaphase-arresting agent (e.g. colchicine or Colcemid®). Chromosome preparations are then made from germ cells and stained, and metaphase cells are analysed for chromosome aberrations.

VERIFICATION OF LABORATORY PROFICIENCY

10. Competency in this assay should be established by demonstrating the ability to reproduce expected results for structural chromosomal aberration frequencies in spermatogonia with positive control substances (including weak responses) such as those listed in Table 1 and obtaining negative control frequencies that are consistent with acceptable range of control data in the published literature (e.g. (2)(3)(6)(7)(8)(9)(10)) or with the laboratory's historical control distribution, if available.

DESCRIPTION OF THE METHOD

Preparations

Selection of animal species

11. Commonly used laboratory strains of healthy young adult animals should be employed. Male mice are commonly used; however, males of other appropriate mammalian species may be used when scientifically justified and to allow this test to be run in conjunction with another test method. The scientific justification for using species other than rodents should be provided in the report.

Animal Housing and feeding conditions

12. For rodents, the temperature in the animal room should be 22°C (±3°C). Although the relative humidity ideally should be 50-60%, it should be at least 40% and preferably not exceed 70%

other than during room cleaning. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. The choice of diet may be influenced by the need to ensure a suitable admixture of a test chemical when administered by this route. Rodents should be housed in small groups (no more than five per cage) if no aggressive behaviour is expected, preferably in solid floor cages with appropriate environmental enrichment. Animals may be housed individually if scientifically justified.

Preparation of the animals

13. Healthy young adult male animals (8-12 weeks old at start of treatment) are normally used, and are randomly assigned to the control and treatment groups. The individual animals are identified uniquely using a humane, minimally invasive method (e.g. by ringing, tagging, micro-chipping or biometric identification, but not ear or toe clipping) and acclimated to the laboratory conditions for at least five days. Cages should be arranged in such a way that possible effects due to cage placement are minimised. Cross contamination by the positive control and test chemical should be avoided. At the commencement of the study, the variation between individual animal weights should be minimal and not exceed $\pm 20\%$.

Preparation of doses

14. Solid test chemicals should be dissolved or suspended in appropriate solvents or vehicles or admixed in diet or drinking water prior to dosing of the animals. Liquid test chemicals may be dosed directly or diluted prior to dosing. For inhalation exposures, test chemicals can be administered as gas, vapour, or a solid/liquid aerosol, depending on their physicochemical properties. Fresh preparations of the test chemical should be employed unless stability data demonstrate the acceptability of storage and define the appropriate storage conditions.

Test conditions - Solvent/vehicle

15. The solvent/vehicle should not produce toxic effects at the dose levels used, and should not be capable of chemical reaction with the test chemicals. If other than well-known solvents/vehicles are used, their inclusion should be supported with reference data indicating their compatibility. It is recommended that, wherever possible, the use of an aqueous solvent/vehicle should be considered first. Examples of commonly used compatible solvents/vehicles include water, physiological saline, methylcellulose solution, carboxymethyl cellulose sodium salt solution, olive oil and corn oil. In the absence of historical or published control data showing that no structural chromosomal aberrations and other deleterious effects are induced by a chosen atypical solvent/vehicle, an initial study should be conducted in order to establish the acceptability of the solvent/vehicle control.

Positive controls

16. Concurrent positive control animals should always be used unless the laboratory has demonstrated proficiency in the conduct of the test and has used the test routinely in the recent past (e.g. within the last 5 years). When a concurrent positive control group is not included,

scoring controls (fixed and unstained slides) should be included in each experiment. These can be obtained by including within the scoring of the study appropriate reference samples that have been obtained and stored from a separate positive control experiment conducted periodically (e.g. every 6-18 months) in the laboratory where the test is performed; for example, during proficiency testing and on a regular basis thereafter, where necessary.

17. Positive control substances should reliably produce a detectable increase in the frequencies of cells with structural chromosomal aberrations over the spontaneous levels. Positive control doses should be chosen so that the effects are clear but do not immediately reveal the identity of the coded samples to the scorer. Examples of positive control substances are included in Table 1.

Table 1: Examples of positive control substances.

Substances [CAS No] (reference no)
Cyclophosphamide (monohydrate) [CAS no. 50-18-0 (CAS no. 6055-19-2)] (9)
Cyclohexylamine [CAS no. 108-91-8] (7)
Mitomycin C [CAS no. 50-07-7] (6)
Monomeric acrylamide [CAS 79-06-1] (10)
Triethylenemelamine [CAS 51-18-3] (8)

Negative controls

18. Negative control animals, treated with solvent or vehicle alone, and otherwise treated in the same way as the treatment groups, should be included for every sampling time. In the absence of historical or published control data showing that no chromosomal aberrations or other deleterious effects are induced by the chosen solvent/vehicle, untreated control animals also should be included for every sampling time in order to establish acceptability of the vehicle control.

PROCEDURE

Number of animals

19. Group sizes at study initiation should be established with the aim of providing a minimum of 5 male animals per group. This number of animals per group is considered to be sufficient to provide adequate statistical power (i.e. generally able to detect at least a doubling in chromosomal aberration frequency when the negative control level is 1.0% or greater with 80% probability at a significance level of 0.05) (3) (11). As a guide to typical maximum animal requirements, a study at two sampling times with three dose groups and a concurrent negative control group, plus a positive control group (each composed of five animals per group), would require 45 animals.

Treatment schedule

20. Test chemicals are usually administered once (i.e. as a single treatment); other dose regimens may be used, provided they are scientifically justified.
21. In the highest dose group two sampling times after treatment are used. Since the time required for uptake and metabolism of the test chemical(s), as well as its effect on cell cycle kinetics, can affect the optimum time for chromosomal aberration detection, one early and one late sampling time approximately 24 and 48 hours after treatment are used. For doses other than the highest dose, an early sampling time of 24 hours (less than or equal to the cell cycle time of B spermatogonia and thus optimising the probability of scoring first post-treatment metaphases) after treatment should be taken, unless another sampling time is known to be more appropriate and justified.
22. Other sampling times may be used. For example in the case of chemicals that exert S-independent effects, earlier sampling times (i.e. less than 24 hr) may be appropriate.
23. A repeat dose treatment regimen can be used, such as in conjunction with a test on another endpoint that uses a 28 day administration period (e.g., TM B.58); however, additional animal groups would be required to accommodate different sampling times. Accordingly, the appropriateness of such a schedule needs to be justified scientifically on a case-by-case basis.
24. Prior to euthanasia, animals are injected intraperitoneally with an appropriate dose of a metaphase arresting chemical (e.g. Colcemid® or colchicine). Animals are sampled at an appropriate interval thereafter. For mice and rats, this interval is approximately 3 - 5 hours.

Dose levels

25. If a preliminary range-finding study is performed because there are no suitable data already available to aid in dose selection, it should be performed in the same laboratory, using the same species, strain, and treatment regimen to be used in the main study, according to recommendations for conducting dose range-finding studies (12). This study should aim to identify the maximum tolerated dose (MTD), defined as the dose inducing slight toxic effects relative to the duration of the study period (for example, abnormal behaviour or reactions, minor body weight depression or hematopoietic system cytotoxicity) but not death or evidence of pain, suffering or distress necessitating euthanasia of the animals (13).
26. The highest dose may also be defined as a dose that produces some indication of toxicity in the spermatogonial cells (e.g. a reduction in the ratio of spermatogonial mitoses to first and second meiotic metaphases). This reduction should not exceed 50%.
27. Test chemicals with specific biological activities at low non-toxic doses (such as hormones and mitogens), and chemicals which exhibit saturation of toxicokinetic properties may be exceptions to the dose-setting criteria and should be evaluated on a case-by-case basis.

28. In order to obtain dose response information, a complete study should include a negative control group (paragraph 18) and a minimum of three dose levels generally separated by a factor of 2, but by no greater than 4. If the test chemical does not produce toxicity in a range-finding study or based on existing data, the highest dose for a single administration should be 2000 mg/kg body weight. However, if the test chemical does cause toxicity, the MTD should be the highest dose administered, and the dose levels used should preferably cover a range from the maximum to a dose producing little or no toxicity. When target tissue (i.e. testis) toxicity is observed at all dose levels tested, further study at non-toxic doses is advisable. Studies intending to more fully characterise the quantitative dose-response information may require additional dose groups. For certain types of test chemicals (e.g. human pharmaceuticals) covered by specific requirements, these limits may vary. If the test chemical does produce toxicity, the limit dose plus two lower doses (as described above) should be selected. The limit dose for an administration period of 14 days or more is 1000 mg/kg body weight/day, and for administration periods of less than 14 days, the limit dose is 2000 mg/kg/body weight/day.

Administration of doses

29. The anticipated route of human exposure should be considered when designing an assay. Therefore, routes of exposure such as dietary, drinking water, topical subcutaneous, intravenous, oral (by gavage), inhalation, or implantation may be chosen as justified. In any case, the route should be chosen to ensure adequate exposure of the target tissue. Intraperitoneal injection is not normally recommended unless scientifically justified since it is not usually a physiologically relevant route of human exposure. If the test chemical is admixed in diet or drinking water, especially in case of single dosing, care should be taken that the delay between food and water consumption and sampling should be sufficient to allow detection of the effects (see paragraph 33). The maximum volume of liquid that can be administered by gavage or injection at one time depends on the size of the test animal. The volume should not normally exceed 1 ml/100g body weight except in the case of aqueous solutions where a maximum of 2 ml/100g body weight may be used. The use of volumes greater than this (if permitted by animal welfare legislation) should be justified. Variability in test volume should be minimised by adjusting the concentration to ensure a constant volume in relation to body weight at all dose levels.

Observations

30. General clinical observations of the test animals should be made and clinical signs recorded at least once a day, preferably at the same time(s) each day and considering the peak period of anticipated effects after dosing. At least twice daily, all animals should be observed for morbidity and mortality. All animals should be weighed at study initiation, at least once a week during repeated-dose studies, and at euthanasia. In studies of at least one-week duration, measurements of food consumption should be made at least weekly. If the test chemical is administered via the drinking water, water consumption should be measured at each change of

water and at least weekly. Animals exhibiting non-lethal indicators of excess toxicity should be euthanised prior to completion of the test period (13).

Chromosome preparation

31. Immediately after euthanasia, germ cell suspensions are obtained from one, or both, testes, exposed to hypotonic solution and fixed following established protocols (e.g. (2) (14) (15). The cells are then spread on slides and stained (16) (17). All slides should be coded so that their identity is not available to the scorer.

Analysis

32. At least 200 well spread metaphases should be scored for each animal (3) (11). If the historical negative control frequency is $< 1\%$, more than 200 cells/animal should be scored to increase the statistical power (3). Staining methods that permit the identification of the centromere should be used.
33. Chromosome and chromatid-type aberrations should be recorded separately and classified by sub-types (breaks, exchanges). Gaps should be recorded, but not considered, when determining whether a chemical induces significant increases in the incidence of cells with chromosomal aberrations. Procedures in use in the laboratory should ensure that analysis of chromosomal aberrations is performed by well-trained scorers. Recognising that slide preparation procedures often result in the breakage of a proportion of metaphases with a resulting loss of chromosomes, the cells scored should, therefore, contain a number of centromeres not less than $2n \pm 2$, where n is the haploid number of chromosomes for that species.
34. Although the purpose of the test is to detect structural chromosomal aberrations, it is important to record the frequencies of polyploid cells and cells with endoreduplicated chromosomes when these events are seen (see Paragraph 44).

DATA AND REPORTING

Treatment of results

35. Individual animal data should be presented in tabular form. For each animal the number of cells with structural chromosomal aberration(s) and the number of chromosome aberrations per cell should be evaluated. Chromatid- and chromosome-type aberrations classified by sub-types (breaks, exchanges) should be listed separately with their numbers and frequencies for experimental and control groups. Gaps are recorded separately. The frequency of gaps is reported but generally not included in the analysis of the total structural chromosomal aberration frequency. Percentage of polyploidy and cells with endoreduplicated chromosomes are reported when seen.
36. Data on toxicity and clinical signs (as per Paragraph 30) should be reported.

Acceptability Criteria

37. The following criteria determine the acceptability of a test.

- Concurrent negative control is consistent with published norms for historical negative control data, which are generally expected to be $> 0\%$ and $\leq 1.5\%$ cells with chromosomal aberrations, and the laboratory's historical control data if available (see Paragraphs 10 and 18).
- Concurrent positive controls induce responses that are consistent with published norms for historical positive control data, or the laboratory's historical positive control database, if available, and produce a statistically significant increase compared with the negative control (see Paragraphs 17, 18).
- Adequate numbers of cells and doses have been analysed (see Paragraphs 28 and 32).
- The criteria for the selection of top dose are consistent with those described in Paragraphs 25, and 26.

38. If both mitosis and meiosis are observed, the ratio of spermatogonial mitoses to first and second meiotic metaphases should be determined as a measure of cytotoxicity for all treated and negative control animals in a total sample of 100 dividing cells per animal. If only mitosis is observed, the mitotic index should be determined in at least 1000 cells for each animal.

Evaluation and interpretation of results

39. At least three treated dose groups should be analysed in order to provide sufficient data for dose-response analysis.

40. Providing that all acceptability criteria are fulfilled, a test chemical is considered a clear positive if:

- at least one of the test doses exhibits a statistically significant increase compared with the concurrent negative control;
- the increase is dose-related at least at one sampling time; and,
- any of the results are outside acceptable range of negative control data, or the distribution of the laboratory's historical negative control data (e.g. Poisson-based 95% control limit) if available.

The test chemical is then considered able to induce chromosomal aberrations in spermatogonial cells of the test animals. Recommendations for the most appropriate statistical methods can also be found in the literature (11) (18). Statistical tests used should consider the animal as the experimental unit.

41. Providing that all acceptability criteria are fulfilled, a test chemical is considered a clear negative if:

- none of the test doses exhibits a statistically significant increase compared with the concurrent negative control;

- there is no dose-related increase in any experimental condition; and,
- all results are within acceptable range of negative control data, or the laboratory's historical negative control data (e.g. Poisson-based 95% control limit), if available.

The test chemical is then considered unable to induce chromosomal aberrations in the spermatogonial cells of the test animals. Recommendations for the most appropriate statistical methods can also be found in the literature (11) (18). A negative result does not exclude the possibility that the chemical may induce chromosomal aberrations at later developmental phases not studied, or gene mutations.

42. There is no requirement for verification of a clear positive or clear negative response.
43. If the response is not clearly negative or positive, and in order to assist in establishing the biological relevance of a result (e.g. a weak or borderline increase), the data should be evaluated by expert judgment and/or further investigations using the existing experimental data, such as consideration whether the positive result is outside the acceptable range of negative control data, or the laboratory's historical negative control data (19).
44. In rare cases, even after further investigations, the data set will preclude making a conclusion of positive or negative results, and will therefore be concluded as equivocal.
45. An increase in the number of polyploid cells may indicate that the test chemical has the potential to inhibit mitotic processes and to induce numerical chromosomal aberrations (20). An increase in the number of cells with endoreduplicated chromosomes may indicate that the test chemical has the potential to inhibit cell cycle progress (21) (22), which is a different mechanism of inducing numerical chromosome changes than inhibition of mitotic processes (see Paragraph 2). Therefore incidence of polyploid cells and cells with endoreduplicated chromosomes should be recorded separately.

Test report

46. The test report should include the following information:

Summary.

Test chemical:

- source, lot number, limit date for use, if available;
- stability of the test chemical itself, if known;
- solubility and stability of the test chemical in solvent, if known;
- measurement of pH, osmolality, and precipitate in the culture medium to which the test chemical was added, as appropriate.

Mono-constituents substance:

- physical appearance, water solubility, and additional relevant physicochemical properties;
- chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc.

Multi-constituent substance, UVCBs and mixtures:

- characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents.

Test chemical preparation:

- justification for choice of vehicle;
- solubility and stability of the test chemical in solvent/vehicle.
- preparation of dietary, drinking water or inhalation formulations;
- analytical determinations on formulations (e.g. stability, homogeneity, nominal concentrations when conducted).

Test animals:

- species/strain used and justification for use;
- number and age of animals;
- source, housing conditions, diet, etc.;
- method for uniquely identifying the animals
- for short-term studies: individual weight of the animals at the start and end of the test; for studies longer than one week: individual body weights during the study and food consumption. Body weight range, mean and standard deviation for each group should be included.

Test conditions:

- positive and negative (vehicle/solvent) control data;
- data from range finding study, if conducted;
- rationale for dose level selection;
- rationale for route of administration;
- details of test chemical preparation;
- details of the administration of the test chemical;
- rationale for sacrifice times;

- methods for measurement of animal toxicity, including, where available, histopathological or hematological analyses and the frequency with which animal observations and body weights were taken;
- methods for verifying that the test chemical reached the target tissue, or general circulation, if negative results are obtained;
- actual dose (mg/kg body weight/day) calculated from diet/drinking water test chemical concentration (ppm) and consumption, if applicable;
- details of food and water quality;
- detailed description of treatment and sampling schedules and justifications for the choices;
- method of euthanasia;
- method of analgesia (where used)
- procedures for isolating tissues;
- identity of metaphase arresting chemical, its concentration and duration of treatment;
- methods of slide preparation;
- criteria for scoring aberrations;
- number of cells analysed per animal;
- criteria for considering studies as positive, negative or equivocal.

Results:

- animal condition prior to and throughout the test period, including signs of toxicity;
- body and organ weights at sacrifice (if multiple treatments are employed, body weights taken during the treatment regimen);
- signs of toxicity;
- mitotic index;
- ratio of spermatogonial mitoses cells to first and second meiotic metaphases, or other evidence of exposure to the target tissue;
- type and number of aberrations, given separately for each animal;
- total number of aberrations per group with means and standard deviations;
- number of cells with aberrations per group with means and standard deviations;
- dose-response relationship, where possible;
- statistical analyses and methods applied;
- concurrent negative control data;

- historical negative control data with ranges, means, standard deviations, and 95% confidence interval (where available), or published historical negative control data used for acceptability of the test results;
- concurrent positive control data;
- changes in ploidy, if seen, including frequencies of polyploidy and/or endoreduplicated cells.

Discussion of the results

Conclusion

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Appendix

DEFINITIONS

Aneuploidy: any deviation from the normal diploid (or haploid) number of chromosomes by a single chromosome or more than one, but not by entire set(s) of chromosomes (polyploidy).

Centromere: Region(s) of a chromosome with which spindle fibers are associated during cell division, allowing orderly movement of daughter chromosomes to the poles of the daughter cells.

Chemical: A substance or a mixture

Chromosome diversity: diversity of chromosome shapes (e.g. metacentrique, acrocentriques, etc) and sizes.

Chromatid-type aberration: structural chromosome damage expressed as breakage of single chromatids or breakage and reunion between chromatids.

Chromosome-type aberration: structural chromosome damage expressed as breakage, or breakage and reunion, of both chromatids at an identical site.

Clastogen: any chemical which causes structural chromosomal aberrations in populations of cells or organisms.

Gap: an achromatic lesion smaller than the width of one chromatid, and with minimum misalignment of the chromatids.

Genotoxic: a general term encompassing all types of DNA or chromosome damage, including breaks, deletions, adducts, nucleotides modifications and linkages, rearrangements, mutations, chromosome aberrations, and aneuploidy. Not all types of genotoxic effects result in mutations or stable chromosome damage.

Mitotic index (MI): the ratio of cells in metaphase divided by the total number of cells observed in a population of cells; an indication of the degree of proliferation of that population.

Mitosis: division of the cell nucleus usually divided into prophase, prometaphase, metaphase, anaphase, and telophase.

Mutagenic: produces a heritable change of DNA base-pair sequence(s) in genes or of the structure of chromosomes (chromosome aberrations).

Numerical abnormality: a change in the number of chromosomes from the normal number characteristic of the animals utilised.

Polyploidy: a multiple of the haploid chromosome number (n) other than the diploid number (i.e., $3n$, $4n$ and so on).

Structural aberration: a change in chromosome structure detectable by microscopic examination of the metaphase stage of cell division, observed as deletions and fragments, exchanges.

Test chemical: Any substance or mixture tested using this test method.

UVCB: Chemical Substances of Unknown or Variable Composition, Complex Reaction Products and Biological Materials"

(5) In Part B, Chapter B.40 is replaced by the following:

"B.40 *IN VITRO* SKIN CORROSION: TRANSCUTANEOUS ELECTRICAL RESISTANCE TEST METHOD (TER)

INTRODUCTION

1. This test method (TM) is equivalent to OECD test guideline (TG) 430 (2015). Skin corrosion refers to the production of irreversible damage to the skin manifested as visible necrosis through the epidermis and into the dermis, following the application of a test chemical [as defined by the United Nations (UN) Globally Harmonized System of Classification and Labelling of Chemicals (GHS) (1) and the European Union (EU) Regulation 1272/2008 on Classification, Labelling and Packaging of Substances and Mixtures (CLP)¹]. This updated test method B.40 provides an *in vitro* procedure allowing the identification of non-corrosive and corrosive substances and mixtures in accordance with UN GHS (1) and CLP
2. The assessment of skin corrosivity has typically involved the use of laboratory animals (TM B.4, equivalent to OECD TG 404 originally adopted in 1981, and revised in 1992, 2002 and 2015) (2). In addition to the present TM B.40, other *in vitro* test methods for testing of skin corrosion potential of chemicals have been validated and adopted as TM B.40bis (equivalent to OECD TG 431) (3) and TM B.65 (equivalent to OECD TG 435) (4), that are also able to identify sub-categories of corrosive chemicals when required. Several validated *in vitro* test methods have been adopted as TM B.46 (equivalent to OECD TG 439) (5), to be used for the testing of skin irritation. An OECD guidance document on Integrated Approaches to Testing and Assessment (IATA) for Skin Corrosion and Irritation describes several modules which group various information sources and analysis tools and provides guidance on (i) how to integrate and use existing testing and non-testing data for the assessment of skin irritation and skin corrosion potentials of chemicals and (ii) proposes an approach when further testing is needed (6).
3. This test method addresses the human health endpoint skin corrosion. It is based on the rat skin transcutaneous electrical resistance (TER) test method, which utilises skin discs to identify corrosives by their ability to produce a loss of normal *stratum corneum* integrity and barrier function. The corresponding OECD test guideline was originally adopted in 2004 and updated in 2015 to refer to the IATA guidance document.

¹ Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006, OJ L 353/1, 31.12.2008

4. In order to evaluate *in vitro* skin corrosion testing for regulatory purposes, pre-validation studies (7) followed by a formal validation study of the rat skin TER test method for assessing skin corrosion were conducted (8) (9) (10) (11). The outcome of these studies led to the recommendation that the TER test method (designated the Validated Reference Method – VRM) could be used for regulatory purposes for the assessment of *in vivo* skin corrosivity (12) (13) (14).
5. Before a proposed similar or modified *in vitro* TER test method for skin corrosion other than the VRM can be used for regulatory purposes, its reliability, relevance (accuracy), and limitations for its proposed use should be determined to ensure its similarity to the VRM, in accordance with the requirements of the Performance Standards (PS) (15). OECD Mutual Acceptance of Data will only be guaranteed after any proposed new or updated test method following the PS have been reviewed and included in the corresponding OECD test guideline.

DEFINITIONS

6. Definitions used are provided in the Appendix.

INITIAL CONSIDERATIONS

7. A validation study (10) and other published studies (16) (17) have reported that the rat skin TER test method is able to discriminate between known skin corrosives and non-corrosives with an overall sensitivity of 94% (51/54) and specificity of 71% (48/68) for a database of 122 substances.
8. This test method addresses *in vitro* skin corrosion. It allows the identification of non-corrosive and corrosive test chemicals in accordance with the UN GHS/CLP. A limitation of this test method, as demonstrated by the validation studies (8) (9) (10) (11), is that it does not allow the sub-categorisation of corrosive substances and mixtures in accordance with the UN GHS/ CLP. The applicable regulatory framework will determine how this test method will be used. While this test method does not provide adequate information on skin irritation, it should be noted that TM B.46 specifically addresses the health effect skin irritation *in vitro* (5). For a full evaluation of local skin effects after a single dermal exposure, the OECD Guidance Document on IATA should be consulted (6).
9. A wide range of chemicals representing mainly substances has been tested in the validation underlying this test method and the empirical database of the validation study amounted to 60 substances covering a wide range of chemical classes (8) (9). On the basis of the overall data available, the test method is applicable to a wide range of chemical classes and physical states including liquids, semi-solids, solids and waxes. However, since for specific physical states test items with suitable reference data are not readily available, it should be noted that a comparably small number of waxes and corrosive solids were assessed during validation. The liquids may be aqueous or non-aqueous; solids may be soluble or insoluble in water. In cases where

evidence can be demonstrated on the non-applicability of the test method to a specific category of substances, the test method should not be used for that specific category of substances. In addition, this test method is assumed to be applicable to mixtures as an extension of its applicability to substances. However, due to the fact that mixtures cover a wide spectrum of categories and composition, and that only limited information is currently available on the testing of mixtures, in cases where evidence can be demonstrated on the non-applicability of the test method to a specific category of mixtures (*e.g.* following a strategy as proposed by Eskes *et al.*, 2012) (18), the test method should not be used for that specific category of mixtures. Before use of the test method on a mixture for generating data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed, when there is a regulatory requirement for testing of the mixture. Gases and aerosols have not been assessed yet in validation studies (8) (9). While it is conceivable that these can be tested using the TER test method, the current test method does not allow testing of gases and aerosols.

PRINCIPLE OF THE TEST

10. The test chemical is applied for up to 24 hours to the epidermal surfaces of skin discs in a two-compartment test system in which the skin discs function as the separation between the compartments. The skin discs are taken from humanely killed rats aged 28-30 days. Corrosive chemicals are identified by their ability to produce a loss of normal *stratum corneum* integrity and barrier function, which is measured as a reduction in the TER below a threshold level (16) (see paragraph 32). For rat skin TER, a cut-off value of 5k Ω has been selected based on extensive data for a wide range of substances where the vast majority of values were either clearly well above (often > 10 k Ω), or well below (often < 3 k Ω) this value (16). Generally, test chemicals that are non-corrosive in animals but are irritant or non-irritant do not reduce the TER below this cut-off value. Furthermore, use of other skin preparations or other equipment may alter the cut-off value, necessitating further validation.
11. A dye-binding step is incorporated into the test procedure for confirmation testing of positive results in the TER including values around 5 k Ω . The dye-binding step determines if the increase in ionic permeability is due to physical destruction of the *stratum corneum*. The TER method utilising rat skin has shown to be predictive of *in vivo* corrosivity in the rabbit assessed under TM B.4 (2).

DEMONSTRATION OF PROFICIENCY

12. Prior to routine use of the rat skin TER test method that adheres to this test method, laboratories should demonstrate technical proficiency by correctly classifying the twelve Proficiency Substances recommended in Table 1. In situations where a listed substance is unavailable or where justifiable, another substance for which adequate *in vivo* and *in vitro* reference data are

available may be used (e.g. from the list of reference chemicals (16)) provided that the same selection criteria as described in Table 1 is applied.

Table 1: List of Proficiency Substances¹

Substance	CASRN	Chemical Class ²	UN GHS/CLP Cat. Based on <i>In Vivo</i> Results ³	VRM Cat. Based on <i>In Vitro</i> Results	Physical State	pH ⁴
<i>In Vivo</i> Corrosives						
N,N'-Dimethyl dipropylenetriamine	10563-29-8	organic base	1A	6 x C	L	8.3
1,2-Diaminopropane	78-90-0	organic base	1A	6 x C	L	8.3
Sulfuric acid (10%)	7664-93-9	inorganic acid	(1A)/1B/1C	5 x C 1x NC	L	1.2
Potassium hydroxide (10% aq.)	1310-58-3	inorganic base	(1A)/1B/1C	6 x C	L	13.2
Octanoic (Caprylic) acid	124-07-2	organic acid	1B/1C	4 x C 2 x NC	L	3.6
2-tert-Butylphenol	88-18-6	phenol	1B/1C	4 x C 2 x NC	L	3.9
<i>In Vivo</i> Non-corrosives						
Isostearic acid	2724-58-5	organic acid	NC	6 x NC	L	3.6
4-Amino-1,2,4-triazole	584-13-4	organic base	NC	6 x NC	S	5.5
Phenethyl bromide	103-63-9	electrophile	NC	6 x NC	L	3.6
4-(Methylthio)-benzaldehyde	3446-89-7	electrophile	NC	6 x NC	L	6.8
1,9-Decadiene	1647-16-1	neutral organic	NC	6 x NC	L	3.9
Tetrachloroethylene	127-18-4	neutral organic	NC	6 x NC	L	4.5

Abbreviations: aq = aqueous; CASRN = Chemical Abstracts Service Registry Number; VRM = Validated Reference Method; C = corrosive; NC = not corrosive.

¹The proficiency substances, sorted first by corrosives versus non-corrosives, then by corrosive subcategory and then by chemical class, were selected from the substances used in the ECVAM validation study of the rat skin TER test method (8) (9). Unless otherwise indicated, the substances were tested at the purity level obtained when purchased from a commercial source (8). The selection included, to the extent possible, substances that: (i) are representative of the range of corrosivity responses (e.g. non-corrosives; weak to strong corrosives) that the VRM is capable of measuring or predicting; (ii) are representative of the chemical classes used in the validation study; (iii) reflect the performance characteristics of the VRM; (iv) have chemical structures that are well-defined; (v) induce definitive results in the *in vivo* reference test method; (vi) are commercially available; and (vii) are not associated with prohibitive disposal costs.

²Chemical class assigned by Barratt *et al.* (8).

³The corresponding UN Packing groups are I, II and III, respectively, for the UN GHS/CLP categories 1A, 1B and 1C.

⁴The pH values were obtained from Fentem *et al.* (9) and Barratt *et al.* (8).

PROCEDURE

13. Standard Operating Procedures (SOP) for the rat skin TER skin corrosion test method are available (19). The rat skin TER test methods covered by this test method should comply with the following conditions:

Animals

14. Rats should be used because the sensitivity of their skin to substances in this test method has been previously demonstrated (12) and is the only skin source that has been formally validated (8) (9). The age (when the skin is collected) and strain of the rat is particularly important to ensure that the hair follicles are in the dormant phase before adult hair growth begins.
15. The dorsal and flank hair from young, approximately 22 day-old, male or female rats (Wistar-derived or a comparable strain), is carefully removed with small clippers. Then, the animals are washed by careful wiping, whilst submerging the clipped area in antibiotic solution (containing, for example, streptomycin, penicillin, chloramphenicol, and amphotericin, at concentrations effective in inhibiting bacterial growth). Animals are washed with antibiotics again on the third or fourth day after the first wash and are used within 3 days of the second wash, when the *stratum corneum* has recovered from the hair removal.

Preparation of the skin discs

16. Animals are humanely killed when 28-30 days old; this age is critical. The dorso-lateral skin of each animal is then removed and stripped of excess subcutaneous fat by carefully peeling it away from the skin. Skin discs, with a diameter of approximately 20-mm each, are removed. The skin may be stored before discs are used where it is shown that positive and negative control data are equivalent to that obtained with fresh skin.
17. Each skin disc is placed over one of the ends of a PTFE (polytetrafluoroethylene) tube, ensuring that the epidermal surface is in contact with the tube. A rubber 'O' ring is press-fitted over the end of the tube to hold the skin in place and excess tissue is trimmed away. The rubber 'O' ring is then carefully sealed to the end of the PTFE tube with petroleum jelly. The tube is supported by a spring clip inside a receptor chamber containing MgSO₄ solution (154 mM) (Figure 1). The skin disc should be fully submerged in the MgSO₄ solution. As many as 10-15 skin discs can be obtained from a single rat skin. Tube and 'O' ring dimensions are shown in Figure 2.
18. Before testing begins, the TER of two skin discs are measured as a quality control procedure for each animal skin. Both discs should give electrical resistance values greater than 10 kΩ for the remainder of the discs to be used for the test method. If the resistance value is less than 10 kΩ, the remaining discs from that skin should be discarded.

Application of the test chemical and control substances

19. Concurrent positive and negative controls should be used for each run (experiment) to ensure adequate performance of the experimental model. Skin discs from a single animal should be used in each run (experiment). The suggested positive and negative control test chemicals are 10M hydrochloric acid and distilled water, respectively.
20. Liquid test chemicals (150 μ l) are applied uniformly to the epidermal surface inside the tube. When testing solid materials, a sufficient amount of the solid is applied evenly to the disc to ensure that the whole surface of the epidermis is covered. Deionised water (150 μ l) is added on top of the solid and the tube is gently agitated. In order to achieve maximum contact with the skin, solids may need to be warmed to 30⁰ C to melt or soften the test chemical, or ground to produce a granular material or powder.
21. Three skin discs are used for each test and control chemical in each testing run (experiment). Test chemicals are applied for 24 hours at 20-23⁰ C. The test chemical is removed by washing with a jet of tap water at up to room temperature until no further material can be removed.

TER measurements

22. The skin impedance is measured as TER by using a low-voltage, alternating current Wheatstone bridge (18). General specifications of the bridge are 1-3 Volt operating voltage, a sinus or rectangular shaped alternating current of 50 - 1000 Hz, and a measuring range of at least 0.1 -30 k Ω . The databridge used in the validation study measured inductance, capacitance and resistance up to values of 2000H, 2000 μ F, and 2 M Ω , respectively at frequencies of 100Hz or 1kHz, using series or parallel values. For the purposes of the TER corrosivity assay measurements are recorded in resistance, at a frequency of 100 Hz and using series values. Prior to measuring the electrical resistance, the surface tension of the skin is reduced by adding a sufficient volume of 70% ethanol to cover the epidermis. After a few seconds, the ethanol is removed from the tube and the tissue is then hydrated by the addition of 3 ml MgSO₄ solution (154mM). The databridge electrodes are placed on either side of the skin disc to measure the resistance in k Ω /skin disc (Figure 1). Electrode dimensions and the length of the electrode exposed below the crocodile clips are shown in Figure 2. The clip attached to the inner electrode is rested on the top of the PTFE tube during resistance measurement to ensure that a consistent length of electrode is submerged in the MgSO₄ solution. The outer electrode is positioned inside the receptor chamber so that it rests on the bottom of the chamber. The distance between the spring clip and the bottom of the PTFE tube is maintained as a constant (Figure 2), because this distance affects the resistance value obtained. Consequently, the distance between the inner electrode and the skin disc should be constant and minimal (1-2 mm).
23. If the measured resistance value is greater than 20 k Ω , this may be due to the remains of the test chemical coating the epidermal surface of the skin disc. Further removal of this coating can be attempted, for example, by sealing the PTFE tube with a gloved thumb and shaking it for

approximately 10 seconds; the MgSO_4 solution is discarded and the resistance measurement is repeated with fresh MgSO_4 .

24. The properties and dimensions of the test apparatus and the experimental procedure used may influence the TER values obtained. The 5 k Ω corrosive threshold was developed from data obtained with the specific apparatus and procedure described in this test method. Different threshold and control values may apply if the test conditions are altered or a different apparatus is used. Therefore, it is necessary to calibrate the methodology and resistance threshold values by testing a series of Proficiency Substances chosen from the substances used in the validation study (8) (9), or from similar chemical classes to the substances being investigated. A set of suitable Proficiency Substances is identified in Table 1.

Dye Binding Methods

25. Exposure of certain non-corrosive materials can result in a reduction of resistance below the cut-off of 5 k Ω allowing the passage of ions through the *stratum corneum*, thereby reducing the electrical resistance (9). For example, neutral organics and substances that have surface-active properties (including detergents, emulsifiers and other surfactants) can remove skin lipids making the barrier more permeable to ions. Thus, if TER values produced by such chemicals are less than or around 5 k Ω in the absence of visually perceptible damage of the skin discs, an assessment of dye penetration should be carried out on the control and treated tissues to determine if the TER values obtained were the result of increased skin permeability, or skin corrosion (7) (9). In case of the latter where the *stratum corneum* is disrupted, the dye sulforhodamine B, when applied to the skin surface rapidly penetrates and stains the underlying tissue. This particular dye is stable to a wide range of substances and is not affected by the extraction procedure described below.

Sulforhodamine B dye application and removal

26. Following TER assessment, the magnesium sulphate is discarded from the tube and the skin is carefully examined for obvious damage. If there is no obvious major damage (*e.g.* perforation), 150 μl of a 10% (w/v) dilution in distilled water of the dye sulforhodamine B (Acid Red 52; C.I. 45100; CAS number 3520-42-1), is applied to the epidermal surface of each skin disc for 2 hours. These skin discs are then washed with tap water at up to room temperature for approximately 10 seconds to remove any excess/unbound dye. Each skin disc is carefully removed from the PTFE tube and placed in a vial (*e.g.* a 20-ml glass scintillation vial) containing deionised water (8 ml). The vials are agitated gently for 5 minutes to remove any additional unbound dye. This rinsing procedure is then repeated, after which the skin discs are removed and placed into vials containing 5ml of 30% (w/v) sodium dodecyl sulphate (SDS) in distilled water and are incubated overnight at 60 $^{\circ}\text{C}$.
27. After incubation, each skin disc is removed and discarded and the remaining solution is centrifuged for 8 minutes at 21 $^{\circ}\text{C}$ (relative centrifugal force $\sim 175 \times g$). A 1ml sample of the

supernatant is diluted 1 in 5 (v/v) [*i.e.* 1ml + 4ml] with 30% (w/v) SDS in distilled water. The optical density (OD) of the solution is measured at 565 nm.

Calculation of dye content

28. The sulforhodamine B dye content per disc is calculated from the OD values (9) (sulforhodamine B dye molar extinction coefficient at 565nm = 8.7×10^4 ; molecular weight = 580). The dye content is determined for each skin disc by the use of an appropriate calibration curve and mean dye content is then calculated for the replicates.

Acceptability Criteria

29. The mean TER results are accepted if the concurrent positive and negative control values fall within the acceptable ranges for the method in the testing laboratory. The acceptable resistance ranges for the methodology and apparatus described above are given in the following table:

Control	Substance	Resistance range (k Ω)
Positive	10M Hydrochloric acid	0.5 - 1.0
Negative	Distilled water	10 - 25

30. The mean dye binding results are accepted on condition that concurrent control values fall within the acceptable ranges for the method. Suggested acceptable dye content ranges for the control substances for the methodology and apparatus described above are given in the following table:

Control	Substance	Dye content range (μ g/disc)
Positive	10M Hydrochloric acid	40 - 100
Negative	Distilled water	15 - 35

Interpretation of results

31. The cut-off TER value distinguishing corrosive from non-corrosive test chemicals was established during test method optimisation, tested during a pre-validation phase, and confirmed in a formal validation study.
32. The prediction model for rat skin TER skin corrosion test method (9) (19), associated with the UN GHS/CLP classification system, is given below:

The test chemical is considered to be non-corrosive to skin:

- i) if the mean TER value obtained for the test chemical is greater than ($>$) 5 k Ω , or
- ii) the mean TER value obtained for the test chemical is less than or equal to ($\leq$) 5 k Ω , and
 - the skin discs show no obvious damage(*e.g.* perforation), and
 - the mean disc dye content is less than ($<$) the mean disc dye content of the 10M HCl positive control obtained concurrently (see paragraph 30 for positive control values).

The test chemical is considered to be corrosive to skin:

- i) if the mean TER value obtained for the test chemical is less than or equal to ($\leq$) 5 k Ω and the skin discs are obviously damaged(e.g. perforated), or
 - ii) the mean TER value obtained for the test chemical is less than or equal to ($\leq$) 5 k Ω , and
 - the skin discs show no obvious damage(e.g. perforation), but
 - the mean disc dye content is greater than or equal to ($\geq$) the mean disc dye content of the 10M HCl positive control obtained concurrently (see paragraph 30 for positive control values).
33. A testing run (experiment) composed of at least three replicate skin discs should be sufficient for a test chemical when the classification is unequivocal. However, in cases of borderline results, such as non-concordant replicate measurements and/or mean TER equal to 5 ± 0.5 k Ω , a second independent testing run (experiment) should be considered, as well as a third one in case of discordant results between the first two testing runs (experiments).

DATA AND REPORTING

Data

34. Resistance values (k Ω) and dye content values (μ g/disc), where appropriate, for the test chemical, as well as for positive and negative controls should be reported in tabular form, including data for each individual replicate disc in each testing run (experiment) and mean values $\pm$ SD. All repeat experiments should be reported. Observed damage in the skin discs should be reported for each test chemical.

Test report

35. The test report should include the following information:

Test Chemical and Control Substances:

- Mono-constituent substance: chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc;
- Multi-constituent substance, UVCB and mixture: characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physico-chemical properties of the constituents;
- Physical appearance, water solubility, and additional relevant physico-chemical properties;
- Source, lot number if available;
- Treatment of the test chemical/control substance prior to testing, if applicable (e.g. warming, grinding);
- Stability of the test chemical, limit date for use, or date for re-analysis if known;
- Storage conditions.

Test Animals:

- Strain and sex used;
- Age of the animals when used as donor animals;
- Source, housing condition, diet, etc.;
- Details of the skin preparation.

Test Conditions:

- Calibration curves for test apparatus;
- Calibration curves for dye binding test performance, band pass used for measuring OD values, and OD linearity range of measuring device (*e.g.* spectrophotometer), if appropriate;
- Details of the test procedure used for TER measurements;
- Details of the test procedure used for the dye binding assessment, if appropriate;
- Test doses used, duration of exposure period(s) and temperature(s) of exposure;
- Details on washing procedure used after the exposure period;
- Number of replicate skin discs used per test chemical and controls (positive and negative control);
- Description of any modification of the test procedure;
- Reference to historical data of the model. This should include, but is not limited to:
 - i) Acceptability of the positive and negative control TER values (in k Ω) with reference to positive and negative control resistance ranges
 - ii) Acceptability of the positive and negative control dye content values (in $\mu\text{g}/\text{disc}$) with reference to positive and negative control dye content ranges
 - iii) Acceptability of the test results with reference to historical variability between skin disc replicates
- Description of decision criteria/prediction model applied.

Results:

- Tabulation of data from the TER and dye binding assays (if appropriate) for individual test chemicals and controls, for each testing run (experiment) and each skin disc replicate (individual animals and individual skin samples), means, SDs and CVs;
- Description of any effects observed;
- The derived classification with reference to the prediction model/decision criteria used.

Discussion of the results

Conclusions

LITERATURE

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- (3) Chapter B.40bis of this Annex, *In Vitro* Skin Model.
- (4) Chapter B.65 of this Annex, *In Vitro* Membrane Barrier Test Method.
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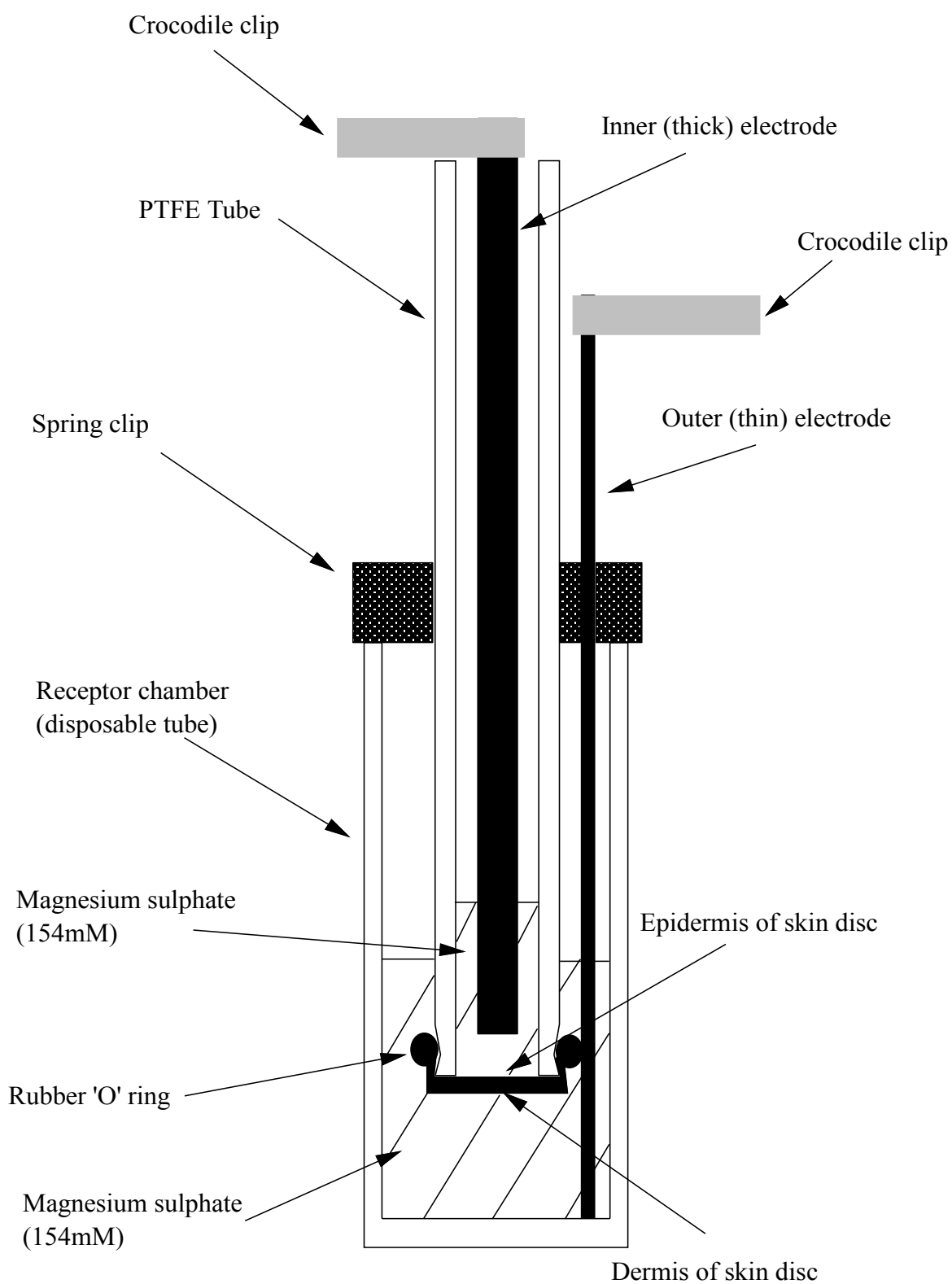
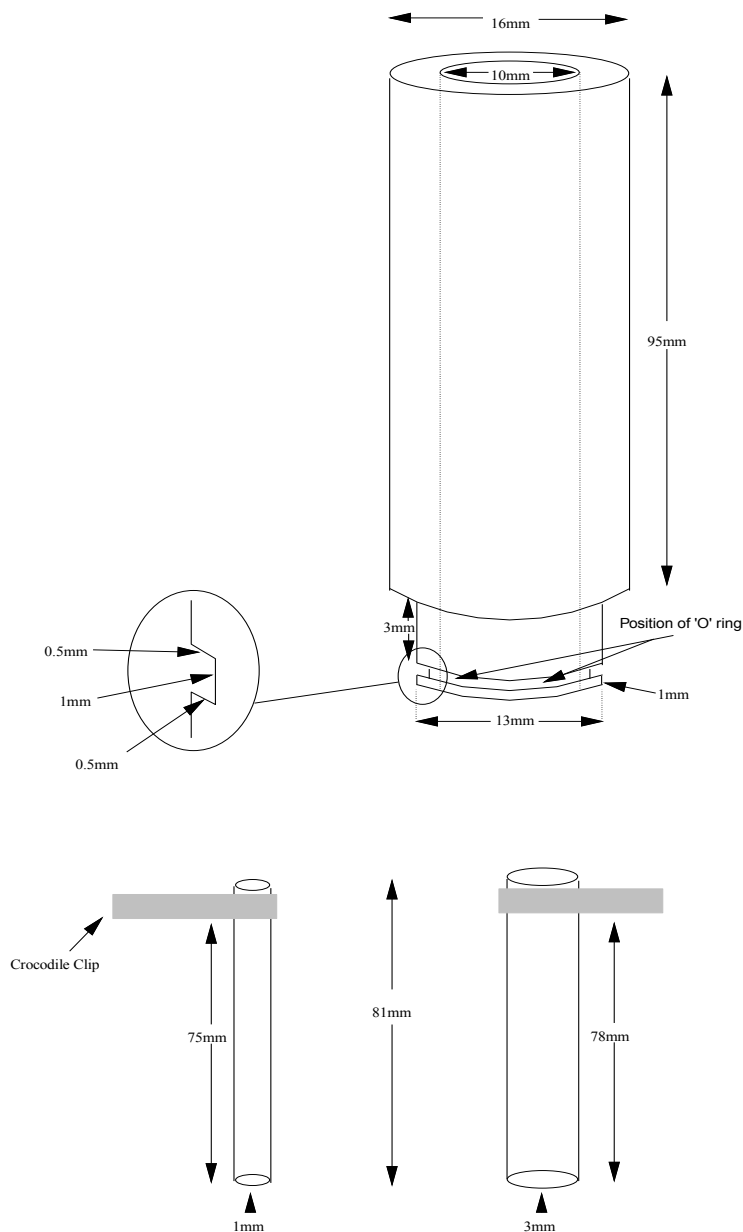
FIGURE 1: APPARATUS FOR THE RAT SKIN TER ASSAY

FIGURE 2: DIMENSIONS OF THE POLYTETRAFLUOROETHYLENE (PTFE) AND RECEPTOR TUBES AND ELECTRODES USED



Critical factors of the apparatus shown above:

- The inner diameter of the PTFE tube,
- The length of the electrodes relative to the PTFE tube and receptor tube, such that the skin disc should not be touched by the electrodes and that a standard length of electrode is in contact with the MgSO_4 solution,
- The amount of MgSO_4 solution in the receptor tube should give a depth of liquid, relative to the level in the PTFE tube, as shown in Figure 1,

- The skin disc should be fixed well enough to the PTFE tube, such that the electrical resistance is a true measure of the skin properties.

Appendix

DEFINITIONS

Accuracy: The closeness of agreement between test method results and accepted reference values. It is a measure of test method performance and one aspect of relevance. The term is often used interchangeably with “concordance” to mean the proportion of correct outcomes of a test method (20).

C: Corrosive.

Chemical: A substance or a mixture.

Concordance: A measure of test method performance for test methods that give a categorical result, and is one aspect of relevance. The term is sometimes used interchangeably with accuracy, and is defined as the proportion of all chemicals tested that are correctly classified as positive or negative. Concordance is highly dependent on the prevalence of positives in the types of test chemical being examined (20).

GHS (Globally Harmonized System of Classification and Labelling of Chemicals (UN)): A system proposing the classification of chemicals (substances and mixtures) according to standardised types and levels of physical, health and environmental hazards, and addressing corresponding communication elements, such as pictograms, signal words, hazard statements, precautionary statements and safety data sheets, so that to convey information on their adverse effects with a view to protect people (including employers, workers, transporters, consumers and emergency responders) and the environment (1).

IATA: Integrated Approach on Testing and Assessment.

Mixture: A mixture or solution composed of two or more substances.

Mono-constituent substance: A substance, defined by its quantitative composition, in which one main constituent is present to at least 80% (w/w).

Multi-constituent substance: A substance, defined by its quantitative composition, in which more than one main constituent is present in a concentration $\geq 10\%$ (w/w) and $< 80\%$ (w/w). A multi-constituent substance is the result of a manufacturing process. The difference between mixture and multi-constituent substance is that a mixture is obtained by blending of two or more substances without chemical reaction. A multi-constituent substance is the result of a chemical reaction.

NC: Non corrosive.

OD: Optical Density.

PC: Positive Control, a replicate containing all components of a test system and treated with a substance known to induce a positive response. To ensure that variability in the positive control response across time can be assessed, the magnitude of the positive response should not be excessive.

Performance standards (PS): Standards, based on a validated test method, that provide a basis for evaluating the comparability of a proposed test method that is mechanistically and functionally similar. Included are; (i) essential test method components; (ii) a minimum list of Reference Chemicals selected from among the chemicals used to demonstrate the acceptable performance of the validated test method; and (iii) the similar levels of reliability and accuracy, based on what was obtained for the validated test method, that the proposed test method should demonstrate when evaluated using the minimum list of Reference Chemicals.

Relevance: Description of relationship of the test method to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the test method correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of the accuracy (concordance) of a test method (20).

Reliability: Measures of the extent that a test method can be performed reproducibly within and between laboratories over time, when performed using the same protocol. It is assessed by calculating intra- and inter-laboratory reproducibility (20).

Sensitivity: The proportion of all positive/active chemicals that are correctly classified by the test method. It is a measure of accuracy for a test method that produces categorical results, and is an important consideration in assessing the relevance of a test method (20).

Skin corrosion *in vivo*: The production of irreversible damage of the skin; namely, visible necrosis through the epidermis and into the dermis, following the application of a test chemical for up to four hours. Corrosive reactions are typified by ulcers, bleeding, bloody scabs, and, by the end of observation at 14 days, by discoloration due to blanching of the skin, complete areas of alopecia, and scars. Histopathology should be considered to evaluate questionable lesions.

Specificity: The proportion of all negative/inactive chemicals that are correctly classified by the test method. It is a measure of accuracy for a test method that produces categorical results and is an important consideration in assessing the relevance of a test method (20).

Substance: A chemical element and its compounds in the natural state or obtained by any production process, including any additive necessary to preserve its stability and any impurities deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the substance or changing its composition.

(Testing) run: A single test chemical concurrently tested in a minimum of three replicate skin discs.

Test chemical: Any substance or mixture tested using this test method.

Transcutaneous Electrical Resistance (TER): is a measure of the electrical impedance of the skin, as a resistance value in kilo Ohms. A simple and robust method of assessing barrier function by recording the passage of ions through the skin using a Wheatstone bridge apparatus.

UVCB: Substances of unknown or variable composition, complex reaction products or biological materials. "

(6) In Part B, Chapter B.40bis is replaced by the following:

"B.40bis *IN VITRO* SKIN CORROSION: RECONSTRUCTED HUMAN EPIDERMIS (RhE) TEST METHOD

INTRODUCTION

1. This test method (TM) is equivalent to OECD test guideline (TG) 431 (2016). Skin corrosion refers to the production of irreversible damage to the skin manifested as visible necrosis through the *epidermis* and into the *dermis*, following the application of a test chemical [as defined by the United Nations (UN) Globally Harmonized System of Classification and Labelling of Chemicals (GHS) (1) and the European Union (EU) Regulation 1272/2008 on Classification, Labelling and Packaging of Substances and Mixtures (CLP)¹]. This updated test method B.40bis provides an *in vitro* procedure allowing the identification of non-corrosive and corrosive substances and mixtures in accordance with UN GHS and CLP. It also allows a partial sub-categorisation of corrosives.
2. The assessment of skin corrosion potential of chemicals has typically involved the use of laboratory animals (TM B.4, equivalent to OECD TG 404; originally adopted in 1981 and revised in 1992, 2002 and 2015) (2). In addition to the present test method B.40bis, two other *in vitro* test methods for testing corrosion potential of chemicals have been validated and adopted as TM B.40 (equivalent to OECD TG 430) (3) and TM B.65 (equivalent to OECD TG 435) (4). Furthermore the *in vitro* TM B.46 (equivalent to OECD TG 439) (5) has been adopted for testing skin irritation potential. A OECD guidance document on Integrated Approaches to Testing and Assessment (IATA) for Skin Corrosion and Irritation describes several modules which group information sources and analysis tools, and provides guidance on (i) how to integrate and use existing testing and non-testing data for the assessment of skin irritation and skin corrosion potentials of chemicals and (ii) proposes an approach when further testing is needed (6).
3. This test method addresses the human health endpoint skin corrosion. It makes use of reconstructed human *epidermis* (RhE) (obtained from human derived non-transformed epidermal keratinocytes) which closely mimics the histological, morphological, biochemical and physiological properties of the upper parts of the human skin, *i.e.* the *epidermis*. The corresponding OECD test guideline was originally adopted in 2004 and updated in 2013 to

¹ Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006, OJ L 353/1, 31.12.2008

include additional test methods using the RhE models and the possibility to use the methods to support the sub-categorisation of corrosive chemicals, and updated in 2015 to refer to the IATA guidance document and introduce the use of an alternative procedure to measure viability.

4. Four validated commercially available RhE models are included in this test method. Prevalidation studies (7), followed by a formal validation study for assessing skin corrosion (8)(9)(10) have been conducted (11) (12) for two of these commercially available test models, EpiSkin™ Standard Model (SM) and EpiDerm™ Skin Corrosivity Test (SCT) (EPI-200) (referred to in the following text as the Validated Reference Methods - VRMs). The outcome of these studies led to the recommendation that the two VRMs mentioned above could be used for regulatory purposes for distinguishing corrosive (C) from non-corrosive (NC) substances, and that the EpiSkin™ could moreover be used to support sub-categorisation of corrosive substances (13)(14)(15). Two other commercially available *in vitro* skin corrosion RhE test models have shown similar results to the EpiDerm™ VRM according to PS-based validation (16)(17)(18). These are the SkinEthic™ RHE¹ and epiCS® (previously named EST-1000) that can also be used for regulatory purposes for distinguishing corrosive from noncorrosive substances (19)(20). Post validation studies performed by the RhE model producers in the years 2012 to 2014 with a refined protocol correcting interferences of unspecific MTT reduction by the test chemicals improved the performance of both discrimination of C/NC as well as supporting subcategorisation of corrosives (21)(22). Further statistical analyses of the post-validation data generated with EpiDerm™ SCT, SkinEthic™ RHE and EpiCS® have been performed to identify alternative predictions models that improved the predictive capacity for sub-categorisation (23).
5. Before a proposed similar or modified *in vitro* RhE test method for skin corrosion other than the VRMs can be used for regulatory purposes, its reliability, relevance (accuracy), and limitations for its proposed use should be determined to ensure its similarity to the VRMs, in accordance with the requirements of the Performance Standards (PS) (24) set out in accordance with the principles of OECD guidance document No 34 (25). The Mutual Acceptance of Data will only be guaranteed after any proposed new or updated test method following the PS have been reviewed and included in the corresponding test guideline. The test models included in that test guideline can be used to address countries' requirements for test results on *in vitro* test method for skin corrosion, while benefiting from the Mutual Acceptance of Data.

DEFINITIONS

¹ The abbreviation RhE (=Reconstructed human Epidermis) is used for all models based on RhE technology. The abbreviation RHE as used in conjunction with the SkinEthic™ model means the same, but, as part of the name of this specific test method as marketed, is spelled all in capitals.

6. Definitions used are provided in Appendix 1.

INITIAL CONSIDERATIONS

7. This test method allows the identification of non-corrosive and corrosive substances and mixtures in accordance with the UN GHS and CLP. This test method further supports the sub-categorisation of corrosive substances and mixtures into optional sub-category 1A, in accordance with the UN GHS (1), as well as a combination of sub-categories 1B and 1C (21)(22)(23). A limitation of this test method is that it does not allow discriminating between skin corrosive sub-category 1B and sub-category 1C in accordance with the UN GHS and CLP due to the limited set of well-known *in vivo* corrosive sub-category 1C chemicals. EpiSkin™, EpiDerm™ SCT, SkinEthic™ RHE and epiCS® test models are able to sub-categorise (i.e. 1A versus 1B-and-1C versus NC)
8. A wide range of chemicals representing mainly individual substances has been tested in the validation supporting the test models included in this test method when they are used for identification of non-corrosives and corrosives; the empirical database of the validation study amounted to 60 chemicals covering a wide range of chemical classes (8)(9)(10). Testing to demonstrate sensitivity, specificity, accuracy and within-laboratory-reproducibility of the assay for sub-categorisation was performed by the test method developers and results were reviewed by the OECD (21) (22) (23). On the basis of the overall data available, the test method is applicable to a wide range of chemical classes and physical states including liquids, semi-solids, solids and waxes. The liquids may be aqueous or non-aqueous; solids may be soluble or insoluble in water. Whenever possible, solids should be ground to a fine powder before application; no other prior treatment of the sample is required. In cases where evidence can be demonstrated on the non-applicability of test models included in this test method to a specific category of test chemicals, they should not be used for that specific category of test chemicals. In addition, this test method is assumed to be applicable to mixtures as an extension of its applicability to substances. However, due to the fact that mixtures cover a wide spectrum of categories and composition, and that only limited information is currently available on the testing of mixtures, in cases where evidence can be demonstrated on the non-applicability of the test method to a specific category of mixtures (*e.g.* following a strategy as proposed in (26)), the test method should not be used for that specific category of mixtures. Before use of the test method on a mixture for generating data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed, when there is a regulatory requirement for testing of the mixture. Gases and aerosols have not been assessed yet in validation studies (8)(9)(10). While it is conceivable that these can be tested using RhE technology, the current test method does not allow testing of gases and aerosols.
9. Test chemicals absorbing light in the same range as MTT formazan and test chemicals able to directly reduce the vital dye MTT (to MTT formazan) may interfere with the tissue viability

measurements and need the use of adapted controls for corrections. The type of adapted controls that may be required will vary depending on the type of interference produced by the test chemical and the procedure used to measure MTT formazan (see paragraphs 25-31).

10. While this test method does not provide adequate information on skin irritation, it should be noted that TM B.46 specifically addresses the health effect skin irritation *in vitro* and is based on the same RhE test system, though using another protocol (5). For a full evaluation of local skin effects after a single dermal exposure, the OECD Guidance Document on Integrated Approaches for Testing and Assessment should be consulted (6). This IATA approach includes the conduct of *in vitro* tests for skin corrosion (such as described in this test method) and skin irritation before considering testing in living animals. It is recognised that the use of human skin is subject to national and international ethical considerations and conditions.

PRINCIPLE OF THE TEST

11. The test chemical is applied topically to a three-dimensional RhE model, comprised of non-transformed, human-derived epidermal keratinocytes, which have been cultured to form a multi-layered, highly differentiated model of the human *epidermis*. It consists of organised basal, spinous and granular layers, and a multi-layered *stratum corneum* containing intercellular lamellar lipid layers representing main lipid classes analogous to those found *in vivo*.
12. The RhE test method is based on the premise that corrosive chemicals are able to penetrate the *stratum corneum* by diffusion or erosion, and are cytotoxic to the cells in the underlying layers. Cell viability is measured by enzymatic conversion of the vital dye MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide, Thiazolyl blue tetrazolium bromide; CAS number 298-93-1], into a blue formazan salt that is quantitatively measured after extraction from tissues (27). Corrosive chemicals are identified by their ability to decrease cell viability below defined threshold levels (see paragraphs 35 and 36). The RhE-based skin corrosion test method has shown to be predictive of *in vivo* skin corrosion effects assessed in rabbits according to the TM B.4 (2).

DEMONSTRATION OF PROFICIENCY

13. Prior to routine use of any of the four validated RhE test models that adhere to this test method, laboratories should demonstrate technical proficiency by correctly classifying the twelve Proficiency Substances listed in Table 1. In case of the use of a method for sub-classification, also the correct sub-categorisation should be demonstrated. In situations where a listed substance is unavailable or where justifiable, another substance for which adequate *in vivo* and *in vitro* reference data are available may be used (e.g. from the list of reference chemicals (24)) provided that the same selection criteria as described in Table 1 is applied.

Table 1: List of Proficiency Substances¹

Substance	CASRN	Chemical Class ²	UN GHS/CLP Cat. Based on <i>In Vivo</i> results ³	VRM Cat. Based on <i>In Vitro</i> results ⁴	MTT Reducer ⁵	Physical State
Sub-category 1A <i>In Vivo</i> Corrosives						
Bromoacetic acid	79-08-3	Organic acid	1A	(3) 1A	--	S
Boron trifluoride dihydrate	13319-75-0	Inorganic acid	1A	(3) 1A	--	L
Phenol	108-95-2	Phenol	1A	(3) 1A	--	S
Dichloroacetyl chloride	79-36-7	Electrophile	1A	(3) 1A	--	L
Combination of sub-categories 1B-and-1C <i>In Vivo</i> Corrosives						
Glyoxylic acid monohydrate	563-96-2	Organic acid	1B-and-1C	(3) 1B-and-1C	--	S
Lactic acid	598-82-3	Organic acid	1B-and-1C	(3) 1B-and-1C	--	L
Ethanolamine	141-43-5	Organic base	1B	(3) 1B-and-1C	Y	Viscous
Hydrochloric acid (14.4%)	7647-01-0	Inorganic acid	1B-and-1C	(3) 1B-and-1C	--	L
<i>In Vivo</i> Non Corrosives						
Phenethyl bromide	103-63-9	Electrophile	NC	(3) NC	Y	L
4-Amino-1,2,4-triazole	584-13-4	Organic base	NC	(3) NC	--	S
4-(methylthio)-benzaldehyde	3446-89-7	Electrophile	NC	(3) NC	Y	L
Lauric acid	143-07-7	Organic acid	NC	(3) NC	--	S

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; VRM = Validated Reference Method; NC = Not Corrosive; Y = yes; S = solid; L = liquid

¹ The proficiency substances, sorted first by corrosives versus non-corrosives, then by corrosive sub-category and then by chemical class, were selected from the substances used in the ECVAM validation studies of EpiSkin™ and EpiDerm™ (8) (9) (10) and from post-validation studies based on data provided by EpiSkin™ (22), EpiDerm™, SkinEthic™ and epiCS® developers (23). Unless otherwise indicated, the substances were tested at the purity level obtained when purchased from a commercial source (8) (10). The selection includes, to the extent possible, substances that: (i) are representative of the range of corrosivity responses (e.g. noncorrosives; weak to strong corrosives) that the VRMs are capable of measuring or predicting; (ii) are representative of the chemical classes used in the validation studies; (iii) have chemical structures that are well-defined; (iv) induce reproducible results in the VRM; (v) induce definitive results in the *in vivo* reference test method; (vi) are commercially available; and (vii) are not associated with prohibitive disposal costs.

² Chemical class assigned by Barratt *et al.* (8).

³ The corresponding UN Packing groups are I, II and III, respectively, for the UN GHS/CLP 1A, 1B and 1C.

⁴ The VRM *in vitro* predictions reported in this table were obtained with the EpiSkin™ and the EpiDerm™ test models (VRMs) during post-validation testing performed by the test method developers.

⁵ The viability values obtained in the ECVAM Skin Corrosion Validation Studies were not corrected for direct MTT reduction (killed controls were not performed in the validation studies). However, the post-validation data generated by the test method developers that are presented in this table were acquired with adapted controls (23).

14. As part of the proficiency exercise, it is recommended that the user verifies the barrier properties of the tissues after receipt as specified by the RhE model manufacturer. This is particularly important if tissues are shipped over long distance/time periods. Once a test method has been successfully established and proficiency in its use has been demonstrated, such verification will not be necessary on a routine basis. However, when using a test method routinely, it is recommended to continue to assess the barrier properties in regular intervals.

PROCEDURE

15. The following is a generic description of the components and procedures of the RhE test models for skin corrosion assessment covered by this test method. The RhE models endorsed as scientifically valid for use within this test method, *i.e.* the EpiSkin™ (SM), EpiDerm™ (EPI-200), SkinEthic™ RHE and epiCS® models (16)(17)(19)(28)(29)(30)(31)(32)(33), can be obtained from commercial sources. Standard Operating Procedures (SOPs) for these four RhE models are available (34)(35)(36)(37), and their main test method components are summarised in Appendix 2. It is recommended that the relevant SOP be consulted when implementing and using one of these models in the laboratory. Testing with the four RhE test models covered by this test method should comply with the following:

RHE TEST METHOD COMPONENTS

General Conditions

16. Non-transformed human keratinocytes should be used to reconstruct the epithelium. Multiple layers of viable epithelial cells (basal layer, *stratum spinosum*, *stratum granulosum*) should be present under a functional *stratum corneum*. The *stratum corneum* should be multi-layered containing the essential lipid profile to produce a functional barrier with robustness to resist rapid penetration of cytotoxic benchmark chemicals, *e.g.* sodium dodecyl sulphate (SDS) or Triton X-100. The barrier function should be demonstrated and may be assessed either by determination of the concentration at which a benchmark chemical reduces the viability of the tissues by 50% (IC₅₀) after a fixed exposure time, or by determination of the exposure time required to reduce cell viability by 50% (ET₅₀) upon application of the benchmark chemical at a specified, fixed concentration (see paragraph 18). The containment properties of the RhE model should prevent the passage of material around the *stratum corneum* to the viable tissue, which would lead to poor modelling of skin exposure. The RhE model should be free of contamination by bacteria, viruses, mycoplasma, or fungi.

Functional Conditions

Viability

17. The assay used for quantifying tissue viability is the MTT-assay (27). The viable cells of the RhE tissue construct reduce the vital dye MTT into a blue MTT formazan precipitate, which is then extracted from the tissue using isopropanol (or a similar solvent). The OD of the extraction

solvent alone should be sufficiently small, i.e., $OD < 0.1$. The extracted MTT formazan may be quantified using either a standard absorbance (OD) measurement or an HPLC/UPLC-spectrophotometry procedure (38). The RhE model users should ensure that each batch of the RhE model used meets defined criteria for the negative control. An acceptability range (upper and lower limit) for the negative control OD values should be established by the RhE model developer/supplier. Acceptability ranges for the negative control OD values for the four validated RhE test models included in this test method are given in Table 2. An HPLC/UPLC-Spectrophotometry user should use the negative control OD ranges provided in Table 2 as the acceptance criterion for the negative control. It should be documented that the tissues treated with negative control are stable in culture (provide similar OD measurements) for the duration of the exposure period.

Table 2: Acceptability ranges for negative control OD values to control batch quality

	Lower acceptance limit	Upper acceptance limit
EpiSkin™ (SM)	> 0.6	< 1.5
EpiDerm™ SCT (EPI-200)	> 0.8	< 2.8
SkinEthic™ RHE	> 0.8	< 3.0
epiCS®	> 0.8	< 2.8

Barrier function

18. The *stratum corneum* and its lipid composition should be sufficient to resist the rapid penetration of certain cytotoxic benchmark chemicals (e.g. SDS or Triton X-100), as estimated by IC_{50} or ET_{50} (Table 3). The barrier function of each batch of the RhE model used should be demonstrated by the RhE model developer/vendor upon supply of the tissues to the end user (see paragraph 21).

Morphology

19. Histological examination of the RhE model should be performed demonstrating multi-layered human *epidermis*-like structure containing *stratum basale*, *stratum spinosum*, *stratum granulosum* and *stratum corneum* and exhibits lipid profile similar to lipid profile of human epidermis. Histological examination of each batch of the RhE model used demonstrating appropriate morphology of the tissues should be provided by the RhE model developer/vendor upon supply of the tissues to the end user (see paragraph 21).

Reproducibility

20. Test method users should demonstrate reproducibility of the test methods over time with the positive and negative controls. Furthermore, the test method should only be used if the RhE model developer/supplier provides data demonstrating reproducibility over time with corrosive and non-corrosive chemicals from e.g. the list of Proficiency Substances (Table 1). In case of

the use of a test method for subcategorisation, the reproducibility with respect to subcategorisation should also be demonstrated.

Quality control (QC)

21. The RhE model should only be used if the developer/supplier demonstrates that each batch of the RhE model used meets defined production release criteria, among which those for *viability* (paragraph 17), *barrier function* (paragraph 18) and *morphology* (paragraph 19) are the most relevant. These data are provided to the test method users, so that they are able to include this information in the test report. Only results produced with QC accepted tissue batches can be accepted for reliable prediction of corrosive classification. An acceptability range (upper and lower limit) for the IC₅₀ or the ET₅₀ is established by the RhE model developer/supplier. The acceptability ranges for the four validated test models are given in Table 3.

Table 3: QC batch release criteria

	Lower acceptance limit	Upper acceptance limit
EpiSkin™ (SM) (18 hours treatment with SDS) (33)	IC ₅₀ = 1.0 mg/ml	IC ₅₀ = 3.0 mg/ml
EpiDerm™ SCT (EPI-200) (1% Triton X-100) (34)	ET ₅₀ = 4.0 hours	ET ₅₀ = 8.7 hours
SkinEthic™ RHE (1% Triton X-100) (35)	ET ₅₀ = 4.0 hours	ET ₅₀ = 10.0 hours
epiCS® (1% Triton X-100) (36)	ET ₅₀ = 2.0 hours	ET ₅₀ = 7.0 hours

Application of the Test Chemical and Control Chemicals

22. At least two tissue replicates should be used for each test chemical and controls for each exposure time. For liquid as well as solid chemicals, sufficient amount of test chemical should be applied to uniformly cover the epidermis surface while avoiding an infinite dose, *i.e.* a minimum of 70 µl/cm² or 30 mg/cm² should be used. Depending on the models, the epidermis surface should be moistened with deionised or distilled water before application of solid chemicals, to improve contact between the test chemical and the epidermis surface (34)(35)(36)(37). Whenever possible, solids should be tested as a fine powder. The application method should be appropriate for the test chemical (see *e.g.* references (34-37)). At the end of the exposure period, the test chemical should be carefully washed from the *epidermis* with an aqueous buffer, or 0.9% NaCl. Depending on which of the four validated RhE test model is used, two or three exposure periods are used per test chemical (for all four valid RhE models: 3 min and 1 hour; for EpiSkin™ an additional exposure time of 4 hours). Depending on the RhE

test model used and the exposure period assessed, the incubation temperature during exposure may vary between room temperature and 37°C.

23. Concurrent negative and positive controls (PC) should be used in each run to demonstrate that viability (with negative controls), barrier function and resulting tissue sensitivity (with the PC) of the tissues are within a defined historical acceptance range. The suggested PC chemicals are glacial acetic acid or 8N KOH depending upon the RhE model used. It should be noted that 8N KOH is a direct MTT reducer that might require adapted controls as described in paragraphs 25 and 26. The suggested negative controls are 0.9% (w/v) NaCl or water.

Cell Viability Measurements

24. The MTT assay, which is a quantitative assay, should be used to measure cell viability under this test method (27). The tissue sample is placed in MTT solution of appropriate concentration (0.3 or 1 mg/ml) for 3 hours. The precipitated blue formazan product is then extracted from the tissue using a solvent (e.g. isopropanol, acidic isopropanol), and the concentration of formazan is measured by determining the OD at 570 nm using a filter band pass of maximum ± 30 nm, or by an HPLC/UPLC- spectrophotometry procedure (see paragraphs 30 and 31)(38).
25. Test chemicals may interfere with the MTT assay, either by direct reduction of the MTT into blue formazan, and/or by colour interference if the test chemical absorbs, naturally or due to treatment procedures, in the same OD range of formazan (570 ± 30 nm, mainly blue and purple chemicals). Additional controls should be used to detect and correct for a potential interference from these test chemicals such as the non-specific MTT reduction (NSMTT) control and the non-specific colour (NSC) control (see paragraphs 26 to 30). This is especially important when a specific test chemical is not completely removed from the tissue by rinsing or when it penetrates the *epidermis*, and is therefore present in the tissues when the MTT viability test is performed. Detailed description of how to correct direct MTT reduction and interferences by colouring agents is available in the SOPs for the test models (34)(35)(36)(37).
26. To identify direct MTT reducers, each test chemical should be added to freshly prepared MTT medium (34) (35) (36) (37). If the MTT mixture containing the test chemical turns blue/purple, the test chemical is presumed to directly reduce the MTT, and further functional check on non-viable epidermis should be performed, independently of using the standard absorbance (OD) measurement or an HPLC/UPLC-spectrophotometry procedure. This additional functional check employs killed tissues that possess only residual metabolic activity but absorb the test chemical in similar amount as viable tissues. Each MTT reducing chemical is applied on at least two killed tissue replicates per exposure time, which undergo the whole skin corrosion test. The true tissue viability is then calculated as the percent tissue viability obtained with living tissues exposed to the MTT reducer minus the percent non-specific MTT reduction obtained with the killed tissues exposed to the same MTT reducer, calculated relative to the negative control run concurrently to the test being corrected (%NSMTT).

27. To identify potential interference by coloured test chemicals or test chemicals that become coloured when in contact with water or isopropanol and decide on the need for additional controls, spectral analysis of the test chemical in water (environment during exposure) and/or isopropanol (extracting solution) should be performed. If the test chemical in water and/or isopropanol absorbs light in the range of 570 ± 30 nm, further colorant controls should be performed or, alternatively, an HPLC/UPLC- spectrophotometry procedure should be used in which case these controls are not required (see paragraphs 30 and 31). When performing the standard absorbance (OD) measurement, each interfering coloured test chemical is applied on at least two viable tissue replicates per exposure time, which undergo the entire skin corrosion test but are incubated with medium instead of MTT solution during the MTT incubation step to generate a non-specific colour (NSC_{living}) control. The NSC_{living} control needs to be performed concurrently per exposure time per coloured test chemical (in each run) due to the inherent biological variability of living tissues. The true tissue viability is then calculated as the percent tissue viability obtained with living tissues exposed to the interfering test chemical and incubated with MTT solution minus the percent nonspecific colour obtained with living tissues exposed to the interfering test chemical and incubated with medium without MTT, run concurrently to the test being corrected (%NSC_{living}).
28. Test chemicals that are identified as producing both direct MTT reduction (see paragraph 26) and colour interference (see paragraph 27) will also require a third set of controls, apart from the NSMTT and NSC_{living} controls described in the previous paragraphs, when performing the standard absorbance (OD) measurement. This is usually the case with darkly coloured test chemicals interfering with the MTT assay (e.g., blue, purple, black) because their intrinsic colour impedes the assessment of their capacity to directly reduce MTT as described in paragraph 26. These test chemicals may bind to both living and killed tissues and therefore the NSMTT control may not only correct for potential direct MTT reduction by the test chemical, but also for colour interference arising from the binding of the test chemical to killed tissues. This could lead to a double correction for colour interference since the NSC_{living} control already corrects for colour interference arising from the binding of the test chemical to living tissues. To avoid a possible double correction for colour interference, a third control for non-specific colour in killed tissues (NSC_{killed}) needs to be performed. In this additional control, the test chemical is applied on at least two killed tissue replicates per exposure time, which undergo the entire testing procedure but are incubated with medium instead of MTT solution during the MTT incubation step. A single NSC_{killed} control is sufficient per test chemical regardless of the number of independent tests/runs performed, but should be performed concurrently to the NSMTT control and, where possible, with the same tissue batch. The true tissue viability is then calculated as the percent tissue viability obtained with living tissues exposed to the test chemical minus %NSMTT minus %NSC_{living} plus the percent non-specific colour obtained with killed tissues exposed to the interfering test chemical and incubated with medium without MTT, calculated relative to the negative control run concurrently to the test being corrected (%NSC_{killed}).

29. It is important to note that non-specific MTT reduction and non-specific colour interferences may increase the readouts of the tissue extract above the linearity range of the spectrophotometer. On this basis, each laboratory should determine the linearity range of their spectrophotometer with MTT formazan (CAS # 57360-69-7) from a commercial source before initiating the testing of test chemicals for regulatory purposes. In particular, the standard absorbance (OD) measurement using a spectrophotometer is appropriate to assess direct MTT-reducers and colour interfering test chemicals when the ODs of the tissue extracts obtained with the test chemical without any correction for direct MTT reduction and/or colour interference are within the linear range of the spectrophotometer or when the uncorrected percent viability obtained with the test chemical already defined it as a corrosive (see paragraphs 35 and 36). Nevertheless, results for test chemicals producing %NSMTT and/or %NSC_{liVing} > 50% of the negative control should be taken with caution.
30. For coloured test chemicals which are not compatible with the standard absorbance (OD) measurement due to too strong interference with the MTT assay, the alternative HPLC/UPLC-spectrophotometry procedure to measure MTT formazan may be employed (see paragraph 31) (37). The HPLC/UPLC-spectrophotometry system allows for the separation of the MTT formazan from the test chemical before its quantification (38). For this reason, NSC_{liVing} or NSC_{killed} controls are never required when using HPLC/UPLC-spectrophotometry, independently of the chemical being tested. NSMTT controls should nevertheless be used if the test chemical is suspected to directly reduce MTT or has a colour that impedes the assessment of the capacity to directly reduce MTT (as described in paragraph 26). When using HPLC/UPLC-spectrophotometry to measure MTT formazan, the percent tissue viability is calculated as percent MTT formazan peak area obtained with living tissues exposed to the test chemical relative to the MTT formazan peak obtained with the concurrent negative control. For test chemicals able to directly reduce MTT, true tissue viability is calculated as the percent tissue viability obtained with living tissues exposed to the test chemical minus %NSMTT. Finally, it should be noted that direct MTT-reducers that may also be colour interfering, which are retained in the tissues after treatment and reduce MTT so strongly that they lead to ODs (using standard OD measurement) or peak areas (using UPLC/HPLC- spectrophotometry) of the tested tissue extracts that fall outside of the linearity range of the spectrophotometer cannot be assessed, although these are expected to occur in only very rare situations.
31. HPLC/UPLC-spectrophotometry may be used also with all types of test chemicals (coloured, non-coloured, MTT-reducers and non-MTT reducers) for measurement of MTT formazan (38). Due to the diversity of HPLC/UPLC-spectrophotometry systems, qualification of the HPLC/UPLC- spectrophotometry system should be demonstrated before its use to quantify MTT formazan from tissue extracts by meeting the acceptance criteria for a set of standard qualification parameters based on those described in the U.S. Food and Drug Administration guidance for industry on bio-analytical method validation (38)(39). These key parameters and their acceptance criteria are shown in Appendix 4. Once the acceptance criteria defined in Appendix 4 have been met, the HPLC/UPLC-spectrophotometry system is considered qualified

and ready to measure MTT formazan under the experimental conditions described in this test method.

Acceptability Criteria

32. For each test method using valid RhE models, tissues treated with the negative control should exhibit OD reflecting the quality of the tissues as described in table 2 and should not be below historically established boundaries. Tissues treated with the PC, *i.e.* glacial acetic acid or 8N KOH, should reflect the ability of the tissues to respond to a corrosive chemical under the conditions of the test model (see Appendix 2). The variability between tissue replicates of test chemical and/or control chemicals should fall within the accepted limits for each valid RhE model requirements (see Appendix 2) (*e.g.* the difference of viability between the two tissue replicates should not exceed 30%). If either the negative control or PC included in a run fall out of the accepted ranges, the run is considered as not qualified and should be repeated. If the variability of test chemicals falls outside of the defined range, its testing should be repeated.

Interpretation of Results and Prediction Model

33. The OD values obtained for each test chemical should be used to calculate percentage of viability relative to the negative control, which is set at 100%. In case HPLC/UPLC-spectrophotometry is used, the percent tissue viability is calculated as percent MTT formazan peak area obtained with living tissues exposed to the test chemical relative to the MTT formazan peak obtained with the concurrent negative control. The cut-off percentage cell viability values distinguishing corrosive from non-corrosive test chemical (or discriminating between different corrosive sub-categories) are defined below in paragraphs 35 and 36 for each of the test models covered by this test method and should be used for interpreting the results.
34. A single testing run composed of at least two tissue replicates should be sufficient for a test chemical when the resulting classification is unequivocal. However, in cases of borderline results, such as non-concordant replicate measurements, a second run may be considered, as well as a third one in case of discordant results between the first two runs.
35. The prediction model for the EpiSkin™ skin corrosion test model (9)(34)(22), associated with the UN GHS/CLP classification system, is shown in Table 4:

Table 4: EpiSkin™ prediction model

Viability measured after exposure time points (t=3, 60 and 240 minutes)	Prediction to be considered
< 35% after 3 min exposure	Corrosive: • Optional sub-category 1A*

$\geq 35\%$ after 3 min exposure AND $< 35\%$ after 60 min exposure OR $\geq 35\%$ after 60 min exposure AND $< 35\%$ after 240 min exposure	Corrosive: • A combination of optional sub-categories 1B-and-1C
$\geq 35\%$ after 240 min exposure	Non-corrosive

*) According to the data generated in view of assessing the usefulness of the RhE test models for supporting sub-categorisation, it was shown that around 22 % of the sub-category 1A results of the EpiSkin™ test model may actually constitute sub-category 1B or sub-category 1C substances/mixtures (i.e. overclassifications) (see Appendix 3).

36. The prediction models for the EpiDerm™ SCT (10)(23)(35), the SkinEthic™ RHE (17)(18) (23) (36), and the epiCS® (16)(23)(37) skin corrosion test models, associated with the UN GHS/CLP classification system, are shown in Table 5:

Table 5: EpiDerm™ SCT, SkinEthic™ RHE and epiCS®

Viability measured after exposure time points (t=3 and 60 minutes)	Prediction to be considered
STEP 1 for EpiDerm™ SCT, for SkinEthic™ RHE and epiCS®	
$< 50\%$ after 3 min exposure	Corrosive
$\geq 50\%$ after 3 min exposure AND $< 15\%$ after 60 min exposure	Corrosive
$\geq 50\%$ after 3 min exposure AND $\geq 15\%$ after 60 min exposure	Non-corrosive
STEP 2 for EpiDerm™ SCT - for substances/mixtures identified as Corrosive in step 1	
$< 25\%$ after 3 min exposure	Optional sub-category 1A *
$\geq 25\%$ after 3 min exposure	A combination of optional sub-categories 1B and 1C
STEP 2 for SkinEthic™ RHE - for substances/mixtures identified as Corrosive in step 1	
$< 18\%$ after 3 min exposure	Optional sub-category 1A *
$\geq 18\%$ after 3 min exposure	A combination of optional sub-categories 1B and 1C
STEP 2 for epiCS® - for substances/mixtures identified as Corrosive in step 1	
$< 15\%$ after 3 min exposure	Optional sub-category 1A *
$\geq 15\%$ after 3 min exposure	A combination of optional sub-categories 1B and 1C

DATA AND REPORTING

Data

37. For each test, data from individual tissue replicates (*e.g.* OD values and calculated percentage cell viability for each test chemical, including classification) should be reported in tabular form, including data from repeat experiments as appropriate. In addition, means and ranges of viability and CVs between tissue replicates for each test should be reported. Observed interactions with MTT reagent by direct MTT reducers or coloured test chemicals should be reported for each tested chemical.

Test report

38. The test report should include the following information:

Test Chemical and Control Chemicals:

- Mono-constituent substance: chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc;
- Multi-constituent substance, UVCB and mixture: characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents;
- Physical appearance, water solubility, and any additional relevant physicochemical properties;
- Source, lot number if available;
- Treatment of the test chemical/control substance prior to testing, if applicable (*e.g.* warming, grinding);
- Stability of the test chemical, limit date for use, or date for re-analysis if known;
- Storage conditions.

*RhE model and protocol used and rationale for it (if applicable)**Test Conditions:*

- RhE model used (including batch number);
- Calibration information for measuring device (*e.g.* spectrophotometer), wavelength and band pass (if applicable) used for quantifying MTT formazan, and linearity range of measuring device;
- Description of the method used to quantify MTT formazan;
- Description of the qualification of the HPLC/UPLC-spectrophotometry system, if applicable;
- Complete supporting information for the specific RhE model used including its performance. This should include, but is not limited to:
 - i) Viability;

- ii) Barrier function;
- iii) Morphology;
- iv) Reproducibility and predictive capacity;
- v) Quality controls (QC) of the model;

- Reference to historical data of the model. This should include, but is not limited to acceptability of the QC data with reference to historical batch data;
- Demonstration of proficiency in performing the test method before routine use by testing of the proficiency substances.

Test Procedure:

- Details of the test procedure used (including washing procedures used after exposure period);
- Doses of test chemical and control chemicals used;
- Duration of exposure period(s) and temperature(s) of exposure;
- Indication of controls used for direct MTT-reducers and/or colouring test chemicals, if applicable;
- Number of tissue replicates used per test chemical and controls (PC, negative control, and NSMTT, NSCliving and NSCKilled, if applicable), per exposure time;
- Description of decision criteria/prediction model applied based on the RhE model used;
- Description of any modifications of the test procedure (including washing procedures).

Run and Test Acceptance Criteria:

- Positive and negative control mean values and acceptance ranges based on historical data;
- Acceptable variability between tissue replicates for positive and negative controls;
- Acceptable variability between tissue replicates for test chemical.

Results:

- Tabulation of data for individual test chemicals and controls, for each exposure period, each run and each replicate measurement including OD or MTT formazan peak area, percent tissue viability, mean percent tissue viability, differences between replicates, SDs and/or CVs if applicable;
- If applicable, results of controls used for direct MTT-reducers and/or colouring test chemicals including OD or MTT formazan peak area, %NSMTT, %NSCliving, %NSCKilled, differences between tissue replicates, SDs and/or CVs (if applicable), and final correct percent tissue viability;
- Results obtained with the test chemical(s) and control chemicals in relation to the defined run and test acceptance criteria;

- Description of other effects observed;
- The derived classification with reference to the prediction model/decision criteria used.

Discussion of the results

Conclusions

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Appendix 1

DEFINITIONS

Accuracy: The closeness of agreement between test method results and accepted reference values. It is a measure of test method performance and one aspect of relevance. The term is often used interchangeably with “concordance” to mean the proportion of correct outcomes of a test method (25).

Cell viability: Parameter measuring total activity of a cell population *e.g.* as ability of cellular mitochondrial dehydrogenases to reduce the vital dye MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Thiazolyl blue), which depending on the endpoint measured and the test design used, correlates with the total number and/or vitality of living cells.

Chemical: A substance or a mixture.

Concordance: This is a measure of test method performance for test methods that give a categorical result, and is one aspect of relevance. The term is sometimes used interchangeably with accuracy, and is defined as the proportion of all chemicals tested that are correctly classified as positive or negative. Concordance is highly dependent on the prevalence of positives in the types of test chemical being examined (25).

ET₅₀: Can be estimated by determination of the exposure time required to reduce cell viability by 50% upon application of the benchmark chemical at a specified, fixed concentration, see also IC₅₀.

GHS (Globally Harmonized System of Classification and Labelling of Chemicals): A system proposing the classification of chemicals (substances and mixtures) according to standardized types and levels of physical, health and environmental hazards, and addressing corresponding communication elements, such as pictograms, signal words, hazard statements, precautionary statements and safety data sheets, so that to convey information on their adverse effects with a view to protect people (including employers, workers, transporters, consumers and emergency responders) and the environment (1).

HPLC: High Performance Liquid Chromatography.

IATA: Integrated Approach on Testing and Assessment.

IC₅₀: Can be estimated by determination of the concentration at which a benchmark chemical reduces the viability of the tissues by 50% (IC₅₀) after a fixed exposure time, see also ET₅₀.

Infinite dose: Amount of test chemical applied to the *epidermis* exceeding the amount required to completely and uniformly cover the *epidermis* surface. **Mixture:** A mixture or solution composed of two or more substances in which they do not react.

Mono-constituent substance: A substance, defined by its quantitative composition, in which one main constituent is present to at least 80% (w/w).

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Thiazolyl blue tetrazolium bromide.

Multi-constituent substance: A substance, defined by its quantitative composition, in which more than one main constituent is present in a concentration > 10% (w/w) and < 80% (w/w). A multi-constituent substance is the result of a manufacturing process. The difference between mixture and multi-constituent substance is that a mixture is obtained by blending of two or more substances without chemical reaction. A multi-constituent substance is the result of a chemical reaction.

NC: Non corrosive.

NSC_{killed} control: Non-Specific Colour control in killed tissues.

NSC_{living} control : Non-Specific Colour control in living tissues.

NSMTT: Non-Specific MTT reduction.

OD: Optical Density

PC: Positive Control, a replicate containing all components of a test system and treated with a chemical known to induce a positive response. To ensure that variability in the positive control response across time can be assessed, the magnitude of the positive response should not be excessive.

Performance standards (PS): Standards, based on a validated test method, that provide a basis for evaluating the comparability of a proposed test method that is mechanistically and functionally similar. Included are; (i) essential test method components; (ii) a minimum list of Reference Chemicals selected from among the chemicals used to demonstrate the acceptable performance of the validated test method; and (iii) the similar levels of reliability and accuracy, based on what was obtained for the validated test method, that the proposed test method should demonstrate when evaluated using the minimum list of Reference Chemicals (25).

Relevance: Description of relationship of the test method to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the test method correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of the accuracy (concordance) of a test method (25).

Reliability: Measures of the extent that a test method can be performed reproducibly within and between laboratories over time, when performed using the same protocol. It is assessed by calculating intra- and inter-laboratory reproducibility (25).

Run: A run consists of one or more test chemicals tested concurrently with a negative control

and with a PC.

Sensitivity: The proportion of all positive/active chemicals that are correctly classified by the test method. It is a measure of accuracy for a test method that produces categorical results, and is an important consideration in assessing the relevance of a test method (25).

Skin corrosion *in vivo*: The production of irreversible damage of the skin; namely, visible necrosis through the *epidermis* and into the dermis, following the application of a test chemical for up to four hours. Corrosive reactions are typified by ulcers, bleeding, bloody scabs, and, by the end of observation at 14 days, by discoloration due to blanching of the skin, complete areas of alopecia, and scars. Histopathology should be considered to evaluate questionable lesions.

Specificity: The proportion of all negative/inactive chemicals that are correctly classified by the test method. It is a measure of accuracy for a test method that produces categorical results and is an important consideration in assessing the relevance of a test method (25).

Substance: A chemical element and its compounds in the natural state or obtained by any production process, inducing any additive necessary to preserve its stability and any impurities deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the substance or changing its composition. **Test chemical:** Any substance or mixture tested using this test method.

UPLC: Ultra-High Performance Liquid Chromatography.

UVCB: substances of unknown or variable composition, complex reaction products or biological materials.

Appendix 2

MAIN COMPONENTS OF THE RHE TEST MODELS VALIDATED FOR SKIN CORROSION TESTING

Test Model Components	EpiSkin™	EpiDerm™ SCT	SkinEthic™ RHE	epiCS®
Model surface	0.38 cm ²	0.63 cm ²	0.5 cm ²	0.6 cm ²
Number of tissue replicates	At least 2 per exposure time	2-3 per exposure time	At least 2 per exposure time	At least 2 per exposure time
Treatment doses and application	<u>Liquids and viscous</u>: 50 µl ± 3 µl (131.6 µl/cm²) <u>Solids</u>: 20 ± 2 mg (52.6 mg/cm²) + 100 µl ± 5µl NaCl solution (9 g/l) <u>Waxy/sticky</u>: 50 ± 2 mg (131.6 mg/cm²) with a nylon mesh	<u>Liquids</u>: 50 µl (79.4 µl/cm²) with or without a nylon mesh <i>Pre-test compatibility of test chemical with nylon mesh</i> <u>Semisolids</u>: 50 µl (79.4 µl/cm²) <u>Solids</u>: 25 µl H₂O (or more if necessary) + 25 mg (39.7 mg/cm²) <u>Waxes</u>: flat “disc like” piece of ca. 8 mm diameter placed atop the tissue wetted with 15 µl H₂O.	<u>Liquids and viscous</u>: 40 µl ± 3µl (80 µl/cm²) using nylon mesh <i>Pre-test compatibility of test chemical with nylon mesh</i> <u>Solids</u>: 20 µl ± 2µl H₂O + 20 ± 3 mg (40 mg/cm²) <u>Waxy/sticky</u>: 20 ± 3 mg (40 mg/cm²) using nylon mesh	<u>Liquids</u>: 50 µl (83.3 µl/cm²) using nylon mesh <i>Pre-test compatibility of test chemical with nylon mesh</i> <u>Semisolids</u>: 50 µl (83.3 µl/cm²) <u>Solids</u>: 25 mg (41.7 mg/cm²) + 25 µl H₂O (or more if necessary) <u>Waxy</u>: flat “cookie like” piece of ca. 8 mm diameter placed atop the tissue wetted with 15 µl H₂O

Test Model Components	EpiSkin™	EpiDerm™ SCT	SkinEthic™ RHE	epiCS®
Pre-check for direct MTT reduction	50 µl (liquid) or 20 mg (solid) + 2 ml MTT 0.3 mg/ml solution for 180 ± 5 min at 37°C, 5% CO₂, 95% RH → if solution turns blue/purple, water-killed adapted controls should be performed	50 µl (liquid) or 25 mg (solid) + 1 ml MTT 1 mg/ml solution for 60 min at 37°C, 5% CO₂, 95% RH → if solution turns blue/purple, freeze-killed adapted controls should be performed	40 µl (liquid) or 20 mg (solid) + 1 ml MTT 1 mg/ml solution for 180± 15 min at 37°C, 5% CO₂, 95% RH → if solution turns blue/purple, freeze-killed adapted controls should be performed	50 µl (liquid) or 25 mg (solid) + 1 ml MTT 1 mg/ml solution for 60 min at 37°C, 5% CO₂, 95% RH → if solution turns blue/purple, freeze-killed adapted controls should be performed
Pre-check for colour interference	10 µl (liquid) or 10 mg (solid) + 90 µl H₂O mixed for 15 min at RT → if solution becomes coloured, living adapted controls should be performed	50 µl (liquid) or 25 mg (solid) + 300 µl H₂O for 60 min at 37°C, 5% CO₂, 95% RH → if solution becomes coloured, living adapted controls should be performed	40 µl (liquid) or 20mg (solid) + 300 µl H₂O mixed for 60 min at RT → if test chemical is coloured, living adapted controls should be performed	50 µl (liquid) or 25 mg (solid) + 300 µl H₂O for 60 min at 37°C, 5% CO₂, 95% RH → if solution becomes coloured, living adapted controls should be performed
Exposure time and temperature	3 min, 60 min (± 5 min) and 240 min (± 10 min) In ventilated cabinet Room Temperature (RT, 18-28°C)	3 min at RT, and 60 min at 37°C, 5% CO₂, 95% RH	3 min at RT, and 60 min at 37°C, 5% CO₂, 95% RH	3 min at RT, and 60 min at 37°C, 5% CO₂, 95% RH
Rinsing	25 ml 1x PBS (2 ml/throwing)	20 times with a constant soft stream of 1x PBS	20 times with a constant soft stream of 1x PBS	20 times with a constant soft stream of 1x PBS

Test Model Components	EpiSkin™	EpiDerm™ SCT	SkinEthic™ RHE	epiCS®
Negative control	50 µl NaCl solution (9 g/l) Tested with every exposure time	50 µl H ₂ O Tested with every exposure time	40 µl H ₂ O Tested with every exposure time	50 µl H ₂ O Tested with every exposure time
Positive control	50 µl Glacial acetic acid Tested only for 4 hours	50 µl 8N KOH Tested with every exposure time	40 µl 8N KOH Tested only for 1 hour	50 µl 8N KOH Tested with every exposure time
MTT solution	2 ml 0.3 mg/ml	300 µl 1 mg/ml	300 µl 1 mg/ml	300 µl 1 mg/ml
MTT incubation time and temperature	180 min (± 15 min) at 37°C, 5% CO ₂ , 95% RH	180 min at 37°C, 5% CO ₂ , 95% RH	180 min (± 15 min) at 37°C, 5% CO ₂ , 95% RH	180 min at 37°C, 5% CO ₂ , 95% RH
Extraction solvent	500 µl acidified isopropanol (0.04 N HCl in isopropanol) (isolated tissue fully immersed)	2 ml isopropanol (extraction from top and bottom of insert)	1.5 ml isopropanol (extraction from top and bottom of insert)	2 ml isopropanol (extraction from top and bottom of insert)
Extraction time and temperature	Overnight at RT, protected from light	Overnight without shaking at RT or for 120 min with shaking (~120 rpm) at RT	Overnight without shaking at RT or for 120 min with shaking (~120 rpm) at RT	Overnight without shaking at RT or for 120 min with shaking (~120 rpm) at RT
OD reading	570 nm (545 - 595 nm) without reference filter	570 nm (or 540 nm) without reference filter	570 nm (540 - 600 nm) without reference filter	540 - 570 nm without reference filter
Tissue Quality Control	18 hours treatment with SDS 1.0 mg/ml ≤ IC ₅₀ ≤ 3.0 mg/ml	Treatment with 1% Triton X-100 4.08 hours ≤ ET ₅₀ ≤ 8.7 hours	Treatment with 1% Triton X-100 4.0 hours ≤ ET ₅₀ ≤ 10.0 hours	Treatment with 1% Triton X-100 2.0 hours ≤ ET ₅₀ ≤ 7.0 hours

Test Model Components	EpiSkin™	EpiDerm™ SCT	SkinEthic™ RHE	epiCS®
Acceptability Criteria	1. Mean OD of the tissue replicates treated with the negative control (NaCl) should be ≥ 0.6 and ≤ 1.5 for every exposure time 2. Mean viability of the tissue replicates exposed for 4 hours with the positive control (glacial acetic acid), expressed as % of the negative control, should be $\leq 20\%$ 3. In the range 20-100% viability and for $ODs \geq 0.3$, difference of viability between the two tissue replicates should not exceed 30%.	1. Mean OD of the tissue replicates treated with the negative control (H₂O) should be ≥ 0.8 and ≤ 2.8 for every exposure time 2. Mean viability of the tissue replicates exposed for 1 hour with the positive control (8N KOH), expressed as % of the negative control, should be $< 15\%$ 3. In the range 20 - 100% viability, the Coefficient of Variation (CV) between tissue replicates should be $\leq 30\%$	1. Mean OD of the tissue replicates treated with the negative control (H₂O) should be ≥ 0.8 and ≤ 3.0 for every exposure time 2. Mean viability of the tissue replicates exposed for 1 hour (and 4 hours, if applicable) with the positive control (8N KOH), expressed as % of the negative control, should be $< 15\%$ 3. In the range 20-100% viability, and for $ODs \geq 0.3$, difference of viability between the two tissue replicates should not exceed 30%	1. Mean OD of the tissue replicates treated with the negative control (H₂O) should be ≥ 0.8 and ≤ 2.8 for every exposure time 2. Mean viability of the tissue replicates exposed for 1 hour with the positive control (8N KOH), expressed as % of the negative control, should be $< 20\%$ 3. In the range 20-100% viability, and for $ODs \geq 0.3$, difference of viability between the two tissue replicates should not exceed 30%

Appendix 3

PERFORMANCE OF TEST MODELS FOR SUB-CATEGORISATION

The table below provides the performances of the four test models calculated based on a set of 80 chemicals tested by the four test developers. Calculations were performed by the OECD Secretariat, reviewed and agreed by an expert subgroup (21) (23).

EpiSkin™, EpiDerm™, SkinEthic™ and epiCS® test models are able to sub-categorise (i.e. 1A versus 1B-and-1C versus NC)

Performances, overclassification rates, underclassification rates, and accuracy (Predictive capacity) of the four test models based on a set of 80 chemicals all tested over 2 or 3 runs in each test model:

STATISTICS ON PREDICTIONS OBTAINED ON THE ENTIRE SET OF CHEMICALS				
(n= 80 chemicals tested over 2 independent runs for epiCS® or 3 independent runs for EpiDerm™ SCT, EpiSkin™ and SkinEthic™ RHE, i.e. respectively 159* or 240 classifications)				
*one chemical was tested once in epiCS® because of no availability (23)				
	EpiSkin™	EpiDerm™	SkinEthic™	epiCS®
Overclassifications:				
1B-and-1C overclassified 1A	21.50%	29.0%	31.2%	32.8%
NC overclassified 1B-and-1C	20.7%	23.4%	27.0 %	28.4 %
NC overclassified 1A	0.00%	2.7%	0.0 %	0.00%
overclassified Corr.	20.7%	26.1%	27.0%	28.4%
Global overclassification rate (all categories)	17.9%	23.3%	24.5%	25.8%
Underclassifications:				
1A underclassified 1B-and-1C	16.7%	16.7 %	16.7%	12.5 %
1A underclassified NC	0.00%	0.00%	0.00%	0.00%
1B-and-1C underclassified NC	2.2%	0.00%	7.5%	6.6%
Global underclassification rate (all categories)	3.3%	2.5%	5.4%	4.4%
Correct Classifications:				
1A correctly classified	83.3%	83.3%	83.3%	87.5%
1B-and-/1C correctly classified	76.3%	71.0%	61.3%	60.7%

NC correctly classified	79.3%	73.9%	73.0%	71.62%
Overall Accuracy	78.8%	74.2%	70%	69.8%

NC: Non-corrosive

Appendix 4

Key parameters and acceptance criteria for qualification of an HPLC/UPLC-spectrophotometry system for measurement of MTT formazan extracted from RhE tissue

Parameter	Protocol Derived from FDA Guidance (37)(38)	Acceptance Criteria
Selectivity	Analysis of isopropanol, living blank (isopropanol extract from living RhE tissues without any treatment), dead blank (isopropanol extract from killed RhE tissues without any treatment)	$\text{Area}_{\text{interference}} \leq 20\% \text{ of } \text{Area}_{\text{LLOQ}}^1$
Precision	Quality Controls (i.e., MTT formazan at 1.6 µg/ml, 16 µg/ml and 160 µg/ml) in isopropanol (n=5)	$\text{CV} \leq 15\% \text{ or } \leq 20\% \text{ for the LLOQ}$
Accuracy	Quality Controls in isopropanol (n=5)	$\% \text{Dev} \leq 15\% \text{ or } \leq 20\% \text{ for LLOQ}$
Matrix Effect	Quality Controls in living blank (n=5)	$85\% \leq \text{Matrix Effect } \% \leq 115\%$
Carryover	Analysis of isopropanol after an ULOQ ² standard	$\text{Area}_{\text{interference}} \leq 20\% \text{ of } \text{Area}_{\text{LLOQ}}$
Reproducibility (intra-day)	3 independent calibration curves (based on 6 consecutive 1/3 dilutions of MTT formazan in isopropanol starting at ULOQ, i.e., 200 µg/ml); Quality Controls in isopropanol (n=5)	Calibration Curves: $\% \text{Dev} \leq 15\% \text{ or } \leq 20\% \text{ for LLOQ}$ Quality Controls: $\% \text{Dev} \leq 15\% \text{ and } \text{CV} \leq 15\%$
Reproducibility (inter-day)	Day 1: 1 calibration curve and Quality Controls in isopropanol (n=3) Day 2: 1 calibration curve and Quality Controls in isopropanol (n=3) Day 3: 1 calibration curve and Quality Controls in isopropanol (n=3)	
Short Term Stability of MTT Formazan in RhE Tissue Extract	Quality Controls in living blank (n=3) analysed the day of the preparation and after 24 hours of storage at room temperature	$\% \text{Dev} \leq 15\%$
Long Term Stability of MTT Formazan in RhE Tissue Extract, if required	Quality Controls in living blank (n=3) analysed the day of the preparation and after several days of storage at a specified temperature (e.g., 4°C, -20°C, -80°C)	$\% \text{Dev} \leq 15\%$

1LLOQ: Lower Limit of Quantification, defined to cover 1-2% tissue viability, i.e., 0.8 µg/ml.

2ULOQ: Upper Limit of Quantification, defined to be at least two times higher than the highest expected MTT formazan concentration in isopropanol extracts from negative controls i.e., 200 µg/ml."

(7) In Part B, Chapter B.46 is replaced by the following:

"B.46 *IN VITRO* SKIN IRRITATION: RECONSTRUCTED HUMAN *EPIDERMIS* TEST METHOD

INTRODUCTION

1. This test method (TM) is equivalent to OECD test guideline (TG) 439 (2015). Skin irritation refers to the production of reversible damage to the skin following the application of a test chemical for up to 4 hours [as defined by the United Nations (UN) Globally Harmonized System of Classification and Labelling of Chemicals (GHS)]⁽¹⁾ and the European Union (EU) Regulation 1272/2008 on Classification, Labelling and Packaging of Substances and Mixtures (CLP)¹. This test method provides an *in vitro* procedure that may be used for the hazard identification of irritant chemicals (substances and mixtures) in accordance with UN GHS/CLP Category 2 (2). In regions that do not adopt the optional UN GHS Category 3 (mild irritants), this test method can also be used to identify non-classified chemicals. Therefore, depending on the regulatory framework and the classification system in use, this test method may be used to determine the skin irritancy of chemicals either as a stand-alone replacement test for *in vivo* skin irritation testing or as a partial replacement test within a testing strategy (3).
2. The assessment of skin irritation has typically involved the use of laboratory animals [TM B.4, equivalent to OECD TG 404 originally adopted in 1981 and revised in 1992, 2002 and 2015] (4). For the testing of corrosivity, three validated *in vitro* test methods have been adopted as EU TM B.40 (equivalent to OECD TG 430), TM B.40bis (equivalent to OECD TG 431) and TM B.65 (equivalent to OECD TG 435) (5) (6) (7). An OECD guidance document on Integrated Approaches to Testing and Assessment (IATA) for Skin Corrosion and Irritation describes several modules which group information sources and analysis tools, and provides guidance on (i) how to integrate and use existing test and non-test data for the assessment of skin irritation and skin corrosion potentials of chemicals and (ii) proposes an approach when further testing is needed (3).
3. This test method addresses the human health endpoint skin irritation. It is based on the *in vitro* test system of reconstructed human *epidermis* (RhE), which closely mimics the biochemical and physiological properties of the upper parts of the human skin, *i.e.* the

¹ Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006, OJ L 353/1, 31.12.2008

epidermis. The RhE test system uses human derived non-transformed keratinocytes as cell source to reconstruct an epidermal model with representative histology and cytoarchitecture. Performance Standards (PS) are available to facilitate the validation and assessment of similar and modified RhE-based test methods, in accordance with the principles of the OECD guidance document No 34 (8) (9). The corresponding test guideline was originally adopted in 2010, updated in 2013 to include additional RhE models, and updated in 2015 to refer to the IATA guidance document and introduce the use of an alternative procedure to measure viability.

4. Pre-validation, optimisation and validation studies have been completed for four commercially available *in vitro* test models (10) (11) (12) (13) (14) (15) (16) (17) (18) (19) (20) (21) (22) (23) (24) (25) (26) (27) (28) based on the RhE test system (sensitivity 80%, specificity 70%, and accuracy 75%). These four test models are included in this TM and are listed in Appendix 2, which also provides information on the type of validation study used to validate the respective test methods. As noted in Appendix 2, the Validated Reference Method (VRM) have been used to develop the present test method and the Performance Standards (8).
5. OECD Mutual Acceptance of Data will only be guaranteed for test models validated according to the Performance Standards (8), if these test models have been reviewed and adopted by OECD. The test models included in this test method and the corresponding OECD TG can be used indiscriminately to address countries' requirements for test results from *in vitro* test methods for skin irritation, while benefiting from the Mutual Acceptance of Data.
6. Definitions of terms used in this document are provided in Appendix 1.

INITIAL CONSIDERATIONS AND LIMITATIONS

7. A limitation of the test method, as demonstrated by the full prospective validation study assessing and characterising RhE test methods (16), is that it does not allow the classification of chemicals to the optional UN GHS Category 3 (mild irritants) (1). Thus, the regulatory framework in member countries will decide how this test method will be used. For the EU, Category 3 has not been taken up in CLP. For a full evaluation of local skin effects after a single dermal exposure, the OECD Guidance Document on Integrated Approaches for Testing and Assessment should be consulted (3). It is recognised that the use of human skin is subject to national and international ethical considerations and conditions.
8. This test method addresses the human health endpoint skin irritation. While this test method does not provide adequate information on skin corrosion, it should be noted that TM B.40bis (equivalent to OECD TG 431) on skin corrosion is based on the same RhE test

system, though using another protocol (6). This test method is based on RhE-models using human keratinocytes, which therefore represent *in vitro* the target organ of the species of interest. It moreover directly covers the initial step of the inflammatory cascade/mechanism of action (cell and tissue damage resulting in localised trauma) that occurs during irritation *in vivo*. A wide range of chemicals has been tested in the validation underlying this test method and the database of the validation study amounted to 58 chemicals in total (16) (18) (23). The test method is applicable to solids, liquids, semi-solids and waxes. The liquids may be aqueous or non-aqueous; solids may be soluble or insoluble in water. Whenever possible, solids should be ground to a fine powder before application; no other pre-treatment of the sample is required. Gases and aerosols have not been assessed yet in a validation study (29). While it is conceivable that these can be tested using RhE technology, the current test method does not allow testing of gases and aerosols.

9. Before use of the test method on a mixture for generating data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed, when there is a regulatory requirement for testing of the mixture. However, due to the fact that mixtures cover a wide spectrum of categories and composition, and that only limited information is currently available on the testing of mixtures, in cases where evidence can be demonstrated on the non-applicability of the test method to a specific category of mixtures (e.g. following a strategy as proposed in Eskes *et al.* 2012 (30)), the test method should not be used for that specific category of mixtures. Similar care should be taken in case specific chemical classes or physico-chemical properties are found not to be applicable to the current test method.
10. Test chemicals absorbing light in the same range as MTT formazan and test chemicals able to directly reduce the vital dye MTT (to MTT formazan) may interfere with the cell viability measurements and need the use of adapted controls for corrections (see paragraphs 28-34).
11. A single testing run composed of three replicate tissues should be sufficient for a test chemical when the classification is unequivocal. However, in cases of borderline results, such as non-concordant replicate measurements and/or mean percent viability equal to $50 \pm 5\%$, a second run should be considered, as well as a third one in case of discordant results between the first two runs.

PRINCIPLE OF THE TEST

12. The test chemical is applied topically to a three-dimensional RhE model, comprised of non-transformed human-derived epidermal keratinocytes, which have been cultured to form a multilayered, highly differentiated model of the human *epidermis*. It consists of organised basal, spinous and granular layers, and a multilayered *stratum corneum*

containing intercellular lamellar lipid layers representing main lipid classes analogous to those found *in vivo*.

13. Chemical-induced skin irritation, manifested mainly by erythema and oedema, is the result of a cascade of events beginning with penetration of the chemicals through the *stratum corneum* where they may damage the underlying layers of keratinocytes and other skin cells. The damaged cells may either release inflammatory mediators or induce an inflammatory cascade which also acts on the cells in the *dermis*, particularly the stromal and endothelial cells of the blood vessels. It is the dilation and increased permeability of the endothelial cells that produce the observed erythema and oedema (29). Notably, the RhE-based test methods, in the absence of any vascularisation in the *in vitro* test system, measure the initiating events in the cascade, e.g. cell / tissue damage (16) (17), using cell viability as readout.
14. Cell viability in RhE models is measured by enzymatic conversion of the vital dye MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Thiazolyl blue; CAS number 298-93-1], into a blue formazan salt that is quantitatively measured after extraction from tissues (31). Irritant chemicals are identified by their ability to decrease cell viability below defined threshold levels (*i.e.* $\leq 50\%$, for UN GHS/CLP Category 2). Depending on the regulatory framework and applicability of the test method, test chemicals that produce cell viabilities above the defined threshold level, may be considered non-irritants (*i.e.* $> 50\%$, No Category).

DEMONSTRATION OF PROFICIENCY

15. Prior to routine use of any of the four validated test models that adhere to this test method (Appendix 2), laboratories should demonstrate technical proficiency, using the ten Proficiency Substances listed in Table 1. In situations where, for instance, a listed substance is unavailable, another substance for which adequate *in vivo* and *in vitro* reference data are available may be used (e.g. from the list of reference chemicals (8)) provided that the same selection criteria as described in Table 1 are applied. Using an alternative proficiency substance should be justified.
16. As part of the proficiency testing, it is recommended that users verify the barrier properties of the tissues after receipt as specified by the RhE model producer. This is particularly important if tissues are shipped over long distance/time periods. Once a test method has been successfully established and proficiency in its use has been acquired and demonstrated, such verification will not be necessary on a routine basis. However, when using a test method routinely, it is recommended to continue to assess the barrier properties at regular intervals.

Table 1: Proficiency Substances¹

Substance	CAS NR	<i>In vivo</i> score ²	Physical state	UN GHS Category
NON-CLASSIFIED SUBSTANCES (UN GHS No Category)				
naphthalene acetic acid	86-87-3	0	Solid	No Cat.
isopropanol	67-63-0	0.3	Liquid	No Cat.
methyl stearate	112-61-8	1	Solid	No Cat.
heptyl butyrate	5870-93-9	1.7	Liquid	No Cat. (Optional Cat. 3) ³
hexyl salicylate	6259-76-3	2	Liquid	No Cat. (Optional Cat. 3) ³
CLASSIFIED SUBSTANCES (UN GHS Category 2)				
cyclamen aldehyde	103-95-7	2.3	Liquid	Cat. 2
1-bromohexane	111-25-1	2.7	Liquid	Cat. 2
potassium hydroxide (5% aq.)	1310-58-3	3	Liquid	Cat. 2
1-methyl-3-phenyl-1-piperazine	5271-27-2	3.3	Solid	Cat. 2
heptanal	111-71-7	3.4	Liquid	Cat. 2

1 The Proficiency Substances are a subset of the substances used in the validation study and the selection is based on the following criteria; (i), the chemicals substances are commercially available; (ii), they are representative of the full range of Draize irritancy scores (from non-irritant to strong irritant); (iii), they have a well-defined chemical structure; (iv), they are representative of the chemical functionality used in the validation process; (v) they provided reproducible *in vitro* results across multiple testing and multiple laboratories; (vi) they were correctly predicted *in vitro*, and (vii) they are not associated with an extremely toxic profile (e.g. carcinogenic or toxic to the reproductive system) and they are not associated with prohibitive disposal costs.

2 *In vivo* score in accordance with TM B.4 (4).

3 Under this test method, the UN GHS optional Category 3 (mild irritants) (1) is considered as No Category.

PROCEDURE

17. The following is a description of the components and procedures of a RhE test method for skin irritation assessment (See also Appendix 3 for parameters related to each test model). Standard Operating Procedures (SOPs) for the four models complying with this test method are available (32) (33) (34) (35).

RHE TEST METHOD COMPONENTS

General conditions

18. Non -transformed human keratinocytes should be used to reconstruct the epithelium. Multiple layers of viable epithelial cells (basal layer, *stratum spinosum*, *stratum granulosum*) should be present under a functional *stratum corneum*. *Stratum corneum* should be multilayered containing the essential lipid profile to produce a functional barrier with robustness to resist rapid penetration of cytotoxic benchmark chemicals, e.g. sodium dodecyl sulphate (SDS) or Triton X-100. The barrier function should be demonstrated and may be assessed either by determination of the concentration at which a benchmark chemical reduces the viability of the tissues by 50% (IC₅₀) after a fixed exposure time, or

by determination of the exposure time required to reduce cell viability by 50% (ET₅₀) upon application of the benchmark chemical at a specified, fixed concentration. The containment properties of the RhE model should prevent the passage of material around the *stratum corneum* to the viable tissue, which would lead to poor modelling of skin exposure. The RhE model should be free of contamination by bacteria, viruses, mycoplasma, or fungi.

Functional conditions

Viability

19. The assay used for quantifying viability is the MTT-assay (31). The viable cells of the RhE tissue construct can reduce the vital dye MTT into a blue MTT formazan precipitate which is then extracted from the tissue using isopropanol (or a similar solvent). The optical density (OD) of the extraction solvent alone should be sufficiently small, *i.e.* OD < 0.1. The extracted MTT formazan may be quantified using either a standard absorbance (OD) measurement or an HPLC/UPLC-spectrophotometry procedure (36). The RhE model users should ensure that each batch of the RhE model used meets defined criteria for the negative control. An acceptability range (upper and lower limit) for the negative control OD values (in the Skin Irritation test method conditions) are established by the RhE model developer/supplier. Acceptability ranges for the four validated RhE models included in this test method are given in Table 2. An HPLC/UPLC-Spectrophotometry user should use the negative control OD ranges provided in Table 2 as the acceptance criterion for the negative control. It should be documented that the tissues treated with the negative control are stable in culture (provide similar viability measurements) for the duration of the test exposure period.

Table 2: Acceptability ranges for negative control OD values of the test models included in this TM

	Lower acceptance limit	Upper acceptance limit
EpiSkin™ (SM)	≥ 0.6	≤ 1.5
EpiDerm™ SIT (EPI-200)	≥ 0.8	≤ 2.8
SkinEthic™ RHE	≥ 0.8	≤ 3.0
LabCyte EPI-MODEL24 SIT	≥ 0.7	≤ 2.5

Barrier function

20. The *stratum corneum* and its lipid composition should be sufficient to resist the rapid penetration of cytotoxic benchmark chemicals, *e.g.* SDS or Triton X-100, as estimated by IC₅₀ or ET₅₀ (Table 3).

Morphology

21. Histological examination of the RhE model should be provided demonstrating human *epidermis*-like structure (including multilayered *stratum corneum*).

Reproducibility

22. The results of the positive and negative controls of the test method should demonstrate reproducibility over time.

Quality control (QC)

23. The RhE model should only be used if the developer/supplier demonstrates that each batch of the RhE model used meets defined production release criteria, among which those for *viability* (paragraph 19), *barrier function* (paragraph 20) and *morphology* (paragraph 21) are the most relevant. These data should be provided to the test method users, so that they are able to include this information in the test report. An acceptability range (upper and lower limit) for the IC₅₀ or the ET₅₀ should be established by the RhE model developer/supplier. Only results produced with qualified tissues can be accepted for reliable prediction of irritation classification. The acceptability ranges for the four test models included in this TM are given in Table 3.

Table 3: QC batch release criteria of the test models included in this TM

	Lower acceptance limit	Upper acceptance limit
EpiSkin™ (SM) (18 hours treatment with SDS) (32)	IC ₅₀ = 1.0 mg/ml	IC ₅₀ = 3.0 mg/ml
EpiDerm™ SIT (EPI-200) (1% Triton X-100) (33)	ET ₅₀ = 4.0 hr	ET ₅₀ = 8.7 hr
SkinEthic™ RHE (1% Triton X-100) (34)	ET ₅₀ = 4.0 hr	ET ₅₀ = 10.0 hr
LabCyte EPI-MODEL24 SIT (18 hours treatment with SDS) (35)	IC ₅₀ = 1.4 mg/ml	IC ₅₀ = 4.0 mg/ml

Application of the Test Chemical and Control Chemicals

24. At least three replicates should be used for each test chemical and for the controls in each run. For liquid as well as solid chemicals, sufficient amount of test chemical should be applied to uniformly cover the *epidermis* surface while avoiding an infinite dose, *i.e.* ranging from 26 to 83 µl/cm² or mg/cm² (see Appendix 3). For solid chemicals, the *epidermis* surface should be moistened with deionised or distilled water before application, to improve contact between the test chemical and the *epidermis* surface. Whenever possible, solids should be tested as a fine powder. A nylon mesh may be used as a spreading aid in some cases (see Appendix 3). At the end of the exposure period, the test chemical should be carefully washed from the *epidermis* surface with aqueous buffer, or 0.9% NaCl. Depending on the RhE test models used, the exposure period ranges between 15 and 60 minutes, and the incubation temperature between 20 and 37°C. These exposure periods and temperatures are optimised for each individual RhE test method and represent the different intrinsic properties of the test models (*e.g.* barrier function) (see Appendix 3).

25. Concurrent negative control (NC) and positive control (PC) should be used in each run to demonstrate that viability (using the NC), barrier function and resulting tissue sensitivity (using the PC) of the tissues are within a defined historical acceptance range. The suggested PC is 5% aqueous SDS. The suggested NCs is either water or phosphate buffered saline (PBS).

Cell Viability Measurements

26. According to the test procedure, it is essential that the viability measurement is not performed immediately after exposure to the test chemical, but after a sufficiently long post-treatment incubation period of the rinsed tissue in fresh medium. This period allows both for recovery from weak cytotoxic effects and for appearance of clear cytotoxic effects. A 42 hours post-treatment incubation period was found optimal during test optimisation of two of the RhE-based test models underlying this test method (11) (12) (13) (14) (15).
27. The MTT assay is a standardised quantitative method which should be used to measure cell viability under this test method. It is compatible with use in a three-dimensional tissue construct. The tissue sample is placed in MTT solution of appropriate concentration (*e.g.* 0.3 - 1 mg/ml) for 3 hours. The MTT is converted into blue formazan by the viable cells. The precipitated blue formazan product is then extracted from the tissue using a solvent (*e.g.* isopropanol, acidic isopropanol), and the concentration of formazan is measured by determining the OD at 570 nm using a filter band pass of maximum ± 30 nm or, by using an HPLC/UPLC-spectrophotometry procedure (see paragraph 34) (36).
28. Optical properties of the test chemical or its chemical action on MTT (*e.g.* chemicals may prevent or reverse the colour generation as well as cause it) may interfere with the assay leading to a false estimate of viability. This may occur when a specific test chemical is not completely removed from the tissue by rinsing or when it penetrates the *epidermis*. If a test chemical acts directly on the MTT (*e.g.* MTT-reducer), is naturally coloured, or becomes coloured during tissue treatment, additional controls should be used to detect and correct for test chemical interference with the viability measurement technique (see paragraphs 29 and 33). Detailed description of how to correct direct MTT reduction and interferences by colouring agents is available in the SOPs for the four validated models included in this test method (32) (33) (34) (35).
29. To identify direct MTT reducers, each test chemical should be added to freshly prepared MTT solution. If the MTT mixture containing the test chemical turns blue/purple, the test chemical is presumed to directly reduce MTT and a further functional check on non-viable RhE tissues should be performed, independently of using the standard absorbance (OD) measurement or an HPLC/UPLC-spectrophotometry procedure. This additional functional check employs killed tissues that possess only residual metabolic activity but absorb the test chemical in a similar way as viable tissues. Each MTT reducing test chemical is

applied on at least two killed tissue replicates which undergo the entire testing procedure to generate a non-specific MTT reduction (NSMTT) (32) (33) (34) (35). A single NSMTT control is sufficient per test chemical regardless of the number of independent tests/runs performed. The true tissue viability is then calculated as the percent tissue viability obtained with living tissues exposed to the MTT reducer minus the percent non-specific MTT reduction obtained with the killed tissues exposed to the same MTT reducer, calculated relative to the negative control run concurrently to the test being corrected (%NSMTT).

30. To identify potential interference by coloured test chemicals or test chemicals that become coloured when in contact with water or isopropanol and decide on the need for additional controls, spectral analysis of the test chemical in water (environment during exposure) and/or isopropanol (extracting solution) should be performed. If the test chemical in water and/or isopropanol absorbs light in the range of 570 ± 30 nm, further colorant controls should be performed or, alternatively, an HPLC/UPLC-spectrophotometry procedure should be used in which case these controls are not required (see paragraphs 33 and 34). When performing the standard absorbance (OD) measurement, each interfering coloured test chemical is applied on at least two viable tissue replicates, which undergo the entire testing procedure but are incubated with medium instead of MTT solution during the MTT incubation step to generate a non-specific colour (NSC_{living}) control. The NSC_{living} control needs to be performed concurrently to the testing of the coloured test chemical and in case of multiple testing, an independent NSC_{living} control needs to be conducted with each test performed (in each run) due to the inherent biological variability of living tissues. The true tissue viability is then calculated as the percent tissue viability obtained with living tissues exposed to the interfering test chemical and incubated with MTT solution minus the percent non-specific colour obtained with living tissues exposed to the interfering test chemical and incubated with medium without MTT, run concurrently to the test being corrected (%NSC_{living}).
31. Test chemicals that are identified as producing both direct MTT reduction (see paragraph 29) and colour interference (see paragraph 30) will also require a third set of controls, apart from the NSMTT and NSC_{living} controls described in the previous paragraphs, when performing the standard absorbance (OD) measurement.. This is usually the case with darkly coloured test chemicals interfering with the MTT assay (e.g. blue, purple, black) because their intrinsic colour impedes the assessment of their capacity to directly reduce MTT as described in paragraph 29. These test chemicals may bind to both living and killed tissues and therefore the NSMTT control may not only correct for potential direct MTT reduction by the test chemical, but also for colour interference arising from the binding of the test chemical to killed tissues. This could lead to a double correction for colour interference since the NSC_{living} control already corrects for colour interference arising from the binding of the test chemical to living tissues. To avoid a possible double correction for

colour interference, a third control for non-specific colour in killed tissues (NSC_{killed}) needs to be performed. In this additional control, the test chemical is applied on at least two killed tissue replicates, which undergo the entire testing procedure but are incubated with medium instead of MTT solution during the MTT incubation step. A single NSC_{killed} control is sufficient per test chemical regardless of the number of independent tests/runs performed, but should be performed concurrently to the NSMTT control and, where possible, with the same tissue batch. The true tissue viability is then calculated as the percent tissue viability obtained with living tissues exposed to the test chemical minus %NSMTT minus %NSC_{living} plus the percent non-specific colour obtained with killed tissues exposed to the interfering test chemical and incubated with medium without MTT, calculated relative to the negative control run concurrently to the test being corrected (%NSC_{killed}).

32. It is important to note that non-specific MTT reduction and non-specific colour interferences may increase the readouts of the tissue extract above the linearity range of the spectrophotometer. On this basis, each laboratory should determine the linearity range of their spectrophotometer with MTT formazan (CAS # 57360-69-7) from a commercial source before initiating the testing of test chemicals for regulatory purposes. The standard absorbance (OD) measurement using a spectrophotometer is appropriate to assess direct MTT-reducers and colour interfering test chemicals when the ODs of the tissue extracts obtained with the test chemical without any correction for direct MTT reduction and/or colour interference are within the linear range of the spectrophotometer or when the uncorrected percent viability obtained with the test chemical is already $\leq 50\%$. Nevertheless, results for test chemicals producing %NSMTT and/or %NSC_{living} $\geq 50\%$ of the negative control should be taken with caution as this is the cut-off used to distinguish classified from not classified chemicals (see paragraph 36).
33. For coloured test chemicals which are not compatible with the standard absorbance (OD) measurement due to too strong interference with the MTT assay, the alternative HPLC/UPLC-spectrophotometry procedure to measure MTT formazan may be employed (see paragraph 34) (36). The HPLC/UPLC-spectrophotometry system allows for the separation of the MTT formazan from the test chemical before its quantification (36). For this reason, NSC_{living} or NSC_{killed} controls are never required when using HPLC/UPLC-spectrophotometry, independently of the chemical being tested. NSMTT controls should nevertheless be used if the test chemical is suspected to directly reduce MTT or has a colour that impedes the assessment of the capacity to directly reduce MTT (as described in paragraph 29). When using HPLC/UPLC-spectrophotometry to measure MTT formazan, the percent tissue viability is calculated as percent MTT formazan peak area obtained with living tissues exposed to the test chemical relative to the MTT formazan peak obtained with the concurrent negative control. For test chemicals able to directly reduce MTT, true tissue viability is calculated as the percent tissue viability obtained with living tissues

exposed to the test chemical minus %NSMTT. Finally, it should be noted that direct MTT-reducers that may also be colour interfering, which are retained in the tissues after treatment and reduce MTT so strongly that they lead to ODs (using standard OD measurement) or peak areas (using UPLC/HPLC-spectrophotometry) of the tested tissue extracts that fall outside of the linearity range of the spectrophotometer cannot be assessed, although these are expected to occur in only very rare situations.

34. HPLC/UPLC-spectrophotometry may be used also with all types of test chemicals (coloured, non-coloured, MTT-reducers and non-MTT reducers) for measurement of MTT formazan (36). Due to the diversity of HPLC/UPLC-spectrophotometry systems, qualification of the HPLC/UPLC-spectrophotometry system should be demonstrated before its use to quantify MTT formazan from tissue extracts by meeting the acceptance criteria for a set of standard qualification parameters based on those described in the U.S. Food and Drug Administration guidance for industry on bio-analytical method validation (36) (37). These key parameters and their acceptance criteria are shown in Appendix 4. Once the acceptance criteria defined in Appendix 4 have been met, the HPLC/UPLC-spectrophotometry system is considered qualified and ready to measure MTT formazan under the experimental conditions described in this test method.

Acceptability Criteria

35. For each test method using valid RhE model batches (see paragraph 23), tissues treated with the negative control should exhibit OD reflecting the quality of the tissues that followed shipment, receipt steps and all protocol processes. Control OD values should not be below historically established boundaries. Similarly, tissues treated with the PC, *i.e.* 5% aqueous SDS, should reflect their ability to respond to an irritant chemical under the conditions of the test method (see Appendix 3 and for further information SOPs of the four test models included in this TG (32) (33) (34) (35)). Associated and appropriate measures of variability between tissue replicates, *i.e.* standard deviations (SD) should fall within the acceptance limits established for the test model used (see Appendix 3).

Interpretation of Results and Prediction Model

36. The OD values obtained with each test chemical can be used to calculate the percentage of viability normalised to the negative control, which is set to 100%. In case HPLC/UPLC-spectrophotometry is used, the percent tissue viability is calculated as percent MTT formazan peak area obtained with living tissues exposed to the test chemical relative to the MTT formazan peak obtained with the concurrent negative control. The cut-off value of percentage cell viability distinguishing irritant from non-classified test chemicals and the statistical procedure(s) used to evaluate the results and identify irritant chemicals should be clearly defined, documented, and proven to be appropriate (see SOPs of the test models for information). The cut-off values for the prediction of irritation are given below:

- The test chemical is identified as requiring classification and labelling according to UN GHS/CLP (Category 2 or Category 1) if the mean percent tissue viability after exposure and post-treatment incubation is less than or equal ($\leq$) to 50%. Since the RhE test models covered by this test method cannot resolve between UN GHS/CLP Categories 1 and 2, further information on skin corrosion will be required to decide on its final classification [see also the OECD Guidance Document on IATA (3)]. In case the test chemical is found to be non-corrosive (e.g. based on TM.40, B.40bis or B.65), and shows tissue viability after exposure and post-treatment incubation is less than or equal ($\leq$) to 50%, the test chemical is considered to be irritant to skin in accordance with UN GHS/CLP Category 2.
- Depending on the regulatory framework in member countries, the test chemical may be considered as non-irritant to skin in accordance with UN GHS/CLP No Category if the tissue viability after exposure and post-treatment incubation is more than ($>$) 50%.

DATA AND REPORTING

Data

37. For each run, data from individual replicate tissues (*e.g.* OD values and calculated percentage cell viability data for each test chemical, including classification) should be reported in tabular form, including data from repeat experiments as appropriate. In addition means $\pm$ SD for each run should be reported. Observed interactions with MTT reagent and coloured test chemicals should be reported for each tested chemical.

Test Report

38. The test report should include the following information:

Test Chemical and Control Chemicals:

- Mono-constituent substance: chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc;
- Multi-constituent substance, UVCB and mixture: characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents;
- Physical appearance, water solubility, and any additional relevant physicochemical properties;
- Source, lot number if available;
- Treatment of the test chemical/control chemicals prior to testing, if applicable (*e.g.* warming, grinding);
- Stability of the test chemical, limit date for use, or date for re-analysis if known;

- Storage conditions.

RhE model and protocol used (and rationale for the choice, if applicable)

Test Conditions:

- RhE model used (including batch number);
- Calibration information for measuring device (*e.g.* spectrophotometer), wavelength and band pass (if applicable) used for quantifying MTT formazan, and linearity range of measuring device; Description of the method used to quantify MTT formazan;
- Description of the qualification of the HPLC/UPLC-spectrophotometry system, if applicable; Complete supporting information for the specific RhE model used including its performance. This should include, but is not limited to;
 - i) Viability;
 - ii) Barrier function;
 - iii) Morphology;
 - iv) Reproducibility and predictivity;
 - v) Quality controls (QC) of the model;
- Reference to historical data of the model. This should include, but is not limited to acceptability of the QC data with reference to historical batch data.
- Demonstration of proficiency in performing the test method before routine use by testing of the proficiency substances.

Test Procedure:

- Details of the test procedure used (including washing procedures used after exposure period); Dose of test chemical and controls used;
- Duration and temperature of exposure and post-exposure incubation period;
- Indication of controls used for direct MTT-reducers and/or colouring test chemicals, if applicable;
- Number of tissue replicates used per test chemical and controls (PC, negative control, and NSMTT, NSC_{living} and NSC_{killed}, if applicable);
- Description of decision criteria/prediction model applied based on the RhE model used;
- Description of any modifications to the test procedure (including washing procedures).

Run and Test Acceptance Criteria:

- Positive and negative control mean values and acceptance ranges based on historical data; Acceptable variability between tissue replicates for positive and negative controls;
- Acceptable variability between tissue replicates for test chemical.

Results:

- Tabulation of data for individual test chemical for each run and each replicate measurement including OD or MTT formazan peak area, percent tissue viability, mean percent tissue viability and SD;
- If applicable, results of controls used for direct MTT-reducers and/or colouring test chemicals including OD or MTT formazan peak area, %NSMTT, %NSC_{living}, %NSC_{killed}, SD, final correct percent tissue viability;
- Results obtained with the test chemical(s) and controls in relation to the defined run and test acceptance criteria;
- Description of other effects observed;
- The derived classification with reference to the prediction model/decision criteria used.

Discussion of the results

Conclusions

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Appendix 1

DEFINITIONS

Accuracy: The closeness of agreement between test method results and accepted reference values. It is a measure of test method performance and one aspect of relevance. The term is often used interchangeably with “concordance” to mean the proportion of correct outcomes of a test method (9).

Cell viability: Parameter measuring total activity of a cell population *e.g.* as ability of cellular mitochondrial dehydrogenases to reduce the vital dye MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Thiazolyl blue), which depending on the endpoint measured and the test design used, correlates with the total number and/or vitality of living cells.

Chemical: means a substance or a mixture.

Concordance: This is a measure of performance for test models that give a categorical result, and is one aspect of relevance. The term is sometimes used interchangeably with accuracy, and is defined as the proportion of all chemicals tested that are correctly classified as positive or negative. Concordance is highly dependent on the prevalence of positives in the types of test chemical being examined (9).

ET₅₀: Can be estimated by determination of the exposure time required to reduce cell viability by 50% upon application of the benchmark chemical at a specified, fixed concentration, see also IC₅₀.

GHS (Globally Harmonized System of Classification and Labelling of Chemicals by the United Nations (UN)): A system proposing the classification of chemicals (substances and mixtures) according to standardised types and levels of physical, health and environmental hazards, and addressing corresponding communication elements, such as pictograms, signal words, hazard statements, precautionary statements and safety data sheets, so that to convey information on their adverse effects with a view to protect people (including employers, workers, transporters, consumers and emergency responders) and the environment (1).

HPLC: High Performance Liquid Chromatography.

IATA: Integrated Approach on Testing and Assessment

IC₅₀: Can be estimated by determination of the concentration at which a benchmark chemical reduces the viability of the tissues by 50% (IC₅₀) after a fixed exposure time, see also ET₅₀.

Infinite dose: Amount of test chemical applied to the *epidermis* exceeding the amount required to completely and uniformly cover the *epidermis* surface.

Mixture: A mixture or a solution composed of two or more substances.

Mono-constituent substance: A substance, defined by its quantitative composition, in which one main constituent is present to at least 80% (w/w).

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Thiazolyl blue tetrazolium bromide.

Multi-constituent substance: A substance, defined by its quantitative composition, in which more than one main constituent is present in a concentration $\geq 10\%$ (w/w) and $< 80\%$ (w/w). A multi-constituent substance is the result of a manufacturing process. The difference between mixture and multi-constituent substance is that a mixture is obtained by blending of two or more substances without chemical reaction. A multi-constituent substance is the result of a chemical reaction.

NSC_{killed}: Non-Specific Colour in killed tissues.

NSC_{living}: Non-Specific Colour in living tissues.

NSMTT: Non-Specific MTT reduction.

Performance standards (PS): Standards, based on a validated test method, that provide a basis for evaluating the comparability of a proposed test method that is mechanistically and functionally similar. Included are; (i) essential test method components; (ii) a minimum list of Reference Chemicals selected from among the chemicals used to demonstrate the acceptable performance of the validated test method; and (iii) the comparable levels of accuracy and reliability, based on what was obtained for the validated test method, that the proposed test method should demonstrate when evaluated using the minimum list of Reference Chemicals (9).

PC: Positive Control, a replicate containing all components of a test system and treated with a chemical known to induce a positive response. To ensure that variability in the positive control response across time can be assessed, the magnitude of the positive response should not be excessive.

Relevance: Description of relationship of the test to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the test correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of the accuracy (concordance) of a test method (9).

Reliability: Measures of the extent that a test method can be performed reproducibly within and between laboratories over time, when performed using the same protocol. It is assessed by calculating intra- and inter-laboratory reproducibility (9).

Replacement test: A test which is designed to substitute for a test that is in routine use and accepted for hazard identification and/or risk assessment, and which has been determined to provide equivalent or improved protection of human or animal health or the environment, as applicable, compared to the accepted test, for all possible testing situations and chemicals

(9).

Run: A run consists of one or more test chemicals tested concurrently with a negative control and with a PC.

Sensitivity: The proportion of all positive/active test chemicals that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results, and is an important consideration in assessing the relevance of a test method (9).

Skin irritation *in vivo*: The production of reversible damage to the skin following the application of a test chemical for up to 4 hours. Skin irritation is a locally arising reaction of the affected skin tissue and appears shortly after stimulation (38). It is caused by a local inflammatory reaction involving the innate (non-specific) immune system of the skin tissue. Its main characteristic is its reversible process involving inflammatory reactions and most of the clinical characteristic signs of irritation (erythema, oedema, itching and pain) related to an inflammatory process.

Specificity: The proportion of all negative/inactive test chemicals that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results and is an important consideration in assessing the relevance of a test method (9).

Substance: A chemical element and its compounds in the natural state or obtained by any production process, inducing any additive necessary to preserve its stability and any impurities deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the substance or changing its composition.

Test chemical: Any substance or mixture tested using this test method..

UPLC: Ultra-High Performance Liquid Chromatography.

UVCB: substances of unknown or variable composition, complex reaction products or biological materials.

Appendix 2

TEST MODELS INCLUDED IN THIS TEST METHOD

Nr.	Test model name	Validation study type	References
1	EpiSkin™	Full prospective validation study (2003-2007). The components of this model were used to define the essential test method components of the original and updated ECVAM PS (39) (40) (21)*. Moreover, the method's data relating to identification of non-classified vs classified substances formed the main basis for defining the specificity and sensitivity values of the original PS*.	(2) (10) (11) (14) (15) (16) (17) (18) (19) (20) (21) (23) (32) (39) (40)
2	EpiDerm™ SIT (EPI-200)	EpiDerm™ (original): Initially the test model underwent full prospective validation together with Nr. 1. from 2003-2007. The components of this model were used to define the essential test methods components of the original and updated ECVAM PS (39) (40) (21)*. EpiDerm™ SIT (EPI-200): A modification of the original EpiDerm™ was validated using the original ECVAM PS (21) in 2008*	(2) (10) (12) (13) (15) (16) (17) (18) (20) (21) (23) (33) (39) (40) (2) (21) (22) (23) (33)
3	SkinEthic™ RHE	Validation study based on the original ECVAM Performance Standards (21) in 2008*.	(2) (21) (22) (23) (31)
4	LabCyte EPI-MODEL24 SIT	Validation study (2011-2012) based on the Performance Standards (PS) of OECD TG 439 (8) which are based on the updated ECVAM PS* (39) (40).	(24) (25) (26) (27) (28) (35) (39) (40) and PS of this TG (8)*

*) The original ECVAM Performance Standards (PS) (21) were developed in 2007 upon completion of the prospective validation study (16) which had assessed the performance of test models Nr 1 and 2 in reference to the classification system as described in the 28th amendment to the EU Dangerous Substances Directive (41). In 2008 the UN GHS (1) and EU CLP were introduced, effectively shifting the cut-off value for distinguishing non-classified from classified substances from an *in vivo* score of 2.0 to 2.3. To adapt to this changed regulatory requirement, the accuracy values and reference chemical list of the ECVAM PS were updated in 2009 (2) (39) (40). As the original PS, also the updated PS were largely based on data from models Nr. 1 and 2 (16), but additionally used data on reference chemicals from model Nr. 3. In 2010, the updated ECVAM PS were used for stipulating the PS related to this TG (8). For the purpose of this test method, EpiSkin™ is considered the VRM, due to the fact that it was used to develop all the criteria of the PS.. Detailed information on the validation studies, a compilation of the data generated as well as background to the necessary adaptations of the PS as a consequence of the UN GHS/CLP implementation can be found in the ECVAM/BfR explanatory background document to the corresponding OECD TG 439 (23).

SIT: Skin Irritation Test

RHE: Reconstructed Human Epidermis

Appendix 3

PROTOCOL PARAMETERS SPECIFIC TO EACH OF THE TEST MODELS INCLUDED IN THIS TEST METHOD

The RhE models do show very similar protocols and notably all use a post-incubation period of 42 hours (32) (33) (34) (35). Variations concern mainly three parameters relating to the different barrier functions of the test models and listed here: A) pre-incubation time and volume, B) Application of test chemicals and C) Post-incubation volume.

	EpiSkin™ (SM)	EpiDerm™ SIT (EPI-200)	SkinEthic RHE™	LabCyte EPI-MODEL24 SIT
A) Pre-incubation				
Incubation time	18- 24 hours	18-24 hours	< 2 hours	15-30 hours
Medium volume	2ml	0.9ml	0.3 or 1ml	0.5ml
B) Test chemical application				
For liquids	10µl (26µl/cm²)	30µl (47µl/cm²)	16µl (32µl/cm²)	25µl (83µl/cm²)
For solids	10mg (26mg/cm²) + DW (5µl)	25mg (39mg/cm²) + DPBS (25µl)	16mg (32mg/cm²) + DW (10µl)	25mg (83mg/cm²) + DW (25µl)
Use of nylon mesh	Not used	If necessary	Applied	Not used
Total application time	15 minutes	60 minutes	42 minutes	15 minutes
Application temperature	RT	a) at RT for 25 minutes b) at 37°C for 35 minutes	RT	RT
C) Post-incubation volume				
Medium volume	2 ml	0.9ml x 2	2 ml	1 ml
D) Maximum acceptable variability				
Standard deviation between tissue replicates	SD≤18	SD≤18	SD≤18	SD≤18

RT: Room temperature

DW: distilled water

DPBS: Dulbecco's Phosphate Buffer Saline

Appendix 4

KEY PARAMETERS AND ACCEPTANCE CRITERIA FOR QUALIFICATION OF AN HPLC/UPLC-SPECTROPHOTOMETRY SYSTEM FOR MEASUREMENT OF MTT FORMAZAN EXTRACTED FROM RHE TISSUES

Parameter	Protocol Derived from FDA Guidance (36) (37)	Acceptance Criteria
Selectivity	Analysis of isopropanol, living blank (isopropanol extract from living RhE tissues without any treatment), dead blank (isopropanol extract from killed RhE tissues without any treatment)	$\text{Area}_{\text{interference}} \leq 20\% \text{ of } \text{Area}_{\text{LLOQ}}^1$
Precision	Quality Controls (i.e. MTT formazan at 1.6 µg/ml, 16 µg/ml and 160 µg/ml) in isopropanol (n=5)	$\text{CV} \leq 15\% \text{ or } \leq 20\% \text{ for the LLOQ}$
Accuracy	Quality Controls in isopropanol (n=5)	$\% \text{Dev} \leq 15\% \text{ or } \leq 20\% \text{ for LLOQ}$
Matrix Effect	Quality Controls in living blank (n=5)	$85\% \leq \text{Matrix Effect } \% \leq 115\%$
Carryover	Analysis of isopropanol after an ULOQ ² standard	$\text{Area}_{\text{interference}} \leq 20\% \text{ of } \text{Area}_{\text{LLOQ}}$
Reproducibility (intra-day)	3 independent calibration curves (based on 6 consecutive 1/3 dilutions of MTT formazan in isopropanol starting at ULOQ, i.e. 200 µg/ml); Quality Controls in isopropanol (n=5)	Calibration Curves: $\% \text{Dev} \leq 15\% \text{ or } \leq 20\% \text{ for LLOQ}$ Quality Controls: $\% \text{Dev} \leq 15\% \text{ and } \text{CV} \leq 15\%$
Reproducibility (inter-day)	Day 1: 1 calibration curve and Quality Controls in isopropanol (n=3) Day 2: 1 calibration curve and Quality Controls in isopropanol (n=3) Day 3: 1 calibration curve and Quality Controls in isopropanol (n=3)	
Short Term Stability of MTT Formazan in RhE Tissue Extract	Quality Controls in living blank (n=3) analysed the day of the preparation and after 24 hours of storage at room temperature	$\% \text{Dev} \leq 15\%$
Long Term Stability of MTT Formazan in RhE Tissue Extract, if required	Quality Controls in living blank (n=3) analysed the day of the preparation and after several days of storage at a specified temperature (e.g. 4°C, -20°C, -80°C)	$\% \text{Dev} \leq 15\%$

¹LLOQ: Lower Limit of Quantification, defined to cover 1-2% tissue viability, i.e. 0.8 µg/ml.

²ULOQ: Upper Limit of Quantification, defined to be at least two times higher than the highest expected MTT formazan concentration in isopropanol extracts from negative controls i.e. 200 µg/ml."

(8) In Part B, the following Chapters are added:

"B.63 REPRODUCTION/DEVELOPMENTAL TOXICITY SCREENING TEST"

INTRODUCTION

1. This test method is equivalent to OECD test guideline (TG) 421 (2016). OECD guidelines for the testing of chemicals are periodically reviewed in the light of scientific progress. The original screening test guideline 421 was adopted in 1995, based on a protocol for a "Preliminary Reproduction Toxicity Screening Test" discussed in two expert meetings, in London in 1990 (1) and in Tokyo in 1992 (2).
2. This test method has been updated with endocrine disruptor relevant endpoints, as a follow up to the high-priority activity initiated at OECD in 1998 to revise existing test guidelines and to develop new test guidelines for the screening and testing of potential endocrine disruptors (3). OECD TG 407 (Repeated Dose 28-Day Oral Toxicity Study in Rodents, Chapter B.7 of this Annex) for example, was enhanced in 2008 by parameters suitable to detect endocrine activity of test chemicals. The objective in updating TG 421 was to include some endocrine disruptor relevant endpoints in screening TGs where the exposure periods cover some of the sensitive periods during development (pre- or early postnatal periods).
3. The selected additional endocrine disruptor relevant endpoints, also part of TG 443 (Extended One Generation Reproductive Toxicity Study, Chapter B.56 of this Annex), were included in TG 421 based on a feasibility study addressing scientific and technical questions related to their inclusion, as well as possible adaptations of the test design needed for their inclusion (4).
4. This test method is designed to generate limited information concerning the effects of a test chemical on male and female reproductive performance such as gonadal function, mating behaviour, conception, development of the conceptus and parturition. It is not an alternative to, nor does it replace the existing test methods B.31, B.34, B.35 or B.56.

INITIAL CONSIDERATIONS

5. This screening test method can be used to provide initial information on possible effects on reproduction and/or development, either at an early stage of assessing the toxicological properties of chemicals, or on chemicals of concern. It can also be used as part of a set of initial screening tests for existing chemicals for which little or no toxicological information is available, as a dose range finding study for more extensive reproduction/developmental studies, or when otherwise considered relevant. In conducting the study, the guiding principles and considerations outlined in the OECD guidance document no 19 on the

recognition, assessment, and use of clinical signs as humane endpoints for experimental animals used in safety evaluations (5) should be followed.

6. This test method does not provide complete information on all aspects of reproduction and development. In particular, it offers only limited means of detecting post-natal manifestations of pre-natal exposure, or effects that may be induced during post-natal exposure. Due (amongst other reasons) to the relatively small numbers of animals in the dose groups, the selectivity of the end points, and the short duration of the study, this method will not provide evidence for definite claims of no effects. Moreover, in the absence of data from other reproduction/developmental toxicity tests, positive results are useful for initial hazard assessment and contribute to decisions with respect to the necessity and timing of additional testing.
7. The results obtained by the endocrine related parameters should be seen in the context of the “OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupting Chemicals” (6). In this Conceptual Framework, the enhanced OECD TG 421 is contained in level 4 as an *in vivo* assay providing data on adverse effects on endocrine relevant endpoints. An endocrine signal might not however be considered sufficient evidence on its own that the test chemical is an endocrine disruptor.
8. This test method assumes oral administration of the test chemical. Modifications may be required if other routes of exposure are used.
9. Before use of the test method on a mixture for generating data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed, when there is a regulatory requirement for testing of the mixture.
10. Definitions used are given in Appendix 1.

PRINCIPLE OF THE TEST

11. The test chemical is administered in graduated doses to several groups of males and females. Males should be dosed for a minimum of four weeks and up to and including the day before scheduled kill (this includes a minimum of two weeks prior to mating, during the mating period and, approximately, two weeks post-mating). In view of the limited pre-mating dosing period in males, fertility may not be a particularly sensitive indicator of testicular toxicity. Therefore, a detailed histological examination of the testes is essential. The combination of a pre-mating dosing period of two weeks and subsequent mating/fertility observations with an overall dosing period of at least four weeks, followed by detailed histopathology of the male gonads, is considered sufficient to enable detection of the majority of effects on male fertility and spermatogenesis.

12. Females should be dosed throughout the study. This includes two weeks prior to mating (with the objective of covering at least two complete oestrous cycles), the variable time to conception, the duration of pregnancy and at least thirteen days after delivery, up to and including the day before scheduled kill.
13. Duration of study, following acclimatisation and pre-dosing oestrous cycle evaluation, is dependent on the female performance and is approximately 63 days, [at least 14 days pre-mating, (up to) 14 days mating, 22 days gestation, 13 days lactation].
14. During the period of administration, the animals are observed closely each day for signs of toxicity. Animals which die or are killed during the test period are necropsied and, at the conclusion of the test, surviving animals are killed and necropsied.

DESCRIPTION OF THE METHOD

Selection of animal species

15. This test method is designed for use with the rat. If the parameters specified within this test method are investigated in another rodent species a detailed justification should be given. In the international validation program for the detection of endocrine disrupters in OECD TG 407 (corresponding to Chapter B.7 of this Annex), the rat was the only species used. Strains with low fecundity or well-known high incidence of developmental defects should not be used. Healthy virgin animals, not subjected to previous experimental procedures, should be used. The test animals should be characterised as to species, strain, sex, weight and age. At the commencement of the study the weight variation of animals used should be minimal and not exceed 20% of the mean weight of each sex. Where the study is conducted as a preliminary study to a long-term or a full-generation study, it is preferable that animals from the same strain and source are used in both studies.

Housing and feeding

16. All procedures should conform to local standards of laboratory animal care. The temperature in the experimental animal room should be 22 °C ($\pm$ 3°). Although the relative humidity should be at least 30% and preferably not exceed 70% other than during room cleaning, the aim should be 50-60%. Lighting should be artificial, the photoperiod being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. The choice of diet may be influenced by the need to ensure a suitable admixture of a test chemical when administered by this method.
17. Animals should be group housed in small groups of the same sex; animals may be housed individually if scientifically justified. For group caging, no more than five animals should be housed per cage. Mating procedures should be carried out in cages suitable for the purpose. Pregnant females should be caged individually and provided with nesting materials. Lactating females will be caged individually with their offspring.

18. The feed should be regularly analysed for contaminants. A sample of the diet should be retained until finalisation of the report.

Preparation of the animals

19. Healthy young adult animals are randomly assigned to the control and treatment groups. Cages should be arranged in such a way that possible effects due to cage placement are minimised. The animals are uniquely identified and kept in their cages for at least five days prior to the start of the study to allow for acclimatisation to the laboratory conditions.

Preparation of doses

20. It is recommended that the test chemical be administered orally unless other routes of administration are considered more appropriate. When the oral route is selected, the test chemical is usually administered by gavage; however, alternatively, test chemicals may be administered via the diet or drinking water.
21. Where necessary, the test chemical is dissolved or suspended in a suitable vehicle. It is recommended that, wherever possible, the use of an aqueous solution/suspension be considered first, followed by consideration of a solution/emulsion in oil (e.g. corn oil) and then by possible solution in other vehicles. For vehicles other than water the toxic characteristics of the vehicle should be known. The stability and homogeneity of the test chemical in the vehicle should be determined.

PROCEDURE

Number and sex of animals

22. It is recommended that each group be started with at least 10 males and 12-13 females. Females will be evaluated pre-exposure for oestrous cyclicity and animals that fail to exhibit typical 4-5 day cycles will not be included in the study; therefore, extra females are recommended in order to yield 10 females per group. Except in the case of marked toxic effects, it is expected that this will provide at least 8 pregnant females per group which normally is the minimum acceptable number of pregnant females per group. The objective is to produce enough pregnancies and offspring to assure a meaningful evaluation of the potential of the test chemical to affect fertility, pregnancy, maternal and suckling behaviour, and growth and development of the F₁ offspring from conception to day 13 post-partum.

Dosage

23. Generally, at least three test groups and a control group should be used. Dose levels may be based on information from acute toxicity tests or on results from repeated dose studies. Except for treatment with the test chemical, animals in the control group should be handled

in an identical manner to the test group subjects. If a vehicle is used in administering the test chemical, the control group should receive the vehicle in the highest volume used.

24. Dose levels should be selected taking into account any existing toxicity and (toxico-) kinetic data available. It should also be taken into account that there may be differences in sensitivity between pregnant and non-pregnant animals. The highest dose level should be chosen with the aim of inducing toxic effects but not death or severe suffering. Thereafter, a descending sequence of dose levels should be selected with a view to demonstrating any dosage related response and no-observed-adverse effects (NOAEL) at the lowest dose level. Two to four fold intervals are frequently optimal for setting the descending dose levels and addition of a fourth test group is often preferable to using very large intervals (e.g. more than a factor of 10) between dosages.
25. In the presence of observed general toxicity (e.g. reduced body weight, liver , heart, lung or kidney effects, etc.) or other changes that may not be toxic responses (e.g. reduced food intake, liver enlargement), observed effects on endocrine sensitive endpoints should be interpreted with caution.

Limit test

26. If an oral study at one dose level of at least 1000 mg/kg body weight/day or, for dietary or drinking water administration, an equivalent percentage in the diet or drinking water, using the procedures described for this study, produces no observable toxic effects and if toxicity would not be expected based upon data from structurally related substances, then a full study using several dose levels may not be considered necessary. The limit test applies except when human exposure indicates the need for a higher oral dose level to be used. For other types of administration, such as inhalation or dermal application, the physical chemical properties of the test chemicals often may dictate the maximum attainable concentration.

Administration of doses

27. The animals are dosed with the test chemical daily for 7 days a week. When the test chemical is administered by gavage, this should be done in a single dose to the animals using a stomach tube or a suitable intubation cannula. The maximum volume of liquid that can be administered at one time depends on the size of the test animal. The volume should not exceed 1 ml/100 g body weight, except in the case of aqueous solutions where 2 ml/100 g body weight may be used. Except for irritating or corrosive test chemicals which will normally reveal exacerbated effects with higher concentrations, variability in test volume should be minimised by adjusting the concentration to ensure a constant volume at all dose levels.
28. For test chemical administered via the diet or drinking water, it is important to ensure that the quantities of the test chemical involved do not interfere with normal nutrition or water

balance. When the test chemical is administered in the diet either a constant dietary concentration (ppm) or a constant dose level in terms of the animals' body weight may be used; the alternative used should be specified. For a test chemical administered by gavage, the dose should be given at similar times each day, and adjusted at least weekly to maintain a constant dose level in terms of animal body weight.

Experimental schedule

29. Dosing of both sexes should begin at least 2 weeks prior to mating, after they have been acclimatised for at least five days and females have been screened for normal oestrous cycles (in a 2 weeks pre-treatment period). The study should be scheduled in such a way that oestrous cycle evaluation begins soon after the animals have attained full sexual maturity. This may vary slightly for different strains of rats in different laboratories, e.g. Sprague Dawley rats 10 weeks of age, Wistar rats about 12 weeks of age. Dams with offspring should be killed on day 13 post-partum, or shortly thereafter. The day of birth (viz. when parturition is complete) is defined as day 0 post-partum. Females showing no-evidence of copulation are killed 24-26 days after the last day of the mating period. Dosing is continued in both sexes during the mating period. Males should further be dosed after the mating period at least until the minimum total dosing period of 28 days has been completed. They are then killed, or, alternatively, are retained and continued to be dosed for the possible conduction of a second mating if considered appropriate.
30. Daily dosing of the parental females should continue throughout pregnancy and at least up to, and including, day 13 post-partum or the day before sacrifice. For studies where the test chemical is administered by inhalation or by the dermal route, dosing should be continued at least up to, and including, day 19 of gestation, and dosing should be re-initiated as soon as possible and not later than PND 4.
31. A diagram of the experimental schedule is given in Appendix 2.

Mating procedure

32. Normally, 1:1 (one male to one female) matings should be used in this study. Exceptions can arise in the case of occasional deaths of males. The female should be placed with the same male until evidence of copulation is observed or two weeks have elapsed. Each morning the females should be examined for the presence of sperm or a vaginal plug. Day 0 of pregnancy is defined as the day on which mating evidence is confirmed (a vaginal plug or sperm is found). In case pairing is unsuccessful, re-mating of females with proven males of the same group could be considered.

Litter size

33. On day 4 after birth, the size of each litter may be adjusted by eliminating extra pups by random selection to yield, as nearly as possible, four or five pups per sex per litter

depending on the normal litter size in the strain of rats used. Blood samples should be collected from two of the surplus pups, pooled, and used for determination of serum T4 levels. Selective elimination of pups, e.g. based upon body weight, or anogenital distance (AGD) is not appropriate. Whenever the number of male or female pups prevents having four or five of each sex per litter, partial adjustment (for example, six males and four females) is acceptable. No pups will be eliminated when litter size will drop below the culling target (8 or 10 pups/litter). If there is only one pup available above the culling target, only one pup will be eliminated and used for blood collection for possible serum T4 assessments.

34. If litter size is not adjusted, two pups per litter are sacrificed on day 4 after birth and blood samples are taken for measurement of serum thyroid hormone concentrations. If possible the two pups per litter should be female pups to reserve male pups for nipple retention evaluations except in the event that removing these pups leaves no remaining females for assessment at termination. No pups will be eliminated when litter size will drop below 8 or 10 pups/litter (depending on the normal litter size in the strain of rats used). If there is only one pup available above the normal litter size, only one pup will be eliminated and used for blood collection for possible serum T4 assessments.

In life observations

Clinical observations

35. Throughout the test period, general clinical observations should be made at least once a day, and more frequently when signs of toxicity are observed. They should be made preferably at the same time(s) each day, considering the peak period of anticipated effects after dosing. Pertinent behavioural changes, signs of difficult or prolonged parturition and all signs of toxicity, including mortality, should be recorded. These records should include time of onset, degree and duration of toxicity signs.

Body weight and food/water consumption

36. Males and females should be weighed on the first day of dosing, at least weekly thereafter, and at termination. During pregnancy, females should be weighed on days 0, 7, 14 and 20 and within 24 hours of parturition (day 0 or 1 post-partum) and at least day 4 and 13 post-partum. These observations should be reported individually for each adult animal.
37. During pre-mating, pregnancy and lactation, food consumption should be measured at least weekly. The measurement of food consumption during mating is optional. Water consumption during these periods should also be measured when the test chemical is administered via drinking water.

Oestrous cycles

38. Oestrous cycles should be monitored before treatment starts to select for the study females with regular cyclicity (see paragraph 22). Vaginal smears should also be monitored daily from the beginning of the treatment period until evidence of mating. If there is concern about acute stress effects that could alter oestrous cycles with the initiation of dosing, laboratories can expose test animals for 2 weeks, then collect vaginal smears daily to monitor oestrous cycle for a minimum of two weeks during the pre-mating period with continued monitoring into the mating period until there is evidence of mating. When obtaining vaginal/cervical cells, care should be taken to avoid disturbance of mucosa, which could induce pseudopregnancy (7) (8).

Offspring parameters

39. The duration of gestation should be recorded and is calculated from day 0 of pregnancy. Each litter should be examined as soon as possible after delivery to establish the number and sex of pups, stillbirths, live births, runts (pups that are significantly smaller than corresponding control pups) and the presence of gross abnormalities.
40. Live pups should be counted and sexed and litters weighed within 24 hours of parturition (day 0 or 1 post-partum) and at least on day 4 and 13 post-partum. In addition to the observations described in paragraph 35, any abnormal behaviour of the offspring should be recorded.
41. The AGD of each pup should be measured on the same postnatal day between PND 0 through PND 4. Pup body weight should be collected on the day the AGD is measured and the AGD should be normalised to a measure of pup size, preferably the cube root of body weight (9). The number of nipples/areolae in male pups should be counted on PND 12 or 13 as recommended in OECD GD 151 (10).

Clinical biochemistry

42. Blood samples from a defined site are taken based on the following schedule:

- from at least two pups per litter on day 4 after birth, if the number of pups allows (see paragraphs 33-34)
- from all dams and at least two pups per litter at termination on day 13, and
- from all adult males, at termination,

All blood samples are stored under appropriate conditions. Blood samples from the day 13 pups and the adult males are assessed for serum levels for thyroid hormones (T4). Further assessment of T4 in blood samples from the dams and day 4 pups is done if relevant. As an option other hormones may be measured if relevant. Pup blood can be pooled by litter for thyroid hormone analyses. Thyroid hormones (T4 and TSH) should preferably be measured as 'total'.

43. The following factors may influence the variability and the absolute concentrations of the hormone determinations:
- time of sacrifice because of diurnal variation of hormone concentrations
 - method of sacrifice to avoid undue stress to the animals that may affect hormone concentrations
 - test kits for hormone determinations that may differ by their standard curves.
44. Plasma samples specifically intended for hormone determination should be obtained at a comparable time of the day. The numerical values obtained when analysing hormone concentrations differ with various commercial assay kits.

Pathology

Gross necropsy

45. At the time of sacrifice or death during the study, the adult animals should be examined macroscopically for any abnormalities or pathological changes. Special attention should be paid to the organs of the reproductive system. The number of implantation sites should be recorded. Vaginal smears should be examined in the morning on the day of necropsy to determine the stage of the oestrous cycle and allow correlation with histopathology of ovaries.
46. The testes and epididymides as well as prostate and seminal vesicles with coagulating glands as a whole, of all male adult animals should be trimmed of any adherent tissue, as appropriate, and their wet weight taken as soon as possible after dissection to avoid drying. In addition, optional organ weights could include levator ani plus bulbocavernosus muscle complex, Cowper's glands and glans penis in males and paired ovaries (wet weight) and uterus (including cervix) in females; if included, these weights should be collected as soon as possible after dissection.
47. Dead pups and pups killed at day 13 post-partum, or shortly thereafter, should, at least, be carefully examined externally for gross abnormalities. Particular attention should be paid to the external reproductive genitals which should be examined for signs of altered development. At day 13 the thyroid from 1 male and 1 female pup per litter should be preserved.
48. The ovaries, testes, accessory sex organs (uterus and cervix, epididymides, prostate, seminal vesicles plus coagulating glands), thyroid and all organs showing macroscopic lesions of all adult animals should be preserved. Formalin fixation is not recommended for routine examination of testes and epididymides. An acceptable method is the use of Bouin's fixative or modified Davidsons for these tissues (11). The tunica albuginea may be gently and shallowly punctured at the both poles of the organ with a needle to permit rapid penetration of the fixative.

Histopathology

49. Detailed histological examination should be performed on the ovaries, testes and epididymides (with special emphasis on stages of spermatogenesis and histopathology of interstitial testicular cell structure) of the animals of the highest dose group and the control group. The other preserved organs including thyroid from pups and adult animals may be examined when necessary. The thyroid weight could be determined after fixation. Trimming should also be done very carefully and only after fixation to avoid tissue damage. Tissue damage could compromise histopathology analysis. Examinations should be extended to the animals of other dosage groups when changes are seen in the highest dose group. The Guidance on histopathology (11) details extra information on dissection, fixation, sectioning and histopathology of endocrine tissues.

DATA AND REPORTING

Data

50. Individual animal data should be provided. Additionally, all data should be summarised in tabular form, showing for each test group the number of animals at the start of the test, the number of animals found dead during the test or killed for humane reasons, the time of any death or humane kill, the number of fertile animals, the number of pregnant females, the number of animals showing signs of toxicity, a description of the signs of toxicity observed, including time of onset, duration, and severity of any toxic effects, the types of histopathological changes, and all relevant litter data. A tabular summary report format that has proven to be very useful for the evaluation of reproductive/developmental effect is given in Appendix 3.
51. Due to the limited dimensions of the study, statistical analyses in the form of tests for "significance" are of limited value for many endpoints, especially reproductive endpoints. If statistical analyses are used then the method chosen should be appropriate for the distribution of the variable examined, and be selected prior to the start of the study. Statistical analysis of AGD and nipple retention should be based on individual pup data, taking litter effects into account. Where appropriate, the litter is the unit of analysis. Statistical analysis of pup body weight should be based on individual pup data, taking litter size into account. Because of the small group size, the use of historic control data (e.g. for litter size), where available, may also be useful as an aid to the interpretation of the study.

Evaluation of results

52. The findings of this toxicity study should be evaluated in terms of the observed effects, necropsy and microscopic findings. The evaluation will include the relationship between the dose of the test chemical and the presence or absence, incidence and severity of abnormalities, including gross lesions, identified target organs, infertility, clinical

abnormalities, affected reproductive and litter performance, body weight changes, effects on mortality and any other toxic effects.

53. Because of the short period of treatment of the male, the histopathology of the testes and epididymides should be considered along with the fertility data, when assessing male reproductive effects. The use of historical control data on reproduction/development (e.g., for litter size, AGD, nipple retention, serum T4 levels), where available, may also be useful as an aid to the interpretation of the study.

54. For quality control it is proposed that historical control data are collected and that for numerical data coefficients of variation are calculated, especially for the parameters linked with endocrine disrupter detection. These data can be used for comparison purposes when actual studies are evaluated.

Test report

55. The test report should include the following information:

Test chemical:

- source, lot number, limit date for use, if available
- stability of the test chemical, if known.

Mono-constituent substance:

- physical appearance, water solubility, and additional relevant physicochemical properties;
- chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc.

Multi-constituent substance, UVCBs and mixtures:

- characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents.

Vehicle (if appropriate):

- justification for choice of vehicle if other than water.

Test animals:

- species/strain used;
- number, age and sex of animals;
- source, housing conditions, diet, etc.;
- individual weights of animals at the start of the test.

- justification for species if not rat

Test conditions:

- rationale for dose level selection;
- details of test chemical formulation/diet preparation, achieved concentrations, stability and homogeneity of the preparation;
- details of the administration of the test chemical;
- conversion from diet/drinking water test chemical concentration (ppm) to the actual dose (mg/kg body weight/day), if applicable;
- details of food and water quality;
- detailed description of the randomisation procedure to select pups for culling, if culled.

Results:

- body weight/body weight changes;
- food consumption, and water consumption if available;
- toxic response data by sex and dose, including fertility, gestation, and any other signs of toxicity;
- gestation length;
- toxic or other effects on reproduction, offspring, post-natal growth, etc.;
- nature, severity and duration of clinical observations (whether reversible or not);
- number of adult females with normal or abnormal oestrous cycle and cycle duration;
- number of live births and post-implantation loss;
- pup body weight data
- AGD of all pups (and body weight on day of AGD measurement)
- nipple retention in male pups,
- thyroid hormone levels, day 13 pups and adult males (and dams and day 4 pups if measured)
- number pups with grossly visible abnormalities, gross evaluation of external genitalia, number of runts;
- time of death during the study or whether animals survived to termination;
- number of implantations, litter size and litter weights at the time of recording;
- body weight at sacrifice and organ weight data for the parental animals;
- necropsy findings;

- detailed description of histopathological findings;
- absorption data (if available);
- statistical treatment of results, where appropriate.

Discussion of results.

Conclusions.

Interpretation of results

56. The study will provide evaluations of reproduction/developmental toxicity associated with administration of repeated doses (see paragraphs 5 and 6). It could provide an indication of the need to conduct further investigations and provides guidance in the design of subsequent studies. OECD Guidance Document 43 should be consulted for aid in the interpretation of reproduction and developmental results (12). OECD Guidance Document No 106 on Histologic Evaluation of Endocrine and Reproductive Tests in Rodents (11) provides information on the preparation and evaluation of (endocrine) organs and vaginal smears that may be helpful for this TG.

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Appendix 1

DEFINITIONS (SEE ALSO OECD GD 150 (6))

Androgenicity is the capability of a chemical to act like a natural androgenic hormone (e.g. testosterone) in a mammalian organism.

Antiandrogenicity is the capability of a chemical to suppress the action of a natural androgenic hormone (e.g. testosterone) in a mammalian organism.

Antioestrogenicity is the capability of a chemical to suppress the action of a natural oestrogenic hormone (e.g. oestradiol 17 β) in a mammalian organism.

Antithyroid activity is the capability of a chemical to suppress the action of a natural thyroid hormone (e.g. T₃) in a mammalian organism.

Chemical is a substance or a mixture.

Developmental toxicity: the manifestation of reproductive toxicity, representing pre-, peri-post-natal, structural, or functional disorders in the progeny.

Dosage is a general term comprising of dose, its frequency and the duration of dosing.

Dose is the amount of test chemical administered. The dose is expressed as weight of test chemical per unit body weight of test animal per day (e.g. mg/kg body weight/day), or as a constant dietary concentration.

Evident toxicity is a general term describing clear signs of toxicity following administration of test chemical. These should be sufficient for hazard assessment and should be such that an increase in the dose administered can be expected to result in the development of severe toxic signs and probable mortality.

Impairment of fertility represents disorders of male or female reproductive functions or capacity.

Maternal toxicity: adverse effects on gravid females, occurring either specifically (direct effect) or not specifically (indirect effect).

NOAEL is the abbreviation for no-observed-adverse effect level. This is the highest dose level where no adverse treatment-related findings are observed due to treatment.

Oestrogenicity is the capability of a chemical to act like a natural oestrogenic hormone (e.g. oestradiol 17 β) in a mammalian organism.

Reproduction toxicity represents harmful effects on the progeny and/or an impairment of male and female reproductive functions or capacity.

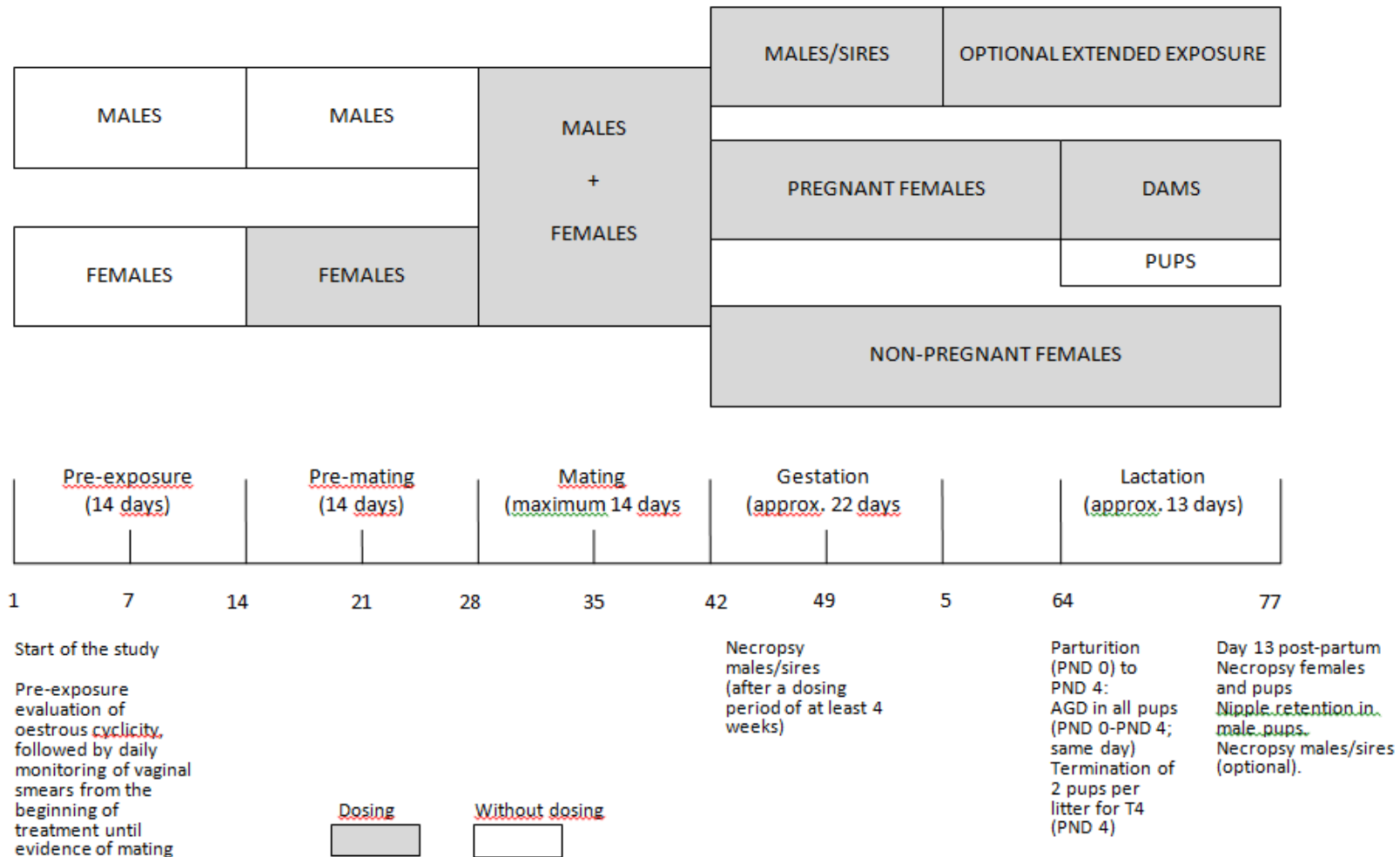
Test chemical is any substance or mixture tested using this test method.

Thyroid activity is the capability of a chemical to act like a natural thyroid hormone (e.g. T₃) in a mammalian organism.

Validation is a scientific process designed to characterise the operational requirements and limitations of a test method and to demonstrate its reliability and relevance for a particular purpose.

Appendix 2

DIAGRAM OF THE EXPERIMENTAL SCHEDULE INDICATING THE MAXIMUM STUDY DURATION, BASED ON A FULL 14-DAY MATING PERIOD



Appendix 3

TABULAR SUMMARY REPORT OF EFFECTS ON REPRODUCTION/DEVELOPMENT

OBSERVATIONS	VALUES				
Dosage (units)	0 (control)	.. .	.. .	.. .	.. .
Pairs started (N)					
Oestrus cycle (at least mean length and frequency of irregular cycles)					
Females showing evidence of copulation (N)					
Females achieving pregnancy (N)					
Conceiving days 1 - 5 (N)					
Conceiving days 6 - . . . ⁽¹⁾ (N)					
Pregnancy $\leq$ 21 days (N)					
Pregnancy = 22 days (N)					
Pregnancy $\geq$ 23 days (N)					
Dams with live young born (N)					
Dams with live young at day 4 pp (N)					
Implants/dam (mean)					
Live pups/dam at birth (mean)					
Live pups/dam at day 4 (mean)					
Sex ratio (m/f) at birth (mean)					
Sex ratio (m/f) at day 4 (mean)					
Litter weight at birth (mean)					
Litter weight at day 4 (mean)					
Pup weight at birth (mean)					

⁽¹⁾ last day of the mating period

Pup weight at the time of AGD measurement (mean males, mean females)					
Pup AGD on the same postnatal day, birth – day 4 (mean males, mean females, note PND)					
Pup weight at day 4 (mean)					
Male pup nipple retention at day 13 (mean)					
Pup weight at day 13 (mean)					
ABNORMAL PUPS					
Dams with 0					
Dams with 1					
Dams with ≥ 2					
LOSS OF OFFSPRING					
Pre-natal/post-implantations (implantations minus live births)					
Females with 0					
Females with 1					
Females with 2					
Females with ≥ 3					
Post-natal (live births minus alive at post-natal day 13)					
Females with 0					
Females with 1					
Females with 2					
Females with ≥ 3					

B.64 COMBINED REPEATED DOSE TOXICITY STUDY WITH THE REPRODUCTION/DEVELOPMENTAL TOXICITY SCREENING TEST

INTRODUCTION

1. This test method is equivalent to OECD test guideline (TG) 422 (2016). OECD guidelines for the Testing of Chemicals are periodically reviewed in the light of scientific progress. The original screening test guideline 422 was adopted in 1996, based on a protocol for a "Combined Repeat Dose and Reproductive/Developmental Screening Test" discussed in two expert meetings, in London in 1990 (1) and in Tokyo in 1992 (2).
2. This test method combines a reproduction/developmental toxicity screening part which is based on experience gained in Member countries from using the original method on existing high production volume chemicals and in exploratory tests with positive control substances (3) (4), and a repeated dose toxicity part, in concordance with OECD test guideline 407 (Repeated Dose 28-Day Oral Toxicity Study in Rodents, corresponding to Chapter B.7 of this Annex).
3. This test method has been updated with endocrine disruptor relevant endpoints, as a follow up to the high-priority activity initiated at OECD in 1998 to revise existing test guidelines and to develop new test guidelines for the screening and testing of potential endocrine disruptors (5). In this context TG 407 (corresponding to Chapter B.7 of this Annex) was enhanced in 2008 by parameters suitable to detect endocrine activity of test chemicals. The objective in updating TG 422 was to include some endocrine disruptor relevant endpoints in screening TGs where the exposure periods cover some of the sensitive periods during development (pre- or early postnatal periods).
4. The selected additional endocrine disruptor relevant endpoints, also part of TG 443 (Extended One Generation Reproductive Toxicity Study, corresponding to Chapter B.56 of this Annex), were included in TG 422 based on a feasibility study addressing scientific and technical questions related to their inclusion, as well as possible adaptations of the test design needed for their inclusion (6).
5. This test method is designed to generate limited information concerning the effects of a test chemical on male and female reproductive performance such as gonadal function, mating behaviour, conception, development of the conceptus and parturition. It is not an alternative to, nor does it replace the existing test methods B.31, B.34, B.35 or B.56.

INITIAL CONSIDERATIONS

6. In the assessment and evaluation of the toxic characteristics of a test chemical the determination of oral toxicity using repeated doses may be carried out after the initial information on toxicity has been obtained by acute testing. This study provides information

on the possible health hazards likely to arise from repeated exposure over a relatively limited period of time. The method comprises the basic repeated dose toxicity study that may be used for chemicals on which a 90-day study is not warranted (e.g. when the production volume does not exceed certain limits) or as a preliminary study to a long-term study. In conducting the study, the guiding principles and considerations outlined in the OECD guidance document n° 19 on the recognition, assessment, and use of clinical signs as humane endpoints for experimental animals used in safety evaluations (7) should be followed.

7. It further comprises a reproduction/developmental toxicity screening test and, therefore, can also be used to provide initial information on possible effects on male and female reproductive performance such as gonadal function, mating behaviour, conception, development of the conceptus and parturition, either at an early stage of assessing the toxicological properties of test chemicals, or on test chemicals of concern. This test method does not provide complete information on all aspects of reproduction and development. In particular, it offers only limited means of detecting postnatal manifestations of prenatal exposure, or effects that may be induced during postnatal exposure. Due (amongst other reasons) to the selectivity of the end points, and the short duration of the study, this method will not provide evidence for definite claims of no reproduction/developmental effects. Moreover, in the absence of data from other reproduction/developmental toxicity tests, positive results are useful for initial hazard assessment and contribute to decisions with respect to the necessity and timing of additional testing.
8. The results obtained by the endocrine related parameters should be seen in the context of the “OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupting Chemicals” (8). In this Conceptual Framework, the enhanced OECD TG 422 is contained in level 4 as an *in vivo* assay providing data on adverse effects on endocrine relevant endpoints. An endocrine signal might not however be considered sufficient evidence on its own that the test chemical is an endocrine disruptor.
9. The test method also places emphasis on neurological effects as a specific endpoint, and the need for careful clinical observations of the animals, so as to obtain as much information as possible, is stressed. The method should identify chemicals with neurotoxic potential, and which may warrant further in-depth investigation of this aspect. In addition, the method may also give a basic indication of immunological effects.
10. In the absence of data from other systemic toxicity, reproduction/developmental toxicity, neurotoxicity and/or immunotoxicity studies, positive results are useful for initial hazard assessment and contribute to decisions with respect to the necessity and timing of additional testing. The test may be particularly useful as part of the OECD Screening Information Data Set (SIDS) for the assessment of existing chemicals for which little or no toxicological information is available and can serve as an alternative to conducting two

separate tests for repeated dose toxicity (OCD TG 407, corresponding to Chapter B.7 of this Annex) and reproduction/developmental toxicity (OECD TG 421, corresponding to Chapter B.63 of this Annex), respectively. It can also be used as a dose range finding study for more extensive reproduction/developmental studies, or when otherwise considered relevant.

11. Generally, it is assumed that there are differences in sensitivity between pregnant and non-pregnant animals. Consequently, it may be more complicated to determine dose levels in this combined test that are adequate to evaluate both general systemic toxicity and specific reproduction/developmental toxicity, rather than when the individual tests are conducted separately. Moreover, interpretation of the test results with respect to general systemic toxicity may be more difficult than when conducting a separate repeated-dose study, especially when serum and histopathology parameters are not evaluated at the same time in the study. Because of these technical complexities, considerable experience in toxicity testing is required for the performance of this combined screening test. On the other hand, apart from the smaller number of animals involved, the combined test may offer a better means of discriminating direct effects on reproduction/development from those that are secondary to other (systemic) effects.
12. In this test, the dosing period is longer than in a conventional 28-day repeated dose study. However, it uses fewer animals of each sex per group when compared with the situation where a conventional 28-day repeated dose study is conducted in addition to a Reproduction/Developmental Toxicity Screening Test.
13. This test method assumes oral administration of the test chemical. Modifications may be required if other routes of exposure are used.
14. Before use of the test method on a mixture for generating data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed, when there is a regulatory requirement for testing of the mixture.
15. Definitions used are given in Appendix 1.

PRINCIPLE OF THE TEST

16. The test chemical is administered in graduated doses to several groups of males and females. Males should be dosed for a minimum of four weeks, up to and including the day before scheduled kill (this includes a minimum of two weeks prior to mating, during the mating period and, approximately, two weeks post mating). In view of the limited pre-mating dosing period in males, fertility may not be a particularly sensitive indicator of testicular toxicity. Therefore, a detailed histological examination of the testes is essential. The combination of a pre-mating dosing period of two weeks and subsequent

mating/fertility observations with an overall dosing period of at least four weeks, followed by detailed histopathology of the male gonads, is considered sufficient to enable detection of the majority of effects on male fertility and spermatogenesis.

17. Females should be dosed throughout the study. This includes two weeks prior to mating (with the objective of covering at least two complete oestrous cycles), the variable time to conception, the duration of pregnancy and at least thirteen days after delivery, up to and including the day before scheduled kill.
18. Duration of study, following acclimatisation and pre-dosing oestrous cycle evaluation, is dependent on the female performance and is approximately 63 days, [at least 14 days pre-mating, (up to) 14 days mating, 22 days gestation, 13 days lactation].
19. During the period of administration, the animals are observed closely each day for signs of toxicity. Animals which die or are killed during the test are necropsied and, at the conclusion of the test, surviving animals are killed and necropsied.

DESCRIPTION OF THE METHOD

Selection of animal species

20. This test method is designed for use with the rat. If the parameters specified within this TG 422 are investigated in another rodent species a detailed justification should be given. In the international validation program for the detection of endocrine disrupters on TG 407, the rat was the only species used. Strains with low fecundity or well-known high incidence of developmental defects should not be used. Healthy virgin animals, not subjected to previous experimental procedures, should be used. The test animals should be characterised as to species, strain, sex, weight and age. At the commencement of the study the weight variation of animals used should be minimal and not exceed $\pm 20\%$ of the mean weight of each sex. Where the study is conducted as a preliminary study to a long-term or a full-generation study, it is preferable that animals from the same strain and source are used in both studies.

Housing and feeding

21. All procedures should conform to local standards of laboratory animal care. The temperature in the experimental animal room should be 22 °C ($\pm 3^\circ$). The relative humidity should be at least 30% and preferably not exceed 70% other than during room cleaning. Lighting should be artificial, the photoperiod being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. The choice of diet may be influenced by the need to ensure a suitable admixture of a test chemical when administered by this method.
22. Animals should be group housed in small groups of the same sex; animals may be housed individually if scientifically justified. For group caging, no more than five animals should

be housed per cage. Mating procedures should be carried out in cages suitable for the purpose. Pregnant females should be caged individually and provided with nesting materials. Lactating females will be caged individually with their offspring.

23. The feed should be regularly analysed for contaminants. A sample of the diet should be retained until finalisation of the report.

Preparation of the animals

24. Healthy young adult animals are randomised and assigned to the treatment groups and cages. Cages should be arranged in such a way that possible effects due to cage placements are minimised. The animals are uniquely identified and kept in their cages for at least five days prior to the start of the study to allow for acclimatisation to the laboratory conditions.

Preparation of doses

25. It is recommended that the test chemical be administered orally unless other routes of administration are considered more appropriate. When the oral route is selected, the test chemical is usually administered by gavage; however, alternatively, test chemicals may also be administered via the diet or drinking water.
26. Where necessary, the test chemical is dissolved or suspended in a suitable vehicle. It is recommended that, wherever possible, the use of an aqueous solution/suspension be considered first, followed by consideration of a solution/suspension in oil (e.g. corn oil) and then by possible solution in other vehicles. For non-aqueous vehicles the toxic characteristics of the vehicle should be known. The stability and homogeneity of the test chemical in the vehicle should be determined.

PROCEDURE

Number and sex of animals

27. It is recommended that each group be started with at least 10 males and 12-13 females. Females will be evaluated pre-exposure for oestrous cyclicity and animals that fail to exhibit typical 4-5 day cycles will not be included in the study; therefore, extra females are recommended in order to yield 10 females per group. Except in the case of marked toxic effects, it is expected that this will provide at least 8 pregnant females per group which normally is the minimum acceptable number of pregnant females per group. The objective is to produce enough pregnancies and offspring to assure a meaningful evaluation of the potential of the test chemical to affect fertility, pregnancy, maternal and suckling behaviour, and growth and development of the F₁ offspring from conception to day 13 post-partum. If interim kills are planned, the number should be increased by the number of animals scheduled to be killed before the completion of the study. Consideration should be given to an additional satellite group of five animals per sex in the control and the top dose group for observation of

reversibility, persistence or delayed occurrence of systemic toxic effects, for at least 14 days post treatment. Animals of the satellite groups will not be mated and, consequently, are not used for the assessment of reproduction/developmental toxicity.

Dosage

28. Generally, at least three test groups and a control group should be used. If there are no suitable general toxicity data available, a range finding study may (animals of the same strain and source) be performed to aid the determination of the doses to be used. Except for treatment with the test chemical, animals in the control group should be handled in an identical manner to the test group subjects. If a vehicle is used in administering the test chemical, the control group should receive the vehicle in the highest volume used.
29. Dose levels should be selected taking into account any existing toxicity and (toxico-) kinetic data available. It should also be taken into account that there may be differences in sensitivity between pregnant and non-pregnant animals. The highest dose level should be chosen with the aim of inducing toxic effects but not death nor obvious suffering. Thereafter, a descending sequence of dose levels should be selected with a view to demonstrating any dosage related response and no adverse effects at the lowest dose level. Two- to four- fold intervals are frequently optimum and addition of a fourth test group is often preferable to using very large intervals (e.g. more than a factor of 10) between dosages.
30. In the presence of observed general toxicity (e.g. reduced body weight, liver , heart, lung or kidney effects, etc.) or other changes that may not be toxic responses (e.g. reduced food intake, liver enlargement), observed effects on endocrine sensitive endpoints should be interpreted with caution.

Limit test

31. If an oral study at one dose level of at least 1000 mg/kg body weight/day or, for dietary administration, an equivalent percentage in the diet, or drinking water (based upon body weight determinations), using the procedures described for this study, produces no observable toxic effects and if toxicity would not be expected based upon data from structurally related substances, then a full study using several dose levels may not be considered necessary. The limit test applies except when human exposure indicates the need for a higher dose level to be used. For other types of administration, such as inhalation or dermal application, the physical chemical properties of the test chemicals often may dictate the maximum attainable exposure.

Administration of doses

32. The animals are dosed with the test chemical daily for 7 days a week. When the test chemical is administered by gavage, this should be done in a single dose to the animals

using a stomach tube or a suitable intubation cannula. The maximum volume of liquid that can be administered at one time depends on the size of the test animal. The volume should not exceed 1 ml/100 g body weight, except in the case of aqueous solutions where 2 ml/100 g body weight may be used. Except for irritating or corrosive test chemicals which will normally reveal exacerbated effects with higher concentrations, variability in test volume should be minimised by adjusting the concentration to ensure a constant volume at all dose levels.

33. For test chemicals administered via the diet or drinking water, it is important to ensure that the quantities of the test chemical involved do not interfere with normal nutrition or water balance. When the test chemical is administered in the diet either a constant dietary concentration (ppm) or a constant dose level in terms of the animals' body weight may be used; the alternative used should be specified. For a test chemical administered by gavage, the dose should be given at similar times each day, and adjusted at least weekly to maintain a constant dose level in terms of animal body weight. Where the combined study is used as a preliminary to a long term or a full reproduction toxicity study, a similar diet should be used in both studies.

Experimental schedule

34. Dosing of both sexes should begin 2 weeks prior to mating, after they have been acclimatised for at least five days and females have been screened for normal oestrous cycles (in a 2 weeks pre-treatment period). The study should be scheduled in such a way that oestrous cycle evaluation begins soon after the animals have attained full sexual maturity. This may vary slightly for different strains of rats in different laboratories, e.g. Sprague Dawley rats 10 weeks of age, Wistar rats about 12 weeks of age. Dams with offspring should be killed on day 13 post-partum, or shortly thereafter. In order to allow for overnight fasting of dams prior to blood collection (if this option is preferred), dams and their offspring need not necessarily be killed on the same day. The day of birth (viz. when parturition is complete) is defined as day 0 post-partum. Females showing no-evidence of copulation are killed 24-26 days after the last day of the mating period. Dosing is continued in both sexes during the mating period. Males should further be dosed after the mating period at least until the minimum total dosing period of 28 days has been completed. They are then killed, or, alternatively, are retained and continued to be dosed for the possible conduction of a second mating if considered appropriate.
35. Daily dosing of the parental females should continue throughout pregnancy and at least up to, and including, day 13 post-partum or the day before sacrifice. For studies where the test chemical is administered by inhalation or by the dermal route, dosing should be continued at least up to, and including, day 19 of gestation, and dosing should be re-initiated as soon as possible and not later than postnatal day (PND) 4.

36. Animals in a satellite group scheduled for follow-up observations, if included, are not mated. They should be kept at least for a further 14 days after the first scheduled kill of dams, without treatment to detect delayed occurrence, or persistence of, or recovery from toxic effects.
37. A diagram of the experimental schedule is given in Appendix 2.

Oestrous cycles

38. Oestrous cycles should be monitored before treatment starts to select for the study females with regular cyclicity (see paragraph 27). Vaginal smears should also be monitored daily from the beginning of the treatment period until evidence of mating. If there is concern about acute stress effects that could alter estrous cycles with the initiation of dosing, laboratories can expose test animals for 2 weeks, then collect vaginal smears daily to monitor estrous cycle for a minimum of two weeks during the pre-mating period with continued monitoring into the mating period until there is evidence of mating. When obtaining vaginal/cervical cells, care should be taken to avoid disturbance of mucosa, which could induce pseudopregnancy (8) (9).

Mating procedure

39. Normally, 1:1 (one male to one female) matings should be used in this study. Exceptions can arise in the case of occasional deaths of males. The female should be placed with the same male until evidence of copulation is observed or two weeks have elapsed. Each morning the females should be examined for the presence of sperm or a vaginal plug. Day 0 of pregnancy is defined as the day on which mating evidence is confirmed (a vaginal plug or sperm is found). In case pairing was unsuccessful, re-mating of females with proven males of the same group could be considered.

Litter size

40. On day 4 after birth, the size of each litter may be adjusted by eliminating extra pups by random selection to yield, as nearly as possible, four or five pups per sex per litter depending on the normal litter size in the strain of rats used. Blood samples should be collected from two of the surplus pups, pooled, and used for determination of serum T4 levels. Selective elimination of pups, e.g. based upon body weight, or anogenital distance (AGD) is not appropriate. Whenever the number of male or female pups prevents having four or five of each sex per litter, partial adjustment (for example, six males and four females) is acceptable. No pups will be eliminated when litter size will drop below the culling target (8 or 10 pups/litter). If there is only one pup available above the culling target, only one pup will be eliminated and used for blood collection for possible serum T4 assessments.

41. If litter size is not adjusted, two pups per litter are sacrificed on day 4 after birth and blood samples are taken for measurement of serum thyroid hormone concentrations. If possible the two pups per litter should be female pups to reserve male pups for nipple retention evaluations, except in the event that removing these pups leaves no remaining females for assessment at termination. No pups will be eliminated when litter size will drop below 8 or 10 pups/litter (depending on the normal litter size in the strain of rats used). If there is only one pup available above the normal litter size, only one pup will be eliminated and used for blood collection for possible serum T4 assessments.

Observations

42. General clinical observations should be made at least once a day, preferably at the same time(s) each day and considering the peak period of anticipated effects after dosing. The health condition of the animals should be recorded. At least twice daily all animals are observed for morbidity and mortality.
43. Once before the first exposure (to allow for within-subject comparisons), and at least once a week thereafter, detailed clinical observations should be made in all parental animals. These observations should be made outside the home cage in a standard arena and preferably at the same time, each day. They should be carefully recorded; preferably using scoring systems, explicitly defined by the testing laboratory. Effort should be made to ensure that variations in the test conditions are minimal and that observations are preferably conducted by observers unaware of the treatment. Signs noted should include, but not be limited to, changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity (e.g. lacrimation, piloerection, pupil size, unusual respiratory pattern). Changes in gait, posture and response to handling as well as the presence of clonic or tonic movements, stereotypies (e.g. excessive grooming, repetitive circling), difficult or prolonged parturition or bizarre behaviour (e.g. self-mutilation, walking backwards) should also be recorded (10).
44. At one time during the study, sensory reactivity to stimuli of different modalities (e.g. auditory, visual and proprioceptive stimuli) (8) (9) (11), assessment of grip strength (12) and motor activity assessment (13) should be conducted in five males and five females, randomly selected from each group. Further details of the procedures that could be followed are given in the respective references. However, alternative procedures than those referenced could also be used. In males, these functional observations should be made towards the end of their dosing period, shortly before scheduled kill but before blood sampling for haematology or clinical chemistry (see paragraphs 53-56, including footnote 1). Females should be in a physiologically similar state during these functional tests and should preferably be tested once during the last week of lactation (e.g., LD 6-13), shortly before scheduled kill. To the extent possible, minimise dams and pups separation times.

45. Functional observations made once towards the end of the study may be omitted when the study is conducted as a preliminary study to a subsequent subchronic (90-day) or long-term study. In that case, the functional observations should be included in this follow-up study. On the other hand, the availability of data on functional observations from this repeated dose study may enhance the ability to select dose levels for a subsequent subchronic or long-term study.
46. As an exception, functional observations may also be omitted for groups that otherwise reveal signs of toxicity to an extent that would significantly interfere with the functional test performance.
47. The duration of gestation should be recorded and is calculated from day 0 of pregnancy. Each litter should be examined as soon as possible after delivery to establish the number and sex of pups, stillbirths, live births, runts (pups that are significantly smaller than corresponding control pups), and the presence of gross abnormalities.
48. Live pups should be counted and sexed and litters weighed within 24 hours of parturition (day 0 or 1 post-partum) and at least on day 4 and day 13 post-partum. In addition to the observations on parent animals (see paragraphs 43 and 44), any abnormal behaviour of the offspring should be recorded.
49. The AGD of each pup should be measured on the same postnatal day between PND 0 through PND 4. Pup body weight should be collected on the day the AGD is measured and the AGD should be normalised to a measure of pup size, preferably the cube root of body weight (14). The number of nipples/areolae in male pups should be counted on PND 12 or 13 as recommended in OECD GD 151 (15).

Body weight and food/water consumption

50. Males and females should be weighed on the first day of dosing, at least weekly thereafter, and at termination. During pregnancy, females should be weighed on days 0, 7, 14 and 20 and within 24 hours of parturition (day 0 or 1 post-partum), and at least day 4 and day 13 post-partum. These observations should be reported individually for each adult animal.
51. During pre-mating, pregnancy and lactation, food consumption should be measured at least weekly. The measurement of food consumption during mating is optional. Water consumption during these periods should also be measured, when the test chemical is administered by that medium.

Haematology

52. Once during the study, the following haematological examinations should be made in five males and five females randomly selected from each group: haematocrit, haemoglobin concentrations, erythrocyte count, reticulocytes, total and differential leucocyte count, platelet count and a measure of blood clotting time/potential. Other determinations that

should be carried out, if the test chemical or its putative metabolites have or are suspected to have oxidising properties include methaemoglobin concentration and Heinz bodies.

53. Blood samples should be taken from a named site. Females should be in a physiologically similar state during sampling. In order to avoid practical difficulties related to the variability in the onset of gestation, blood collection in females may be done at the end of the pre-mating period as an alternative to sampling just prior to, or as part of, the procedure for euthanasia of the animals. Blood samples of males should preferably be taken just prior to, or as part of, the procedure for euthanasia of the animals. Alternatively, blood collection in males may also be done at the end of the pre-mating period when this time point was preferred for females.
54. Blood samples should be stored under appropriate conditions.

Clinical biochemistry

55. Clinical biochemistry determinations to investigate major toxic effects in tissues and, specifically, effects on kidney and liver, should be performed on blood samples obtained from the selected five males and five females of each group. Overnight fasting of the animals prior to blood sampling is recommended¹. Investigations of plasma or serum should include sodium, potassium, glucose, total cholesterol, urea, creatinine, total protein and albumin, at least two enzymes indicative of hepatocellular effects (such as alanin aminotransferase, aspartate aminotransferase and sorbitol dehydrogenase) and bile acids. Measurements of additional enzymes (of hepatic or other origin) and bilirubin may provide useful information under certain circumstances.
56. Blood samples from a defined site are taken based on the following schedule:
- from at least two pups per litter on day 4 after birth, if the number of pups allows (see paragraphs 40-41)
 - from all dams and at least two pups per litter at termination on day 13, and

¹ For a number of measurements in serum and plasma, most notably for glucose, overnight fasting would be preferable. The major reason for this preference is that the increased variability which would inevitably result from non-fasting, would tend to mask more subtle effects and make interpretation difficult. On the other hand, however, overnight fasting may interfere with the general metabolism of the (pregnant) animals, disturbs lactation and nursing behaviour, and, particularly in feeding studies, may disturb the daily exposure to the test chemical. If overnight fasting is adopted, clinical biochemical determinations should be performed after the conduct of functional observations in week 4 of the study for the males. The dams should be retained for an additional day after the pups are removed on e.g. PND 13). Dams should be fasted overnight from lactation day 13-14 and terminal blood used for clinical chemistry parameters.

- from all adult males, at termination

All blood samples are stored under appropriate conditions. Blood samples from the day 13 pups and the adult males are assessed for serum levels for thyroid hormones (T4). Further assessment of T4 in blood samples from the dams and day 4 pups is done if relevant. As an option, other hormones may be measured if relevant. Pup blood can be pooled by litter for thyroid hormone analyses. Thyroid hormones (T4 and TSH) should preferably be measured as 'total'.

57. Optionally, the following urinalysis determinations could be performed in five randomly selected males of each group during the last week of the study using timed urine volume collection; appearance, volume, osmolality or specific gravity, pH, protein, glucose and blood/blood cells.
58. In addition, studies to investigate serum markers of general tissue damage should be considered. Other determinations that should be carried out if the known properties of the test chemical may, or are suspected to, affect related metabolic profiles include calcium, phosphate, fasting triglycerides and fasting glucose, specific hormones, methaemoglobin and cholinesterase. These need to be identified on a case-by-case basis.
59. The following factors may influence the variability and the absolute concentrations of the hormone determinations:
 - time of sacrifice because of diurnal variation of hormone concentrations
 - method of sacrifice to avoid undue stress to the animals that may affect hormone concentrations
 - test kits for hormone determinations that may differ by their standard curves.
60. Plasma samples specifically intended for hormone determination should be obtained at a comparable time of the day. The numerical values obtained when analysing hormone concentrations differ with various commercial assay kits.
61. If historical baseline data are inadequate, consideration should be given to determination of haematological and clinical biochemistry variables before dosing commences or preferably in a set of animals not included in the experimental groups. For females, the data have to be from lactating animals.

PATHOLOGY

Gross necropsy

62. All adult animals in the study should be subjected to a full, detailed gross necropsy which includes careful examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents. Special attention should be paid

to the organs of the reproductive system. The number of implantation sites should be recorded. Vaginal smears should be examined on the day of necropsy to determine the stage of the oestrous cycle and allow correlation with histopathology of female reproductive organs.

63. The testes and epididymides as well as prostate and seminal vesicles with coagulating glands as a whole of all male adult animals should be trimmed of any adherent tissue, as appropriate, and their wet weight taken as soon as possible after dissection to avoid drying. In addition, optional organ weights could include levator ani plus bulbocavernosus muscle complex, Cowper's glands and glans penis in males and paired ovaries (wet weight) and uterus (including cervix) in females; if included, these weights should be collected as soon as possible after dissection. The ovaries, testes, epididymides, accessory sex organs, and all organs showing macroscopic lesions of all adult animals, should be preserved.
64. From all adult males and females and one male and female day 13 pup from each litter thyroid glands should be preserved in the most appropriate fixation medium for the intended subsequent histopathological examination. The thyroid weight could be determined after fixation. Trimming should also be done very carefully and only after fixation to avoid tissue damage. Tissue damage could compromise histopathology analysis. Blood samples should be taken from a named site just prior to or as part of the procedure for euthanasia of the animals, and stored under appropriate conditions (see paragraph 56).
65. In addition, for a least five adult males and females, randomly selected from each group (apart from those found moribund and/or euthanised prior to the termination of the study), the liver, kidneys, adrenals, thymus, spleen, brain and heart should be trimmed of any adherent tissue, as appropriate and their wet weight taken as soon as possible after dissection to avoid drying. The following tissues should be preserved in the most appropriate fixation medium for both the type of tissue and the intended subsequent histopathological examination : all gross lesions, brain (representative regions including cerebrum, cerebellum and pons), spinal cord, eye, stomach, small and large intestines (including Peyer's patches), liver, kidneys, adrenals, spleen, heart, thymus, trachea and lungs (preserved by inflation with fixative and then immersion), gonads (testis and ovaries), accessory sex organs (uterus and cervix, epididymides, prostate, seminal vesicles plus coagulating glands), vagina, urinary bladder, lymph nodes (besides the most proximal draining node, another lymph node should be taken according to the laboratory's experience (16)), peripheral nerve (sciatic or tibial) preferably in close proximity to the muscle, skeletal muscle and bone, with bone marrow (section or, alternatively, a fresh mounted bone marrow aspirate). It is recommended that testes be fixed by immersion in Bouin's or modified Davidson's fixative (16) (17) (18); formalin fixation is not recommended for these tissues. The tunica albuginea may be gently and shallowly punctured at the both poles of the organ with a needle to permit rapid penetration of the fixative. The clinical and other findings may suggest the need to examine additional

tissues. Also any organs considered likely to be target organs based on the known properties of the test chemical should be preserved.

66. The following tissues may give valuable indication for endocrine-related effects: Gonads (ovaries and testes), accessory sex organs (uterus including cervix, epididymides, seminal vesicles with coagulation glands, dorsolateral and ventral prostate), vagina, pituitary, male mammary gland and adrenal gland. Changes in male mammary glands have not been sufficiently documented but this parameter may be very sensitive to substances with estrogenic action. Observation of organs/tissues that are not listed in paragraph 65 is optional.
67. Dead pups and pups killed at day 13 post-partum, or shortly thereafter, should, at least, be carefully examined externally for gross abnormalities. Particular attention should be paid to the external reproductive genitals which should be examined for signs of altered development.

Histopathology

68. Full histopathology should be carried out on the preserved organs and tissues of the selected animals in the control and high dose groups (with special emphasis on stages of spermatogenesis in the male gonads and histopathology of interstitial testicular cell structure). The thyroid gland from pups and from the remaining adult animals may be examined when necessary. These examinations should be extended to animals of other dosage groups, if treatment-related changes are observed in the high dose group. The Guidance on histopathology (10) details extra information on dissection, fixation, sectioning and histopathology of endocrine tissues.
69. All gross lesions should be examined. To aid in the elucidation of NOAELs, target organs in other dose groups should be examined, particularly in groups claimed to show a NOAEL.
70. When a satellite group is used, histopathology should be performed on tissues and organs identified as showing effects in the treated groups.

DATA AND REPORTING

Data

71. Individual animal data should be provided. Additionally, all data should be summarised in tabular form, showing for each test group the number of animals at the start of the test, the number of animals found dead during the test or euthanised for humane reasons, the time of any death or euthanasia, the number of fertile animals, the number of pregnant females, the number of animals showing signs of toxicity, a description of the signs of toxicity observed, including time of onset, duration, and severity of any toxic effects, the types of

histopathological changes, and all relevant litter data. A tabular summary report format, which has proven to be very useful for the evaluation of reproductive/developmental effects, is given in Appendix 3.

72. When possible, numerical results should be evaluated by an appropriate and general acceptable statistical method. Comparisons of the effect along a dose range should avoid the use of multiple t-tests. The statistical methods should be selected during the design of the study. Statistical analysis of AGD and nipple retention should be based on individual pup data, taking litter effects into account. Where appropriate, the litter is the unit of analysis. Statistical analysis of pup body weight should be based on individual pup data, taking litter size into account. Due to the limited dimensions of the study, statistical analyses in the form of tests for "significance" are of limited value for many endpoints, especially reproductive endpoints. Some of the most widely used methods, especially parametric tests for measures of central tendency, are inappropriate. If statistical analyses are used then the method chosen should be appropriate for the distribution of the variable examined and be selected prior to the start of the study.

Evaluation of results

73. The findings of this toxicity study should be evaluated in terms of the observed effects, necropsy and microscopic findings. The evaluation will include the relationship between the dose of the test chemical and the presence or absence, incidence and severity of abnormalities, including gross lesions, identified target organs, infertility, clinical abnormalities, affected reproductive and litter performance, body weight changes, effects on mortality and any other toxic effects.
74. Because of the short period of treatment of the male, the histopathology of the testes and epididymides should be considered along with the fertility data, when assessing male reproduction effects. The use of historic control data on reproduction/development (e.g. for litter size, AGD, nipple retention, serum T4 levels), where available, may also be useful as an aid to the interpretation of the study.
75. For quality control it is proposed that historical control data are collected and that for numerical data coefficients of variation are calculated, especially for the parameters linked with endocrine disrupter detection. These data can be used for comparison purposes when actual studies are evaluated.

Test report

76. The test report should include the following information:

Test chemical:

- source, lot number, limit date for use, if available

- stability of the test chemical, if known.

Mono-constituent substance:

- physical appearance, water solubility, and additional relevant physicochemical properties;
- chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc.

Multi-constituent substance, UVCBs and mixtures:

- characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents.

Vehicle (if appropriate):

- justification for choice of vehicle, if other than water.

Test animals:

- species/strain used;
- number, age and sex of animals;
- source, housing conditions, diet, etc.;
- individual weights of animals at the start of the test.
- justification for species if not rat

Test conditions:

- rationale for dose level selection;
- details of test chemical formulation/diet preparation, achieved concentration, stability and homogeneity of the preparation;
- details of the administration of the test chemical;
- conversion from diet/drinking water test chemical concentration (ppm) to the actual dose (mg/kg body weight/day), if applicable;
- details of food and water quality;
- detailed description of the randomisation procedure to select pups for culling, if culled.

Results:

- body weight/body weight changes;
- food consumption and water consumption, if applicable;
- toxic response data by sex and dose, including fertility, gestation, and any other signs of

- toxicity;
- gestation length;
- toxic or other effects on reproduction, offspring, postnatal growth, etc.;
- nature, severity and duration of clinical observations (whether reversible or not);
- sensory activity, grip strength and motor activity assessments;
- haematological tests with relevant base-line values;
- clinical biochemistry tests with relevant base-line values;
- number of adult females with normal or abnormal oestrous cycle and cycle duration;
- number of live births and post implantation loss;
- number of pups with grossly visible abnormalities; gross evaluation of external genitalia, number of runts;
- time of death during the study or whether animals survived to termination;
- number of implantations, litter size and litter weights at the time of recording;
- pup body weight data
- AGD of all pups (and body weight on day of AGD measurement)
- nipple retention in male pups,
- thyroid hormone levels, day 13 pups and adult males (and dams and day 4 pups if measured)
- body weight at sacrifice and organ weight data for the parental animals;
- necropsy findings;
- a detailed description of histopathological findings;
- absorption data (if available);
- statistical treatment of results, where appropriate.

Discussion of results.

Conclusions.

Interpretation of Results

77. The study will provide evaluations of reproduction/developmental toxicity associated with administration of repeated doses. In particular, since emphasis is placed on both general toxicity and reproduction/developmental toxicity endpoints, the results of the study will allow for the discrimination between reproduction/developmental effects occurring in the absence of general toxicity and those which are only expressed at levels that are also toxic

to parent animals (see paragraphs 7-11). It could provide an indication of the need to conduct further investigations and could provide guidance in the design of subsequent studies. OECD Guidance Document 43 should be consulted for aid in the interpretation of reproduction and developmental results (19). OECD Guidance Document 106 on Histologic Evaluation of Endocrine and Reproductive Tests in Rodents (16) provides information on the preparation and evaluation of (endocrine) organs and vaginal smears that may be helpful for this test method.

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Appendix 1

DEFINITIONS (SEE ALSO (20) OECD GD 150)

Androgenicity is the capability of a chemical to act like a natural androgenic hormone (e.g. testosterone) in a mammalian organism.

Antiandrogenicity is the capability of a chemical to suppress the action of a natural androgenic hormone (e.g. testosterone) in a mammalian organism.

Antioestrogenicity is the capability of a chemical to suppress the action of a natural oestrogenic hormone (e.g. oestradiol 17 β) in a mammalian organism.

Antithyroid activity is the capability of a chemical to suppress the action of a natural thyroid hormone (e.g. T₃) in a mammalian organism.

Chemical is a substance or a mixture.

Developmental toxicity: the manifestation of reproductive toxicity, representing pre-, peri-post-natal, structural, or functional disorders in the progeny.

Dose is the amount of test chemical administered. The dose is expressed as weight of test chemical per unit body weight of test animal per day (e.g. mg/kg body weight/day), or as a constant dietary concentration.

Dosage is a general term comprising dose, its frequency and the duration of dosing.

Evident toxicity is a general term describing clear signs of toxicity following administration of test chemical. These should be sufficient for hazard assessment and should be such that an increase in the dose administered can be expected to result in the development of severe toxic signs and probable mortality.

Impairment of fertility represents disorders of male or female reproductive functions or capacity.

Maternal toxicity: adverse effects on gravid females, occurring either specifically (direct effect) or not specifically (indirect effect) and being related to the gravid state.

NOAEL is the abbreviation for no-observed-adverse-effect level. This is the highest dose level where no adverse treatment-related findings are observed due to treatment.

Oestrogenicity is the capability of a chemical to act like a natural oestrogenic hormone (e.g. oestradiol 17 β) in a mammalian organism.

Reproduction toxicity represents harmful effects on the progeny and/or an impairment of male and female reproductive functions or capacity.

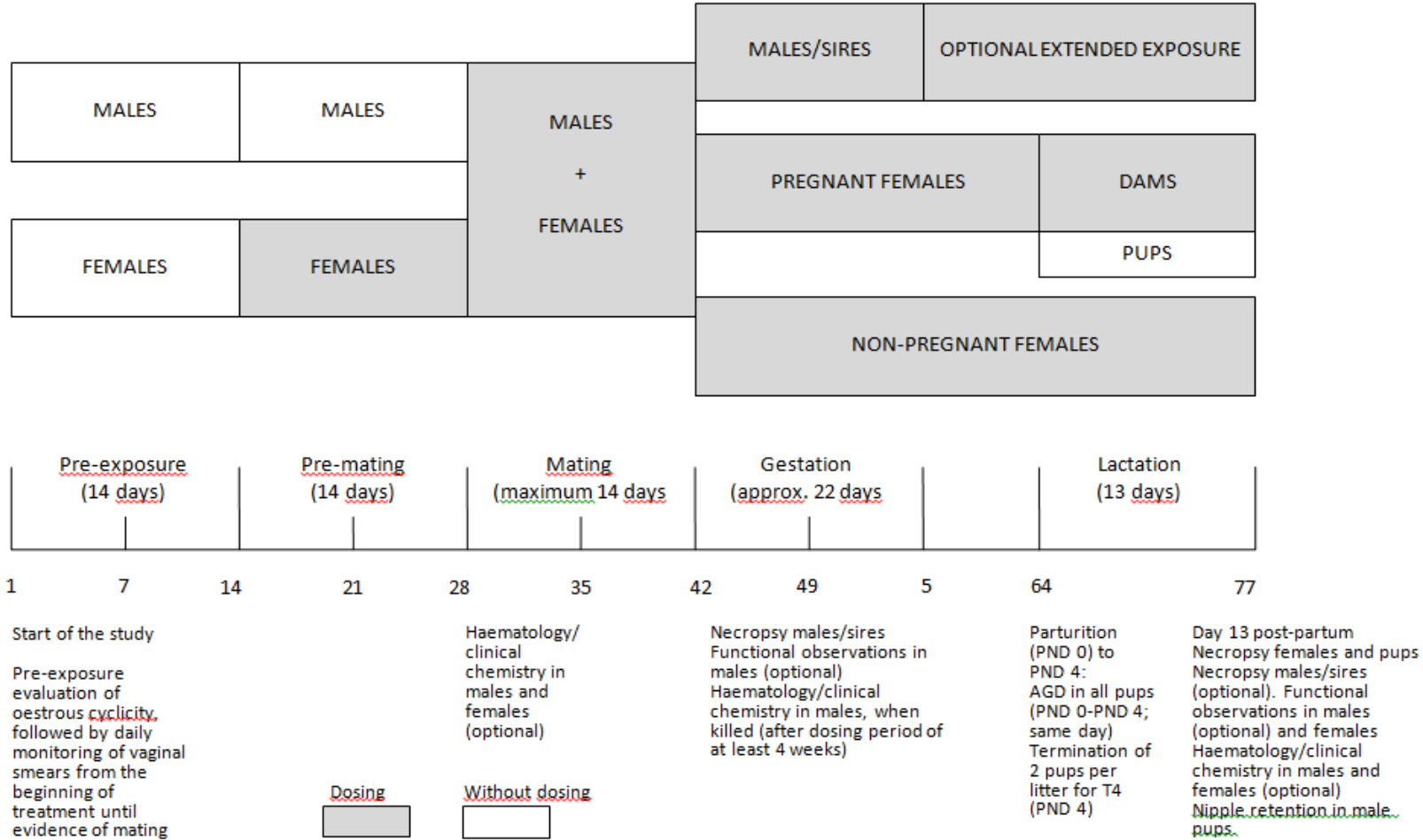
Test chemical is any substance or mixture tested using this test method.

Thyroid activity is the capability of a chemical to act like a natural thyroid hormone (e.g. T₃) in a mammalian organism.

Validation is a scientific process designed to characterise the operational requirements and limitations of a test method and to demonstrate its reliability and relevance for a particular purpose.

Appendix 2

DIAGRAM OF THE EXPERIMENTAL SCHEDULE, INDICATING THE MAXIMUM STUDY DURATION, BASED ON A FULL 14-DAY MATING PERIOD



Appendix 3

TABULAR SUMMARY REPORT OF EFFECTS ON REPRODUCTION/DEVELOPMENT

OBSERVATIONS	VALUES				
Dosage (units).....	0 (control)	...	...	...	...
Pairs started (N)					
Oestrus cycle (at least mean length and frequency of irregular cycles)					
Females showing evidence of copulation (N)					
Females achieving pregnancy (N)					
Conceiving days 1 - 5 (N)					
Conceiving days 6 - ... ⁽¹⁾ (N)					
Pregnancy ≤ 21 days (N)					
Pregnancy = 22 days (N)					
Pregnancy ≥ 23 days (N)					
Dams with live young born (N)					
Dams with live young at day 4 pp (N)					
Implants/dam (mean)					
Live pups/dam at birth (mean)					
Live pups/dam at day 4 (mean)					
Sex ratio (m/f) at birth (mean)					
Sex ratio (m/f) at day 4 (mean)					
Litter weight at birth (mean)					
Litter weight at day 4 (mean)					
Pup weight at birth (mean)					
Pup weight at the time of AGD measurement (mean males, mean females)					
Pup AGD on the same postnatal day, birth- day 4 (mean males, mean females, note PND)					
Pup weight at day 4 (mean)					
Pup weight at day 13 (mean)					
Male pup nipple retention at day 13 (mean)					
ABNORMAL PUPS					

⁽¹⁾ last day of the mating period

Dams with 0					
Dams with 1					
Dams with ≥ 2					
LOSS OF OFFSPRING					
Pre-natal (implantations minus live births)					
Females with 0					
Females with 1					
Females with 2					
Females with ≥ 3					
Post-natal (live births minus alive at post natal day 13)					
Females with 0					
Females with 1					
Females with 2					
Females with ≥ 3					

B.65 *IN VITRO* MEMBRANE BARRIER TEST METHOD FOR SKIN CORROSION

INTRODUCTION

1. This test method is equivalent to OECD test guideline (TG) 435 (2015). Skin corrosion refers to the production of irreversible damage to the skin, manifested as visible necrosis through the epidermis and into the dermis, following the application of a test chemical as defined by the United Nations (UN) Globally Harmonized System of Classification and Labelling of Chemicals (GHS) (1) and the European Union (EU) Regulation 1272/2008 on Classification, Labelling and Packaging of Substances and Mixtures (CLP)¹ This test method, equivalent to the updated OECD test guideline 435 provides an *in vitro* membrane barrier test method that can be used to identify corrosive chemicals. The test method utilises an artificial membrane designed to respond to corrosive chemicals in a manner similar to animal skin *in situ*.
2. Skin corrosivity has traditionally been assessed by applying the test chemical to the skin of living animals and assessing the extent of tissue damage after a fixed period of time (2). Besides the present test method, a number of other *in vitro* test methods have been adopted as alternatives (3)(4) to the standard *in vivo* rabbit skin procedure (Chapter B.4 of this Annex, equivalent to OECD TG 404) used to identify corrosive chemicals (2). The UN GHS tiered testing and evaluation strategy for the assessment and classification of skin corrosivity and the OECD Guidance Document on Integrated Approaches to Testing and Assessment (IATA) for Skin Irritation/Corrosion recommend the use of validated and accepted *in vitro* test methods under modules 3 and 4 (1)(5). The IATA describes several modules which group information sources and analysis tools and (i) provides guidance on how to integrate and use existing test and non-test data for the assessment of the skin irritation and skin corrosion potentials of chemicals and (ii) proposes an approach when further testing is needed, including when negative results are found (5). In this modular approach, positive results from *in vitro* test methods can be used to classify a chemical as corrosive without the need for animal testing, thus reducing and refining the use of animals in and avoiding the pain and distress that might occur if animals were used for this purpose.

¹ Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006, OJ L 353/1, 31.12.2008

3. Validation studies have been completed for the *in vitro* membrane barrier model commercially available as Corrositex® (6)(7)(8), showing an overall accuracy to predict skin corrosivity of 79% (128/163), a sensitivity of 85% (76/89), and a specificity of 70% (52/74) for a database of 163 substances and mixtures (7). Based on its acknowledged validity, this validated reference method (VRM) has been recommended for use as part of a tiered testing strategy for assessing the dermal corrosion hazard potential of chemicals (5)(7). Before an *in vitro* membrane barrier model for skin corrosion can be used for regulatory purposes, its reliability, relevance (accuracy), and limitations for its proposed use should be determined to ensure that it is similar to that of the VRM (9), in accordance with the pre-defined performance standards (PS) (10). The OECD Mutual Acceptance of Data will only be guaranteed after any proposed new or updated method following the PS have been reviewed and included in the equivalent OECD test guideline. Currently, only one *in vitro* method is covered by OECD test guideline 435 and this test method, the commercially available Corrositex® model.
4. Other test methods for skin corrosivity testing are based on the use of reconstituted human skin (OECD TG 431) (3) and isolated rat skin (OECD TG 430) (4). This Test Guideline also provides for subcategorisation of corrosive chemicals into the three UN GHS Sub-categories of corrosivity and the three UN Transport Packing Groups for corrosivity hazard. This Test Guideline was originally adopted in 2006 and updated in 2015 to refer to the IATA guidance document and update the list of proficiency substances.

DEFINITIONS

5. Definitions used are provided in the Appendix.

INITIAL CONSIDERATIONS AND LIMITATIONS

6. The test described in this test method allows the identification of corrosive test chemicals and allows the sub-categorisation of corrosive test chemicals according to UN GHS/CLP (Table 1). In addition, such a test method may be used to make decisions on the corrosivity and non-corrosivity of specific classes of chemicals, *e.g.* organic and inorganic acids, acid derivatives¹, and bases for certain transport testing purposes (7)(11)(12). This test method describes a generic procedure similar to the validated reference test method (7). While this test method does not provide adequate information on skin irritation, it should be noted

¹ “Acid derivative” is a non-specific class designation and is broadly defined as a chemical produced from an acid either directly or by modification or partial substitution. This class includes anhydrides, halo acids, salts, and other types of chemicals.

that TM B.46 (equivalent to OECD TG 439) specifically addresses the health effect skin irritation *in vitro* (13). For a full evaluation of local skin effects after a single dermal exposure, the OECD Guidance Document on Integrated Approaches for Testing Assessment should be consulted (5).

Table 1: The UN GHS Skin Corrosive Category and Subcategories (1)

Corrosive Category (category 1) (for authorities not using subcategories)	Potential Corrosive Subcategories ¹ (for authorities using subcategories, including the CLP Regulation)	Corrosive in ≥ 1 of 3 animals	
		Exposure	Observation
Corrosive	Corrosive subcategory 1A	≤ 3 minutes	≤ 1 hour
	Corrosive subcategory 1B	> 3 minutes / ≤ 1 hour	≤ 14 days
	Corrosive subcategory 1C	> 1 hour / ≤ 4 hours	≤ 14 days

7. A limitation of the validated reference method (7) is that many non-corrosive chemicals and some corrosive chemicals may not qualify for testing, based on the results of the initial compatibility test (see paragraph 13). Aqueous chemicals with a pH in the range of 4.5 to 8.5 often do not qualify for testing; however, 85% of chemicals tested in this pH range were non-corrosive in animal tests (7). The *in vitro* membrane barrier method may be used to test solids (soluble or insoluble in water), liquids (aqueous or non-aqueous), and emulsions. However, test chemicals not causing a detectable change in the compatibility test (*i.e.* colour change in the Chemical Detection System (CDS) of the validated reference test method) cannot be tested with the membrane barrier method and should be tested using other test methods.

PRINCIPLE OF THE TEST

8. The test system comprises two components: a synthetic macromolecular bio-barrier and a chemical detection system (CDS); this test method detects via the CDS membrane barrier damage caused by corrosive test chemicals after the application of the test chemical to the surface of the synthetic macromolecular membrane barrier (7), presumably by the same mechanism(s) of corrosion that operate on living skin.

¹ For the EU, the CLP Regulation applies the three skin corrosion subcategories 1A, 1B and 1C.

9. Penetration of the membrane barrier (or breakthrough) might be measured by a number of procedures or CDS, including a change in the colour of a pH indicator dye or in some other property of the indicator solution below the barrier.
10. The membrane barrier should be determined to be valid, *i.e.* relevant and reliable, for its intended use. This includes ensuring that different preparations are consistent in regard to barrier properties, *e.g.* capable of maintaining a barrier to non-corrosive chemicals, able to categorise the corrosive properties of chemicals across the various UN GHS Sub-categories of corrosivity (1). The classification assigned is based on the time it takes a chemical to penetrate through the membrane barrier to the indicator solution.

DEMONSTRATION OF PROFICIENCY

11. Prior to routine use of the *in vitro* membrane barrier method, adhering to this test method, laboratories should demonstrate technical proficiency by correctly classifying the twelve Proficiency Substances recommended in Table 2. In situations where a listed substance is unavailable or where justifiable, another substance for which adequate *in vivo* and *in vitro* reference data are available may be used (*e.g.* from the list of reference chemicals (10)) provided that the same selection criteria as described in Table 1 is applied.

Table 2: Proficiency Substances¹

Substance ²	CASRN	Chemical Class	<i>In Vivo</i> UN GHS Sub-category ³	<i>In Vitro</i> UN GHS Sub-category ³
Boron trifluoride dihydrate	13319-75-0	Inorganic acids	1A	1A
Nitric acid	7697-37-2	Inorganic acids	1A	1A
Phosphorus pentachloride	10026-13-8	Precursors of inorganic acids	1A	1A
Valeryl chloride	638-29-9	Acid chlorides	1B	1B
Sodium Hydroxide	1310-73-2	Inorganic bases	1B	1B
1-(2-Aminoethyl) piperazine	140-31-8	Aliphatic amines	1B	1B
Benzenesulfonyl chloride	98-09-9	Acid chlorides	1C	1C
N,N-Dimethyl benzylamine	103-83-3	Anilines	1C	1C
Tetraethylenepentamine	112-57-2	Aliphatic amines	1C	1C
Eugenol	97-53-0	Phenols	NC	NC

Nonyl acrylate	2664-55-3	Acrylates/methacrylates	NC	NC
Sodium bicarbonate	144-55-8	Inorganic salts	NC	NC

¹The twelve substances listed above contain three substances from each of the three UN GHS subcategories for corrosive substances and three non-corrosive substances, are readily available from commercial suppliers, and the UN GHS subcategory is based on the results of high-quality *in vivo* testing. These substances are taken from the list of 40 reference substances that are included in the minimum list of chemicals identified for demonstrating the accuracy and reliability of test methods that are structurally and functionally similar to the validated reference test method, and were selected from the 163 reference chemicals that were originally used to validate the reference test method (Corrositex[®]) (7) (10) (14). The goal of this selection process was to include, to the extent possible, chemicals that: were representative of the range of corrosivity responses (e.g. non-corrosives; UN Packing Groups I, II, and III corrosives) that the validated reference test method is capable of measuring or predicting; were representative of the chemical classes used during the validation process; have chemical structures that were well-defined; induced reproducible results in the validated reference test method; induced definitive results in the *in vivo* reference test; were commercially available; and were not associated with prohibitive disposal costs (14).

²Substances tested neat or with purity $\geq 90\%$

³The corresponding UN Packing groups are I, II and III, respectively, for the UN GHS Sub-categories 1A, 1B and 1C. NC; Non-corrosive.

PROCEDURE

- The following paragraphs describe the components and procedures of an artificial membrane barrier test method for corrosivity assessment (7)(15), based on the current VRM, i.e. the commercially available Corrositex[®]. The membrane barrier and the compatibility/indicator and categorisation solutions can be constructed, prepared or obtained commercially such as in the case of the VRM Corrositex[®]. A sample test method protocol for the validated reference test method is available (7). Testing should be performed at ambient temperature (17-25°C) and the components should comply with the following conditions.

Test Chemical Compatibility Test

- Prior to performing the membrane barrier test, a compatibility test is performed to determine if the test chemical is detectable by the CDS. If the CDS does not detect the test chemical, the membrane barrier test method is not suitable for evaluating the potential corrosivity of that particular test chemical and a different test method should be used. The CDS and the exposure conditions used for the compatibility test should reflect the exposure in the subsequent membrane barrier test.

Test Chemical Timescale Category Test

- If appropriate for the test method, a test chemical that has been qualified by the compatibility test should be subjected to a timescale category test, i.e. a screening test to distinguish between weak and strong acids or bases. For example, in the validated reference test method a timescale categorisation test is used to indicate which of two timescales should be used based on whether significant acid or alkaline reserve is detected.

Two different breakthrough timescales should be used for determining corrosivity and UN GHS skin corrosivity Sub-category, based on the acid or alkali reserve of the test chemical.

MEMBRANE BARRIER TEST METHOD COMPONENTS

Membrane Barrier

15. The membrane barrier consists of two components: a proteinaceous macromolecular aqueous gel and a permeable supporting membrane. The proteinaceous gel should be impervious to liquids and solids but can be corroded and made permeable. The fully constructed membrane barrier should be stored under pre-determined conditions shown to preclude deterioration of the gel, *e.g.* drying, microbial growth, shifting, cracking, which would degrade its performance. The acceptable storage period should be determined and membrane barrier preparations not used after that period.
16. The permeable supporting membrane provides mechanical support to the proteinaceous gel during the gelling process and exposure to the test chemical. The supporting membrane should prevent sagging or shifting of the gel and be readily permeable to all test chemicals.
17. The proteinaceous gel, composed of protein, *e.g.* keratin, collagen, or mixtures of proteins, forming a gel matrix, serves as the target for the test chemical. The proteinaceous material is placed on the surface of the supporting membrane and allowed to gel prior to placing the membrane barrier over the indicator solution. The proteinaceous gel should be of equal thickness and density throughout, and with no air bubbles or defects that could affect its functional integrity.

Chemical Detection System (CDS)

18. The indicator solution, which is the same solution used for the compatibility test, should respond to the presence of a test chemical. A pH indicator dye or combination of dyes, *e.g.* cresol red and methyl orange that will show a colour change, in response to the presence of the test chemical, should be used. The measurement system can be visual or electronic.
19. Detection systems that are developed for detecting the passage of the test chemical through the barrier membrane should be assessed for their relevance and reliability in order to demonstrate the range of chemicals that can be detected and the quantitative limits of detection.

TEST PERFORMANCE

Assembly of the Test Method Components

20. The membrane barrier is positioned in a vial (or tube) containing the indicator solution so that the supporting membrane is in full contact with the indicator solution and with no air bubbles present. Care should be taken to ensure that barrier integrity is maintained.

Application of the Test Chemical

21. A suitable amount of the test chemical, *e.g.* 500 µl of a liquid or 500 mg of a finely powdered solid (7), is carefully layered onto the upper surface of the membrane barrier and evenly distributed. An appropriate number of replicates, *e.g.* four (7), is prepared for each test chemical and its corresponding controls (see paragraphs 23 to 25). The time of applying the test chemical to the membrane barrier is recorded. To ensure that short corrosion times are accurately recorded, the application times of the test chemical to the replicate vials are staggered.

Measurement of Membrane Barrier Penetrations

22. Each vial is appropriately monitored and the time of the first change in the indicator solution, *i.e.* barrier penetration, is recorded, and the elapsed time between application and penetration of the membrane barrier determined.

Controls

23. In tests that involve the use of a vehicle or solvent with the test chemical, the vehicle or solvent should be compatible with the membrane barrier system, *i.e.* not alter the integrity of the membrane barrier system, and should not alter the corrosivity of the test chemical. When applicable, solvent (or vehicle) control should be tested concurrently with the test chemical to demonstrate the compatibility of the solvent with the membrane barrier system.
24. A positive (corrosive) control with intermediate corrosivity activity, *e.g.* 110 ± 15 mg sodium hydroxide (UN GHS Corrosive Sub-category 1B) (7), should be tested concurrently with the test chemical to assess if the test system is performing in an acceptable manner. A second positive control that is of the same chemical class as the test chemical may be useful for evaluating the relative corrosivity potential of a corrosive test chemical. Positive control(s) should be selected that are intermediate in their corrosivity (*e.g.* UN GHS Sub-category 1B) in order to detect changes in the penetration time that may be unacceptably longer or shorter than the established reference value, thereby indicating that the test system is not functioning properly. For this purpose, extremely corrosive (UN GHS Sub-category 1A) or non-corrosive chemicals are of limited utility. A corrosive UN GHS Sub-category 1B chemical would allow detection of a too rapid or too slow breakthrough time. A weakly corrosive (UN GHS Sub-category 1C) might be employed as a positive control to measure the ability of the test method to consistently distinguish between weakly corrosive and non-corrosive chemicals. Regardless of the approach used, an acceptable positive control response range should be developed based on the historical range of breakthrough times for the positive control(s) employed, such as the mean $\pm$ 2-3 standard deviations. In each study, the exact breakthrough time should be determined for the positive control so that deviations outside the acceptable range can be detected.

25. A negative (non-corrosive) control, *e.g.* 10% citric acid, 6% propionic acid (7), should also be tested concurrently with the test chemical as another quality control measure to demonstrate the functional integrity of the membrane barrier.

Study Acceptability Criteria

26. According to the established time parameters for each of the UN GHS corrosivity Sub-categories, the time (in minutes) elapsed between application of a test chemical to the membrane barrier and barrier penetration is used to predict the corrosivity of the test chemical. For a study to be considered acceptable, the concurrent positive control should give the expected penetration response time (*e.g.* 8-16 min breakthrough time for sodium hydroxide if used as a positive control), the concurrent negative control should not be corrosive, and, when included, the concurrent solvent control should neither be corrosive nor should it alter the corrosivity potential of the test chemical. Prior to routine use of a method that adheres to this test method, laboratories should demonstrate technical proficiency, using the twelve substances recommended in Table 2. For new “me-too” methods developed under this test method that are structurally and functionally similar to the validated reference method (14) the pre-defined performance standards should be used to demonstrate the reliability and accuracy of the new method prior to its use for regulatory testing (10).

Interpretation of Results and Corrosivity Classification of Test Chemicals

27. The time (in minutes) elapsed between application of the test chemical to the membrane barrier and barrier penetration is used to classify the test chemical in terms of UN GHS corrosive Sub-categories (1) and, if applicable, UN Packing Group (16). Cut-off time values for each of the three corrosive subcategories are established for each proposed test method. Final decisions on cut-off times should consider the need to minimise under-classification of corrosive hazard (*i.e.* false negatives). In the present test guideline, the cut-off times of Corrositex® as described in table 3 should be used as it represents the only test method currently falling within the test guideline (7).

Table 3: Corrositex® prediction model

Mean breakthrough time (min.)		UN GHS prediction ³
Category 1 test chemicals ¹ (determined by the method's categorisation test)	Category 2 test chemicals ² (determined by the method's categorisation test)	
0-3 min.	0-3 min.	Corrosive optional Sub-category 1A
> 3 to 60 min.	> 3 to 30 min.	Corrosive optional Sub-category 1B

> 60 to 240 min.	> 30 to 60 min.	Corrosive optional Sub-category 1C
> 240 min.	> 60 min.	Non-corrosive

¹ Test chemicals with high acid/alkaline reserve (6)

² Test chemicals with low acid/alkaline reserve (6)

³ UN GHS Subcategories 1A, 1B and 1C correspond to UN packing groups I, II and III respectively

DATA AND REPORTING

Data

28. The time (in minutes) elapsed between application and barrier penetration for the test chemical and the positive control(s) should be reported in tabular form as individual replicate data, as well as means $\pm$ the standard deviation for each trial.

Test Report

29. The test report should include the following information:

Test Chemical and Control Substances:

- Mono-constituent substance: chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc;
- Multi-constituent substance, UVCB and mixture: characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents;
- Physical appearance, water solubility, and additional relevant physicochemical properties;
- Source, lot number if available;
- Treatment of the test chemical/control substance prior to testing, if applicable (e.g. warming, grinding);
- Stability of the test chemical, limit date for use, or date for re-analysis if known;
- Storage conditions.

Vehicle:

- Identification, concentration (where appropriate), volume used;
- Justification for choice of vehicle.

In vitro membrane barrier model and protocol used, including demonstrated accuracy and reliability

Test Conditions:

- Description of the apparatus and preparation procedures used;
- Source and composition of the *in vitro* membrane barrier used;
- Composition and properties of the indicator solution;
- Method of detection;
- Test chemical and control substance amounts;
- Number of replicates;
- Description and justification for the timescale categorisation test;
- Method of application;
- Observation times.
- Description of the evaluation and classification criteria applied;
- Demonstration of proficiency in performing the test method before routine use by testing of the proficiency chemicals.

Results:

- Tabulation of individual raw data from individual test and control samples for each replicate;
- Descriptions of other effects observed;
- The derived classification with reference to the prediction model/decision criteria used.

Discussion of the results

Conclusions

LITERATURE

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- (2) Chapter B.4 of this Annex, Acute Dermal Irritation, Corrosion.
- (3) Chapter B.40bis of this Annex, *In vitro* skin corrosion: reconstructed human epidermis (RHE) test method.
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Appendix

DEFINITIONS

Accuracy: The closeness of agreement between test method results and accepted reference values. It is a measure of test method performance and one aspect of relevance. The term is often used interchangeably with “concordance” to mean the proportion of correct outcomes of a test method (9).

Chemical: A substance or a mixture.

Chemical Detection System (CDS): A visual or electronic measurement system with an indicator solution that responds to the presence of a test chemical, *e.g.* by a change in a pH indicator dye, or combination of dyes, that will show a colour change in response to the presence of the test chemical or by other types of chemical or electrochemical reactions.

Concordance: This is a measure of test method performance for test methods that give a categorical result, and is one aspect of relevance. The term is sometimes used interchangeably with accuracy, and is defined as the proportion of all chemicals tested that are correctly classified as positive or negative. Concordance is highly dependent on the prevalence of positives in the types of test chemical being examined (9).

GHS (Globally Harmonized System of Classification and Labelling of Chemicals): A system proposing the classification of chemicals (substances and mixtures) according to standardised types and levels of physical, health and environmental hazards, and addressing corresponding communication elements, such as pictograms, signal words, hazard statements, precautionary statements and safety data sheets, so that to convey information on their adverse effects with a view to protect people (including employers, workers, transporters, consumers and emergency responders) and the environment (1).

IATA: Integrated Approach on Testing and Assessment.

Mixture: A mixture or solution composed of two or more substances.

Mono-constituent substance: A substance, defined by its quantitative composition, in which one main constituent is present to at least 80% (w/w).

Multi-constituent substance: A substance, defined by its quantitative composition, in which more than one main constituent is present in a concentration $\geq 10\%$ (w/w) and $< 80\%$ (w/w). A multi-constituent substance is the result of a manufacturing process. The difference between mixture and multi-constituent substance is that a mixture is obtained by blending of two or more substances without chemical reaction. A multi-constituent substance is the result of a chemical reaction.

NC: Non corrosive.

Performance standards: Standards, based on a validated test method, that provide a basis for evaluating the comparability of a proposed test method that is mechanistically and

functionally similar. Included are (i) essential test method components; (ii) a minimum list of Reference Chemicals selected from among the chemicals used to demonstrate the acceptable performance of the validated test method; and (iii) the similar levels of reliability and accuracy, based on what was obtained for the validated test method, that the proposed test method should demonstrate when evaluated using the minimum list of Reference Chemicals (9).

Relevance: Description of relationship of the test method to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the test method correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of the accuracy (concordance) of a test method (9).

Reliability: Measures of the extent that a test method can be performed reproducibly within and between laboratories over time, when performed using the same protocol. It is assessed by calculating intra- and inter-laboratory reproducibility (9).

Sensitivity: The proportion of all positive/active chemicals that are correctly classified by the test method. It is a measure of accuracy for a test method that produces categorical results, and is an important consideration in assessing the relevance of a test method (9).

Skin corrosion *in vivo*: The production of irreversible damage of the skin; namely, visible necrosis through the *epidermis* and into the dermis, following the application of a test chemical for up to four hours. Corrosive reactions are typified by ulcers, bleeding, bloody scabs, and, by the end of observation at 14 days, by discoloration due to blanching of the skin, complete areas of alopecia, and scars. Histopathology should be considered to evaluate questionable lesions.

Specificity: The proportion of all negative/inactive chemicals that are correctly classified by the test method. It is a measure of accuracy for a test method that produces categorical results and is an important consideration in assessing the relevance of a test method (9).

Substance: A chemical element and its compounds in the natural state or obtained by any production process, inducing any additive necessary to preserve its stability and any impurities deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the substance or changing its composition.

Test chemical: Any substance or mixture tested using this test method.

UVCB: Substances of unknown or variable composition, complex reaction products or biological materials.

B.66 STABLY TRANSFECTED TRANSACTIVATION *IN VITRO* ASSAYS TO DETECT ESTROGEN RECEPTOR AGONISTS AND ANTAGONISTS

GENERAL INTRODUCTION

OECD Performance-Based Test Guideline

1. This test method is equivalent to OECD test guideline (TG) 455 (2016). TG 455 is a performance-based test guideline (PBTG), describing the methodology of Stably Transfected Transactivation *In Vitro* Assays to detect Estrogen Receptor Agonists and Antagonists (ER TA assays). It comprises several mechanistically and functionally similar test methods for the identification of estrogen receptor (i.e. ER α , and/or ER β) agonists and antagonists and should facilitate the development of new similar or modified test methods in accordance with the principles for validation set forth in the OECD Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment (1). The fully validated reference test methods (Appendix 2 and Appendix 3) that provide the basis for this PBTG are:
 - The Stably Transfected TA (STTA) assay (2) using the (h) ER α -HeLa-9903 cell line; and
 - The VM7Luc ER TA assay (3) using the VM7Luc4E2 cell line¹ which predominately expresses hER α with some contribution from hER β (4)(5).

For the development and validation of similar assays for the same hazard endpoint, performance standards (PS) (6) (7) are available and should be used. They allow for timely amendment of PBTG 455 so that new similar assays can be added to an updated PBTG; however, similar assays will only be added after review and agreement by OECD that performance standards are met. The assays included in TG 455 can be used indiscriminately to address OECD member countries' requirements for test results on estrogen receptor transactivation while benefiting from the OECD Mutual Acceptance of Data.

Background and principles of the assays included in this test method

¹ Before June 2016, this cell line was designated as BG1Luc cell line. BG-1 cells were originally described by Geisinger et al. (1998) (35) and were later characterized by researchers at the National Institute of Environmental Health Sciences (NIEHS) (36). Relatively recently, it was discovered that there exist two different variants of BG-1 cells being used by researchers, BG-1 Fr and BG-1 NIEHS. In-depth analysis, including DNA testing, of these two BG-1 variant cell lines carried out by Li and coworkers (2014) (37) showed that the BG-1 Fr was unique and that the BG-1 NIEHS, i.e. the original cell line used to develop the assay, was not the BG1 human ovarian carcinoma cell line, but was instead a variant of the MCF7 human breast cancer cell line. The cell line used in the assay, originally referred to as BG1Luc4E2 (38), will now be designated as VM7Luc4E2 ("V" = variant; "M7" = MCF7 cells). Likewise, the assay will now be designated as the VM7Luc ER TA. While this changes the origin of the cell line upon which the assay is based, it does not affect published validation studies nor the utility and application of this assay for screening of estrogenic/anti-estrogenic chemicals.

2. The OECD initiated a high-priority activity in 1998 to revise existing, and to develop new test guidelines for the screening and testing of potential endocrine disrupting chemicals. The OECD conceptual framework (CF) for testing and assessment of potential endocrine disrupting chemicals was revised in 2012. The original and revised CFs are included as Annexes in the OECD Guidance Document on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption (8). The CF comprises five levels, each level corresponding to a different level of biological complexity. The ER Transactivation (TA) assays described in this test method are level 2, which includes "*in vitro assays providing data about selected endocrine mechanism(s)/pathway(s)*". This test method is for *in vitro* Transactivation (TA) assays designed to identify estrogen receptor (ER) agonists and antagonists.
3. The interaction of estrogens with ERs can affect transcription of estrogen-controlled genes, which can lead to the induction or inhibition of cellular processes, including those necessary for cell proliferation, normal fetal development, and reproductive function (9)(10)(11). Perturbation of normal estrogenic systems may have the potential to trigger adverse effects on normal development (ontogenesis), reproductive health and the integrity of the reproductive system.
4. *In vitro* TA assays are based on a direct or indirect interaction of the substances with a specific receptor that regulates the transcription of a reporter gene product. Such assays have been used extensively to evaluate gene expression regulated by specific nuclear receptors, such as ERs (12) (13) (14) (15) (16). They have been proposed for the detection of estrogenic transactivation regulated by the ER (17) (18) (19). There are at least two major subtypes of nuclear ERs, α and β , which are encoded by distinct genes. The respective proteins have different biological functions as well as different tissue distributions and ligand binding affinities (20)(21)(22)(23)(24)(25)(26). Nuclear ER α mediates the classic estrogenic response (27)(28)(29)(30), and therefore most models currently being developed to measure ER activation or inhibition are specific to ER α . The assays are used to identify chemicals that activate (or inhibit) the ER following ligand binding, after which the receptor-ligand complex binds to specific DNA response elements and transactivates a reporter gene, resulting in increased cellular expression of a marker protein. Different reporter responses can be used in these assays. In luciferase based systems, the luciferase enzyme transforms the luciferin substrate to a bioluminescent product that can be quantitatively measured with a luminometer. Other examples of common reporters are fluorescent protein and the *LacZ* gene, which encodes β -galactosidase, an enzyme that can transform the colourless substrate X-gal (5-bromo-4-chloro-indolyl-galactopyranoside) into a blue product that can be quantified with a spectrophotometer. These reporters can be evaluated quickly and inexpensively with commercially available test kits.

5. Validation studies of the STTA and the VM7Luc TA assays have demonstrated their relevance and reliability for their intended purpose (3)(4)(5)(30). Performance standards for luminescence-based ER TA assays using breast cell lines are included in ICCVAM Test Method Evaluation Report on the LUMI-CELL[®] ER (VM7Luc ER TA) Test Method: An *In Vitro* Assay for Identifying Human Estrogen Receptor Agonist and Antagonist Activity of Chemicals (3). These performance standards have been modified to be applicable to both the STTA and VM7Luc TA assays (2).
6. Definitions and abbreviations used in this test method are described in Appendix 1.

Scope and limitations related to the TA assays

7. These assays are being proposed for screening and prioritisation purposes, but can also provide mechanistic information that can be used in a weight of evidence approach. They address TA induced by chemical binding to the ERs in an *in vitro* system. Thus, results should not be directly extrapolated to the complex signalling and regulation of the intact endocrine system *in vivo*.
8. TA mediated by the ERs is considered one of the key mechanisms of endocrine disruption (ED), although there are other mechanisms through which ED can occur, including (i) interactions with other receptors and enzymatic systems within the endocrine system, (ii) hormone synthesis, (iii) metabolic activation and/or inactivation of hormones, (iv) distribution of hormones to target tissues, and (v) clearance of hormones from the body. None of the assays under this test method addresses these modes of action.
9. This test method addresses the ability of chemicals to activate (i.e. act as agonists) and also to suppress (i.e. act as antagonists) ER- dependent transcription. Some chemicals may, in a cell type-dependent manner, display both agonist and antagonist activity and are known as selective estrogen receptor modulators (SERMs). Chemicals that are negative in these assays could be evaluated in an ER binding assay before concluding that the chemical does not bind to the receptor. In addition, the assays are only likely to inform on the activity of the parent molecule bearing in mind the limited metabolising capacities of the *in vitro* cell systems. Considering that only single substances were used during the validation, the applicability to test mixtures has not been addressed. The test method is nevertheless theoretically applicable to the testing of multi-constituent substances, UVCBs and mixtures. Before use of the test method on a multi-constituent substance, UVCB or mixture for generating data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed, when there is a regulatory requirement for testing of the mixture.
10. For informational purposes, Table 1 provides the agonist test results for the 34 substances that were tested in both of the fully validated reference test methods

described in this test method. Of these substances, 26 are classified as definitive ER agonists and 8 negatives based upon published reports, including *in vitro* assays for ER binding and TA, and/or the uterotrophic assay (2)(3)(18)(31)(32)(33)(34). Table 2 provides the antagonist test results for the 15 substances that were tested in both of the fully validated reference test methods described in this test method. Of these substances, 4 are classified as definitive/presumed ER antagonists and 10 negatives based upon published reports, including *in vitro* assays for ER binding and TA (2)(3)(18)(31). In reference to the data summarised in Table 1 and Table 2, there was 100% agreement between the two reference test methods on the classifications of all the substances except for one substance (Mifepristone) for antagonist assay, and each substance was correctly classified as an ER agonist/antagonist or negative. Supplementary information on this group of chemicals as well as additional chemicals tested in the STTA and VM7Luc ER TA assays during the validation studies is provided in the Performance Standards for the ERTA (6)(7), Appendix 2 (Tables 1, 2 and 3).

Table 1: Overview of the Results from STTA and VM7Luc ER TA Assays for Substances Tested in Both Agonist Assays and Classified as ER Agonists (POS) or Negatives (NEG)

	Substance	CASRN	STTA Assay ¹			VM7Luc ER TA Assay ²		Data Source For Classification ⁴		
			ER TA Activity	PC10 Value (M)	PC50 Value ^b (M)	ER TA Activity	EC50 Value ^{b,3} (M)	Other ER TAs ^c	ER Binding	Uterotrophic
1	17 β -estradiol ^a	50-28-2	POS	$<1.00 \times 10^{-11}$	$<1.00 \times 10^{-11}$	POS	5.63×10^{-12}	POS (227/227)	POS	POS
2	17 α -estradiol ^a	57-91-0	POS	7.24×10^{-11}	6.44×10^{-10}	POS	1.40×10^{-9}	POS(11/11)	POS	POS
3	17 α -ethinyl estradiol ^a	57-63-6	POS	$<1.00 \times 10^{-11}$	$<1.00 \times 10^{-11}$	POS	7.31×10^{-12}	POS(22/22)	POS	POS
4	17 β -trenbolone	10161-33-8	POS	1.78×10^{-8}	2.73×10^{-7}	POS	4.20×10^{-8}	POS (2/2)	NT	NT
5	19-nortestosterone ^a	434-22-0	POS	9.64×10^{-9}	2.71×10^{-7}	POS	1.80×10^{-6}	POS(4/4)	POS	POS
6	4-cumylphenol ^a	599-64-4	POS	1.49×10^{-7}	1.60×10^{-6}	POS	3.20×10^{-7}	POS(5/5)	POS	NT
7	4- <i>tert</i> -octylphenol ^a	140-66-9	POS	1.85×10^{-9}	7.37×10^{-8}	POS	3.19×10^{-8}	POS(21/24)	POS	POS
8	Apigenin ^a	520-36-5	POS	1.31×10^{-7}	5.71×10^{-7}	POS	1.60×10^{-6}	POS(26/26)	POS	NT
9	Atrazine ^a	1912-24-9	NEG	-	-	NEG	-	NEG (30/30)	NEG	NT
10	Bisphenol A ^a	80-05-7	POS	2.02×10^{-8}	2.94×10^{-7}	POS	5.33×10^{-7}	POS(65/65)	POS	POS
11	Bisphenol B ^a	77-40-7	POS	2.36×10^{-8}	2.11×10^{-7}	POS	1.95×10^{-7}	POS(6/6)	POS	POS
12	Butylbenzyl phthalate ^a	85-68-7	POS	1.14×10^{-6}	4.11×10^{-6}	POS	1.98×10^{-6}	POS(12/14)	POS	NEG
13	Corticosterone ^a	50-22-6	NEG	-	-	NEG	-	NEG(6/6)	NEG	NT
14	Coumestrol ^a	479-13-0	POS	1.23×10^{-9}	2.00×10^{-8}	POS	1.32×10^{-7}	POS(30/30)	POS	NT
15	Daidzein ^a	486-66-8	POS	1.76×10^{-8}	1.51×10^{-7}	POS	7.95×10^{-7}	POS(39/39)	POS	POS
16	Diethylstilbestrol ^a	56-53-1	POS	$<1.00 \times 10^{-11}$	2.04×10^{-11}	POS	3.34×10^{-11}	POS(42/42)	POS	NT
17	Di-n-butyl phthalate	84-74-2	POS	4.09×10^{-6}		POS	4.09×10^{-6}	POS(6/11)	POS	NEG
18	Ethyl paraben	120-47-8	POS	5.00×10^{-6}	(no PC50)	POS	2.48×10^{-5}	POS		NT
19	Estrone ^a	53-16-7	POS	3.02×10^{-11}	5.88×10^{-10}	POS	2.34×10^{-10}	POS(26/28)	POS	POS
20	Genistein ^a	446-72-0	POS	2.24×10^{-9}	2.45×10^{-8}	POS	2.71×10^{-7}	POS(100/102)	POS	POS
21	Haloperidol	52-86-8	NEG	-	-	NEG	-	NEG (2/2)	NEG	NT
22	Kaempferol ^a	520-18-3	POS	1.36×10^{-7}	1.21×10^{-6}	POS	3.99×10^{-6}	POS(23/23)	POS	NT
23	Kepone ^a	143-50-0	POS	7.11×10^{-7}	7.68×10^{-6}	POS	4.91×10^{-7}	POS(14/18)	POS	NT
24	Ketoconazole	65277-42-1	NEG	-	-	NEG	-	NEG (2/2)	NEG	NT

25	Linuron ^a	330-55-2	NEG	-	-	NEG	-	NEG (8/8)	NEG	NT
26	meso-Hexestrol ^a	84-16-2	POS	$<1.00 \times 10^{-11}$	2.75×10^{-11}	POS	1.65×10^{-11}	POS(4/4)	POS	NT
27	Methyl testosterone ^a	58-18-4	POS	1.73×10^{-7}	4.11×10^{-6}	POS	2.68×10^{-6}	POS(5/6)	POS	NT
28	Morin	480-16-0	POS	5.43×10^{-7}	4.16×10^{-6}	POS	2.37×10^{-6}	POS(2/2)	POS	NT
29	Norethynodrel ^a	68-23-5	POS	1.11×10^{-11}	1.50×10^{-9}	POS	9.39×10^{-10}	POS(5/5)	POS	NT
30	<i>p,p'</i> -Methoxychlor ^a	72-43-5	POS	1.23×10^{-6}	(no PC ₅₀) ^b	POS	1.92×10^{-6}	POS(24/27)	POS	POS
31	Phenobarbital ^a	57-30-7	NEG	-	-	NEG	-	NEG(2/2)	NEG	NT
32	Reserpine	50-55-5	NEG	-	-	NEG	-	NEG(4/4)	NEG	NT
33	Spironolactone ^a	52-01-7	NEG	-	-	NEG	-	NEG(4/4)	NEG	NT
34	Testosterone	58-22-0	POS	2.82×10^{-8}	9.78×10^{-6}	POS	1.75×10^{-5}	POS(5/10)	POS	NT

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; M = molar; EC₅₀ = half maximal effective concentration of test substance; NEG = negative; POS = positive; NT = Not tested; PC₁₀ (and PC₅₀) = the concentration of a test substance at which the response is 10% (or 50 % for PC₅₀) of the response induced by the positive control (E₂, 1nM) in each plate.

^aCommon substances tested in the STTA and VM7Luc ER TA assays that were designated as ER agonists or negatives and used to evaluate accuracy in the VM7Luc ER TA validation study (ICCVAM VM7Luc ER TA Evaluation Report, Table 4-1 (3)).

^bMaximum concentration tested in the absence of limitations due to cytotoxicity or insolubility was 1×10^{-5} M (STTA Assay) and 1×10^{-3} M (VM7Luc ER TA Assay).

^cNumber in parenthesis represents the test results classified as positive (POS) or negative (NEG) over the total number of referenced studies.

¹Values reported in Draft Report of Pre-validation and Inter-laboratory Validation For Stably Transfected Transcriptional Activation (TA) Assay to Detect Estrogenic Activity - The Human Estrogen Receptor Alpha Mediated Reporter Gene Assay Using hER-HeLa-9903 Cell Line (2)

²ICCVAM Test Method Evaluation Report on the LUMI-CELL[®] ER (VM7Luc ER TA) Test Method: An *In Vitro* Method for Identifying ER Agonists and Antagonists (3)

³Mean EC₅₀ values were calculated with values reported by the laboratories of the VM7Luc ER TA validation study (XDS, ECVAM, and Hiyoshi) (3).

⁴Classification as an ER agonist or negative was based upon information in the ICCVAM Background Review Documents (BRD) for ER Binding and TA test methods (31) as well as information obtained from publications published and reviewed after the completion of the ICCVAM BRDs (2) (3) (18) (31) (33) (34).

Notes: Each assay within this test method does not have the same measurements. In some situations the EC₅₀ cannot be calculated because a full dose response curve is not generated. Whilst with the STTA assay, the PC₁₀ value is a key measurement, there may also be further examples where a PC_x will provide useful information.

Table 2: Comparison of Results from STTA and VM7Luc ER TA Assays for Substances Tested in Both Antagonist Assays and Classified as ER Antagonists (POS) or Negatives (NEG)

	Substance ^a	CASRN	ER STTA assay ¹		VM7Luc ER TA assay ²		ER STTA candidate effects ⁴	ICCVAM ⁵ Consensus Classification	MeSH ⁶ Chemical Class	Product Class ⁷
			ER TA Activity	IC ₅₀ Value ^b (M)	ER TA Activity	IC ₅₀ Value ^{b,3} (M)				
1	4-hydroxytamoxifen	68047-06-3	POS	3.97×10^{-9}	POS	2.08×10^{-7}	moderate POS	POS	Hydrocarbon (Cyclic)	Pharmaceutical
2	Dibenzo[a,h] anthracene	53-70-3	POS	No IC ₅₀	POS	No IC ₅₀	POS	PP	Polycyclic Compound	Laboratory Chemical, Natural Product
3	Mifepristone	84371-65-3	POS	5.61×10^{-6}	NEG	-	mild POS	NEG	Steroid	Pharmaceutical
4	Raloxifene HCl	82640-04-8	POS	7.86×10^{-10}	POS	1.19×10^{-9}	moderate POS	POS	Hydrocarbon (Cyclic)	Pharmaceutical
5	Tamoxifen	10540-29-1	POS	4.91×10^{-7}	POS	8.17×10^{-7}	POS	POS	Hydrocarbon (Cyclic)	Pharmaceutical
6	17 β -estradiol	50-28-2	NEG	-	NEG	-	PN	PN	Steroid	Pharmaceutical, Veterinary Agent
7	Apigenin	520-36-5	NEG	-	NEG	-	NEG	NEG	Heterocyclic Compound	Dye, Natural Product, Pharmaceutical Intermediate
8	Atrazine	1912-24-9	NEG	-	NEG	-	NEG	PN	Heterocyclic Compound	Herbicide
9	Di-n-butyl phthalate	84-74-2	NEG	-	NEG	-	NEG	NEG	Ester, Phthalic Acid	Cosmetic Ingredient, Industrial Chemical, Plasticiser
10	Fenarimol	60168-88-9	NEG	-	NEG	-	not tested	PN	Heterocyclic Compound, Pyrimidine	Fungicide
11	Flavone	525-82-6	NEG	-	NEG	-	PN	PN	Flavonoid, Heterocyclic Compound	Natural Product, Pharmaceutical
12	Flutamide	13311-84-7	NEG	-	NEG	-	NEG	PN	Amide	Pharmaceutical, Veterinary Agent
13	Genistein	446-72-0	NEG	-	NEG	-	PN	NEG	Flavonoid, Heterocyclic Compound	Natural Product, Pharmaceutical
14	p-n-nonylphenol	104-40-5	NEG	-	NEG	-	not tested	NEG	Phenol	Chemical Intermediate
15	Resveratrol	501-36-0	NEG	-	NEG	-	PN	NEG	Hydrocarbon (Cyclic)	Natural Product

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; M = molar; IC₅₀ = half maximal inhibitory concentration of test substance; NEG = negative; PN = presumed negative; POS = positive; PP = presumed positive.

- ^a Common substances tested in the STTA and VM7Luc ER TA assays that were designated as ER antagonists or negatives and used to evaluate accuracy in the VM7Luc ER TA validation study (2) (3).
- ^b Maximum concentration tested in the absence of limitations due to cytotoxicity or insolubility was 1×10^{-3} M (STTA Assay) and 1×10^{-5} M (VM7Luc ER TA Assay).
- ¹ The Validation Report of the Stably transfected Transcriptional Activation Assay to Detect ER mediated activity, Part B (2)
- ² ICCVAM Test Method Evaluation Report on the LUMI-CELL[®] ER (VM7Luc ER TA) Test Method: An *In Vitro* Method for Identifying ER Agonists and Antagonists (3).
- ³ Mean IC₅₀ values were calculated with values reported by the laboratories of the VM7Luc ER TA validation study (XDS, ECVAM, and Hiyoshi) (3).
- ⁴ ER STTA activity assumed from their reported effects known from the CERI historical data of ER receptor binding assay, the uterotrophic assay and information collated from the open literature (2)
- ⁵ Classification as an ER antagonist or negative was based upon information in the ICCVAM Background Review Documents (BRD) for ER Binding and TA assays (31) as well as information obtained from publications published and reviewed after the completion of the ICCVAM BRDs (2) (3) (18) (31).
- ⁶ Substances were assigned to one or more chemical classes using the U.S. National Library of Medicine's Medical Subject Headings (MeSH), an internationally recognised standardised classification scheme (available at <http://www.nlm.nih.gov/mesh>).
- ⁷ Substances were assigned to one or more product classes using the U.S. National Library of Medicine's Hazardous Substances Data Bank (available at <http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB>).

ER TA ASSAY COMPONENTS

Essential Assay Components

11. This test method applies to assays using a stably transfected or endogenous ER α receptor and stably transfected reporter gene construct under the control of one or more estrogen response elements; however, other receptors such as ER β may be present. These are essential assay components.

Controls

12. The basis for the proposed concurrent reference standards for each of agonist and antagonist assay should be described. Concurrent controls (negative, solvent, and positive), as appropriate, serve as an indication that the assay is operative under the test conditions and provide a basis for experiment-to-experiment comparisons; they are usually part of the acceptability criteria for a given experiment (1).

Standard Quality Control Procedures

13. Standard quality control procedures should be performed as described for each assay to ensure the cell line remains stable through multiple passages, remains mycoplasma-free (i.e. free of bacterial contamination), and retains the ability to provide the expected ER-mediated responses over time. Cell lines should be further checked for their correct identity as well as for other contaminants (e.g. fungi, yeast and viruses).

Demonstration of Laboratory Proficiency

14. Prior to testing unknown chemicals with any of the assays under this test method, each laboratory should demonstrate proficiency in using the assay. To demonstrate proficiency, each laboratory should test the 14 proficiency substances listed in Table 3 for the agonist assay and 10 proficiency substances in Table 4 for the antagonist assay. This proficiency testing will also confirm the responsiveness of the test system. The list of proficiency substances is a subset of the reference substances provided in the Performance Standards for the ER TA assays (6). These substances are commercially available, represent the classes of chemicals commonly associated with ER agonist or antagonist activity, exhibit a suitable range of potency expected for ER agonists/antagonists (i.e. strong to weak) and include negatives. Testing of the proficiency substances should be replicated at least twice, on different days. Proficiency is demonstrated by correct classification (positive/negative) of each proficiency substance. Proficiency testing should be repeated by each technician when learning the assays. Dependent on cell type, some of these proficiency substances may behave as SERMs and display activity as both agonists and antagonists. However, the proficiency substances are classified in Tables 3 and 4 by their known predominant activity which should be used for proficiency evaluation.

15. To demonstrate performance and for quality control purposes each laboratory should compile agonist and antagonist historical databases with reference standard (e.g. 17β -estradiol and tamoxifen), positive and negative control chemicals and solvent control (e.g. DMSO) data. As a start, the database should be generated from at least 10 independent agonist (e.g. 17β -estradiol) and 10 independent antagonist (e.g. tamoxifen) runs. Results from future analyses of these reference standards and solvent controls should be added to enlarge the database to ensure consistency and performance of the bioassay by the laboratory over time.

Table 3: List of (14) Proficiency Substances for agonist assay⁸

N ^{o7}	Substance	CASRN	Expected Response ¹	STTA Assay			VM7Luc ER TA Assay		MeSH Chemical Class ⁵	Product Class ⁶
				PC ₁₀ Value (M) ²	PC ₅₀ Value (M) ²	Test Conc. Range (M)	VM7Luc EC ₅₀ Value (M) ³	Highest Conc. for Range Finder (M) ⁴		
14	Diethylstilbestrol	56-53-1	POS	$<1.00 \times 10^{-11}$	2.04×10^{-11}	$10^{-14} - 10^{-8}$	3.34×10^{-11}	3.73×10^{-4}	Hydrocarbon (Cyclic)	Pharmaceutical, Veterinary Agent
12	17 α -estradiol	57-91-0	POS	4.27×10^{-11}	6.44×10^{-10}	$10^{-11} - 10^{-5}$	1.40×10^{-9}	3.67×10^{-3}	Steroid	Pharmaceutical, Veterinary Agent
15	<i>meso</i> -Hexestrol	84-16-2	POS	$<1.00 \times 10^{-11}$	2.75×10^{-11}	$10^{-11} - 10^{-5}$	1.65×10^{-11}	3.70×10^{-3}	Hydrocarbon (Cyclic), Phenol	Pharmaceutical, Veterinary Agent
11	4- <i>tert</i> -Octylphenol	140-66-9	POS	1.85×10^{-9}	7.37×10^{-8}	$10^{-11} - 10^{-5}$	3.19×10^{-8}	4.85×10^{-3}	Phenol	Chemical Intermediate
9	Genistein	446-72-0	POS	2.24×10^{-9}	2.45×10^{-8}	$10^{-11} - 10^{-5}$	2.71×10^{-7}	3.70×10^{-4}	Flavonoid, Heterocyclic Compound	Natural Product, Pharmaceutical
6	Bisphenol A	80-05-7	POS	2.02×10^{-8}	2.94×10^{-7}	$10^{-11} - 10^{-5}$	5.33×10^{-7}	4.38×10^{-3}	Phenol	Chemical Intermediate
2	Kaempferol	520-18-3	POS	1.36×10^{-7}	1.21×10^{-6}	$10^{-11} - 10^{-5}$	3.99×10^{-6}	3.49×10^{-3}	Flavonoid, Heterocyclic Compound	Natural Product
3	Butylbenzyl phthalate	85-68-7	POS	1.14×10^{-6}	4.11×10^{-6}	$10^{-11} - 10^{-5}$	1.98×10^{-6}	3.20×10^{-4}	Carboxylic Acid, Ester, Phthalic Acid	Plasticiser, Industrial Chemical
4	<i>p,p'</i> -Methoxychlor	72-43-5	POS	1.23×10^{-6}	-	$10^{-11} - 10^{-5}$	1.92×10^{-6}	2.89×10^{-3}	Hydrocarbon (Halogenated)	Pesticide, Veterinary Agent
1	Ethyl paraben	120-47-8	POS	5.00×10^{-6}	-	$10^{-11} - 10^{-5}$	2.48×10^{-5}	6.02×10^{-3}	Carboxylic Acid, Phenol	Pharmaceutical, Preservative
17	Atrazine	1912-24-9	NEG	-	-	$10^{-10} - 10^{-4}$	-	4.64×10^{-4}	Heterocyclic Compound	Herbicide
20	Spironolactone	52-01-7	NEG	-	-	$10^{-11} - 10^{-5}$	-	2.40×10^{-3}	Lactone, Steroid	Pharmaceutical
21	Ketoconazole	65277-42-1	NEG	-	-	$10^{-11} - 10^{-5}$	-	9.41×10^{-5}	Heterocyclic Compound	Pharmaceutical
22	Reserpine	50-55-5	NEG	-	-	$10^{-11} - 10^{-5}$	-	1.64×10^{-3}	Heterocyclic Compound, Indole	Pharmaceutical, Veterinary Agent

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; EC₅₀ = half maximal effective concentration of test substance; NEG = negative; POS = positive;

PC10 (and PC50) = the concentration of a test substance at which the response is 10% (or 50 % for PC50) of the response induced by the positive control (E2, 1nM) in each plate.

¹Classification as positive or negative for ER agonist activity was based upon the ICCVAM Background Review Documents (BRD) for ER Binding and TA assays (31) as well as empirical data and other information obtained from referenced studies published and reviewed after the completion of the ICCVAM BRDs (2) (3) (18) (31) (32) (33) (34).

²Values reported in Draft Report of Pre-validation and Inter-laboratory Validation For Stably Transfected Transcriptional Activation (TA) Assay to Detect Estrogenic Activity - The Human Estrogen Receptor Alpha Mediated Reporter Gene Assay Using hER-HeLa-9903 Cell Line (30).

³Mean EC50 values were calculated with values reported by the laboratories of the VM7Luc ER TA validation study (XDS, ECVAM, and Hiyoshi) (3).

⁴Concentrations reported were the highest concentrations tested (range finder) during the validation of the VM7Luc ER TA Assay. If concentrations differed between the laboratories, the highest concentration is reported. See table 4-10 of ICCVAM Test Method Evaluation Report; The LUMI-Cell[®]ER (VM7Luc ER TA) Test Method: An *In Vitro* Assay for Identifying Human Estrogen Receptor Agonist and Antagonist Activity of Chemicals (3).

⁵Substances were assigned into one or more chemical classes using the U.S. National Library of Medicine's Medical Subject Headings (MeSH), an internationally recognised standardised classification scheme (available at: <http://www.nlm.nih.gov/mesh>).

⁶Substances were assigned into one or more product classes using the U.S. National Library of Medicine's Hazardous Substances Database (available at: <http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB>)

⁷From Table 1 (List of Reference Chemicals (22) for Evaluation of ER Agonist Accuracy) of the Performance Standards (6)

⁸If a proficiency substance is no longer commercially available, a substance with the same classification and, comparable potency, mode of action and chemical class can be used.

Table 4: List of (10) Proficiency Substances for antagonist assay

	Substance ^a	CASRN	ER STTA assay ¹			VM7Luc ER TA assay ²			ER STTA ¹ Candidate Effects	ICCVAM ⁵ Consensus Classification	MeSH ⁶ Chemical Class	Product Class ⁷
			ER TA Activity	IC ₅₀ (M)	Test Conc. range (M)	ER TA Activity	IC ₅₀ ³ (M)	Highest Conc. for Range Finder (M) ⁴				
1	4-hydroxytamoxifen	68047-06-3	POS	3.97×10^{-9}	$10^{-12} - 10^{-7}$	POS	2.08×10^{-7}	2.58×10^{-4}	moderate POS	POS	Hydrocarbon (Cyclic)	Pharmaceutical
2	Raloxifene HCl	82640-04-8	POS	7.86×10^{-10}	$10^{-12} - 10^{-7}$	POS	1.19×10^{-9}	1.96×10^{-4}	moderate POS	POS	Hydrocarbon (Cyclic)	Pharmaceutical
3	Tamoxifen	10540-29-1	POS	4.91×10^{-7}	$10^{-10} - 10^{-5}$	POS	8.17×10^{-7}	2.69×10^{-4}	POS	POS	Hydrocarbon (Cyclic)	Pharmaceutical
4	17 β -estradiol	50-28-2	NEG	-	$10^{-9} - 10^{-4}$	NEG	-	3.67×10^{-3}	to be negative*	PN	Steroid	Pharmaceutical, Veterinary Agent
5	Apigenin	520-36-5	NEG	-	$10^{-9} - 10^{-4}$	NEG	-	3.70×10^{-4}	NEG	NEG	Heterocyclic Compound	Dye, Natural Product, Pharmaceutical Intermediate
6	Di-n-butyl phthalate	84-74-2	NEG	-	$10^{-8} - 10^{-3}$	NEG	-	3.59×10^{-3}	NEG	NEG	Ester, Phthalic Acid	Cosmetic Ingredient, Industrial Chemical, Plasticiser
7	Flavone	525-82-6	NEG	-	$10^{-8} - 10^{-3}$	NEG	-	4.50×10^{-4}	to be negative*	PN	Flavonoid, Heterocyclic Compound	Natural Product, Pharmaceutical
8	Genistein	446-72-0	NEG	-	$10^{-9} - 10^{-4}$	NEG	-	3.70×10^{-4}	to be negative*	NEG	Flavonoid, Heterocyclic Compound	Natural Product, Pharmaceutical
9	p-n-nonylphenol	104-40-5	NEG	-	$10^{-9} - 10^{-4}$	NEG	-	4.54×10^{-4}	not tested	NEG	Phenol	Chemical Intermediate
10	Resveratrol	501-36-0	NEG	-	$10^{-8} - 10^{-3}$	NEG	-	4.38×10^{-4}	to be negative*	NEG	Hydrocarbon (Cyclic)	Natural Product

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; M = molar; IC₅₀ = half maximal inhibitory concentration of test substance; NEG = negative; PN = presumed negative; POS = positive.

* classified negative according to literature review (2).

^a Common substances tested in the STTA and VM7Luc ER TA assays that were designated as ER antagonists or negatives and used to evaluate accuracy in the VM7Luc ER TA validation study (2) (3).

¹ The Validation Report of the Stably transfected Transcriptional Activation Assay to Detect ER mediated activity, Part B (2)

² ICCVAM Test Method Evaluation Report on the LUMI-CELL ER (VM7Luc ER TA) Test Method: An *In Vitro* Method for Identifying ER Agonists and Antagonists (3).

³ Mean IC₅₀ values were calculated with values reported by the laboratories of the VM7Luc ER TA validation study (XDS, ECVAM, and Hiyoshi) (3).

⁴ Concentrations reported were the highest concentrations tested (range finder) during the validation of the VM7Luc ER TA Assay. If concentrations differed between the laboratories, the highest concentration is reported. See table 4-11 of ICCVAM Test Method Evaluation Report; The LUMI-Cell[®] ER (VM7Luc ER TA) Test Method: An *In Vitro* Assay for Identifying Human Estrogen Receptor Agonist and Antagonist Activity of Chemicals (3).

⁵ Classification as an ER antagonist or negative was based upon information in the ICCVAM Background Review Documents (BRD) for ER Binding and TA test methods (31) as well as information obtained from publications published and reviewed after the completion of the ICCVAM BRDs (2) (3) (18) (31).

⁶ Substances were assigned to one or more chemical classes using the U.S. National Library of Medicine's Medical Subject Headings (MeSH), an internationally recognised standardised classification scheme (available at <http://www.nlm.nih.gov/mesh>).

⁷ Substances were assigned to one or more product classes using the U.S. National Library of Medicine's Hazardous Substances Data Bank (available at <http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB>).

Test Run Acceptability Criteria

16. Acceptance or rejection of a test run is based on the evaluation of results obtained for the reference standards and controls used for each experiment. Values for the PC₅₀ (EC₅₀) or IC₅₀ for the reference standards should meet the acceptability criteria as provided for the selected assay (for STTA see Appendix 2, for VM7Luc ER TA see Appendix 3), and all positive/negative controls should be correctly classified for each accepted experiment. The ability to consistently conduct the assay should be demonstrated by the development and maintenance of a historical database for the reference standards and controls (see paragraph 15). Standard deviations (SD) or coefficients of variation (CV) for the means of reference standards curve fitting parameters from multiple experiments may be used as a measure of within-laboratory reproducibility. In addition, the following principles regarding acceptability criteria should be met:

- Data should be sufficient for a quantitative assessment of ER activation (for agonist assay) or suppression (for antagonist assay) (i.e. efficacy and potency).
- The mean reporter activity for the reference concentration of reference estrogen should be at least the minimum specified in the assays relative to that of the vehicle (solvent) control to ensure adequate sensitivity. For the STTA and VM7Luc ER TA assays, this is four times that of the mean vehicle control on each plate.
- The concentrations tested should remain within the solubility range of the test chemicals and not demonstrate cytotoxicity.

Analysis of data

17. The defined data interpretation procedure for each assay should be used for classifying a positive and negative response.
18. Meeting the acceptability criteria (paragraph 16) indicates the assay is operating properly, but it does not ensure that any particular test run will produce accurate data. Replicating the results of the first run is the best indication that accurate data were produced. If two runs give reproducible results (e.g. both test run results indicate a test chemical is positive), it is not necessary to conduct a third run.
19. If two runs do not give reproducible results (e.g. a test chemical is positive in one run and negative in the other run), or if a higher degree of certainty is required regarding the outcome of this assay, at least three independent runs should be conducted. In this case the classification is based on the two concordant results out of the three.

General Data Interpretation Criteria

20. There is currently no universally agreed method for interpreting ER TA data. However, both qualitative (e.g. positive/negative) and/or quantitative (e.g. EC₅₀, PC₅₀, IC₅₀) assessments of

ER-mediated activity should be based on empirical data and sound scientific judgment. Where possible, positive results should be characterised by both the magnitude of the effect as compared to the vehicle (solvent) control or reference estrogen and the concentration at which the effect occurs (e.g. an EC₅₀, PC₅₀, RPC_{Max}, IC₅₀ , etc.).

Test Report

21. The test report should include the following information:

Assay:

- assay used;
- control/Reference standard/Test chemical;
- source, lot number, limit date for use, if available;
- stability of the test chemical itself, if known;
- solubility and stability of the test chemical in solvent, if known;
- measurement of pH, osmolality and precipitate in the culture medium to which the test chemical was added, as appropriate.

Mono-constituent substance:

- physical appearance, water solubility, and additional relevant physicochemical properties;
- chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc.

Multi-constituent substance, UVCBs and mixtures:

- characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents.

Solvent/Vehicle:

- characterisation (nature, supplier and lot);
- justification for choice of solvent/vehicle;
- solubility and stability of the test chemical in solvent/vehicle, if known.

Cells:

- type and source of cells:
 - Is ER endogenously expressed? If not, which receptor(s) were transfected?
 - Reporter construct(s) used (including source species);

- Transfection method;
- Selection method for maintenance of stable transfection (where applicable);
- Is the transfection method relevant for stable lines?
- number of cell passages (from thawing);
- passage number of cells at thawing;
- methods for maintenance of cell cultures.

Test conditions:

- solubility limitations;
- description of the methods of assessing viability applied;
- composition of media, CO₂ concentration;
- concentrations of test chemical;
- volume of vehicle and test chemical added;
- incubation temperature and humidity;
- duration of treatment;
- cell density at the start of - and during treatment;
- positive and negative reference standards;
- reporter reagents (product name, supplier and lot);
- criteria for considering test runs as positive, negative or equivocal.

Acceptability check:

- fold inductions for each assay plate and whether they meet the minimum required by the assay based on historical controls;
- actual values for acceptability criteria, e.g. log₁₀EC₅₀, log₁₀PC₅₀, logIC₅₀ and Hillslope values, for concurrent positive controls/reference standards.

Results:

- raw and normalised data;
- the maximum fold induction level;
- cytotoxicity data;
- if it exists, the lowest effective concentration (LEC);
- RPC_{Max}, PC_{Max}, PC₅₀, IC₅₀ and/or EC₅₀ values, as appropriate;

- concentration-response relationship, where possible;
- statistical analyses, if any, together with a measure of error and confidence (e.g. SEM, SD, CV or 95% CI) and a description of how these values were obtained.

Discussion of the results

Conclusion

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Appendix 1

DEFINITIONS AND ABBREVIATIONS

Acceptability criteria: Minimum standards for the performance of experimental controls and reference standards. All acceptability criteria should be met for an experiment to be considered valid.

Accuracy (concordance): The closeness of agreement between assay results and an accepted reference values. It is a measure of assay performance and one aspect of relevance. The term is often used interchangeably with “concordance” to mean the proportion of correct outcomes of a assay (1).

Agonist: A substance that produces a response, e.g. transcription, when it binds to a specific receptor.

Antagonist: A type of receptor ligand or chemical that does not provoke a biological response itself upon binding to a receptor, but blocks or dampens agonist-mediated responses.

Anti-estrogenic activity, the capability of a chemical to suppress the action of 17 β -estradiol mediated through estrogen receptors.

Cell morphology: The shape and appearance of cells grown in a monolayer in a single well of a tissue culture plate. Cells that are dying often exhibit abnormal cell morphology.

CF: The OECD Conceptual Framework for the Testing and Evaluation of Endocrine Disrupters.

Charcoal/dextran treatment: Treatment of serum used in cell culture. Treatment with charcoal/dextran (often referred to as “stripping”) removes endogenous hormones and hormone-binding proteins.

Chemical: A substance or a mixture.

Cytotoxicity: Harmful effects to cell structure or function that can ultimately cause cell death and can be reflected by a reduction in the number of cells present in the well at the end of the exposure period or a reduction of the capacity for a measure of cellular function when compared to the concurrent vehicle control.

CV: Coefficient of variation

DCC-FBS: Dextran-coated charcoal treated fetal bovine serum.

DMEM: Dulbecco’s Modification of Eagle’s Medium

DMSO: Dimethyl sulfoxide

E2: 17 β -estradiol

EC₅₀: The half maximal effective concentration of a test chemical.

ED: Endocrine disruption

hER α : Human estrogen receptor alpha

hER β : Human estrogen receptor beta

EFM: Estrogen-free medium. Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 4.5% charcoal/dextran-treated FBS, 1.9% L-glutamine, and 0.9% Pen-Strep.

ER: Estrogen receptor

ERE: Estrogen response element

Estrogenic activity: The capability of a chemical to mimic 17 β -estradiol in its ability to bind to and activate estrogen receptors. hER α -mediated estrogenic activity can be detected with this test method.

ERTA: Estrogen Receptor Trans Activation

FBS: Fetal bovine serum

HeLa: An immortal human cervical cell line

HeLa9903: A HeLa cell subclone into which hER α and a luciferase reporter gene have been stably transfected

IC₅₀: The half maximal effective concentration of an inhibitory test chemical.

ICCVAM: The Interagency Coordinating Committee on the Validation of Alternative Methods.

Inter-laboratory reproducibility: A measure of the extent to which different qualified laboratories, using the same protocol and testing the same substances, can produce qualitatively and quantitatively similar results. Interlaboratory reproducibility is determined during the prevalidation and validation processes, and indicates the extent to which an assay can be successfully transferred between laboratories, also referred to as between-laboratory reproducibility (1).

Intra-laboratory reproducibility: A determination of the extent that qualified people within the same laboratory can successfully replicate results using a specific protocol at different times. Also referred to as "within-laboratory reproducibility" (1).

LEC: Lowest effective concentration is the lowest concentration of test chemical that produces

a response (i.e. the lowest test chemical concentration at which the fold induction is statistically different from the concurrent vehicle control).

Me-too test: A colloquial expression for an assay that is structurally and functionally similar to a validated and accepted reference test method. Interchangeably used with similar test method.

MT: Metallothionein

MMTV: Mouse Mammary Tumor Virus

OHT: 4-Hydroxytamoxifen

PBTG: Performance-Based Test Guideline

PC (Positive control): a strongly active substance, preferably 17 β -estradiol that is included in all tests to help ensure proper functioning of the assay.

PC₁₀: the concentration of a test chemical at which the measured activity in an agonist assay is 10% of the maximum activity induced by the PC (E2 at 1nM for the STTA assay) in each plate.

PC₅₀: the concentration of a test chemical at which the measured activity in an agonist assay is 50% of the maximum activity induced by the PC (E2 at the reference concentration specified in the test method) in each plate.

PC_{Max}: the concentration of a test chemical inducing the RPC_{Max}

Performance standards: Standards, based on a validated assay, that provide a basis for evaluating the comparability of a proposed assay that is mechanistically and functionally similar. Included are (1) essential assay components; (2) a minimum list of reference chemicals selected from among the chemicals used to demonstrate the acceptable performance of the validated test method; and (3) the comparable levels of accuracy and reliability, based on what was obtained for the validated test method, that the proposed assay should demonstrate when evaluated using the minimum list of reference chemicals (1).

Proficiency substances: A subset of the reference substances included in the Performance Standards that can be used by laboratories to demonstrate technical competence with a standardised test method. Selection criteria for these substances typically include that they represent the range of responses, are commercially available, and have high quality reference data available.

Proficiency: The demonstrated ability to properly conduct an assay prior to testing unknown substances.

Reference estrogen (Positive control, PC): 17 β -estradiol (E2, CAS 50-28-2).

Reference standard: a reference substance used to demonstrate the adequacy of a assay. 17 β -estradiol is the reference standard for the STTA and VM7Luc ER TA assays.

Reference test methods: The assays upon which PBTG 455 is based.

Relevance: Description of relationship of an assay to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the assay correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of the accuracy (concordance) of an assay (1).

Reliability: Measure of the extent that an assay can be performed reproducibly within and between laboratories over time, when performed using the same protocol. It is assessed by calculating intra- and inter-laboratory reproducibility.

RLU: Relative Light Units

RNA: Ribonucleic Acid

RPC_{Max}: maximum level of response induced by a test chemical, expressed as a percentage of the response induced by 1 nM E2 on the same plate

RPMI: RPMI 1640 medium supplemented with 0.9% Pen-Strep and 8.0% fetal bovine serum (FBS)

Run: An individual experiment that evaluates chemical action on the biological outcome of the assay. Each run is a complete experiment performed on replicate wells of cells plated from a common pool of cells at the same time.

Independent run: A separate, independent experiment that evaluates chemical action on the biological outcome of the assay, using cells from a different pool, freshly diluted chemicals, conducted on different days or on the same day by different staff.

SD: Standard deviation.

Sensitivity: The proportion of all positive/active substances that are correctly classified by the assay. It is a measure of accuracy for an assay that produces categorical results, and is an important consideration in assessing the relevance of an assay (1).

Specificity: The proportion of all negative/inactive substances that are correctly classified by the test. It is a measure of accuracy for an assay that produces categorical results, and is an important consideration in assessing the relevance of an assay (1).

Stable transfection: When DNA is transfected into cultured cells in such a way that it is stably integrated into the cells genome, resulting in the stable expression of transfected genes. Clones of stably transfected cells are selected by stable markers (e.g. resistance to G418).

STTA Assay: Stably Transfected Transactivation Assay, the ER α transcriptional activation assay using the HeLa 9903 Cell Line.

Study: The full range of experimental work performed to evaluate a single, specific substance using a specific assay. A study comprises all steps including tests of dilution of test substance in

the test media, preliminary range finding runs, all necessary comprehensive runs, data analyses, quality assurance, cytotoxicity assessments, etc. Completion of a study allows the classification of the test chemical activity on the toxicity target (i.e. active, inactive or inconclusive) that is evaluated by the assay used and an estimate of potency relative to the positive reference chemical.

Substance: Under REACH¹², a substance is defined as a chemical element and its compounds in the natural state or obtained by any manufacturing process, including any additive necessary to preserve its stability and any impurities deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the substance or changing its composition. A very similar definition is used in the context of the UN GHS (1).

TA (Transactivation): The initiation of mRNA synthesis in response to a specific chemical signal, such as a binding of an estrogen to the estrogen receptor

Assay: Within the context of this test method, an assay is one of the methodologies accepted as valid in meeting the outlined performance criteria. Components of assay include, for example, the specific cell line with associated growth conditions, specific media in which the test is conducted, plate set up conditions, arrangement and dilutions of test chemicals along with any other required quality control measures and associated data evaluation steps.

Test chemical: Any substance or mixture tested using this test method.

Transcription: mRNA synthesis

UVCB: Chemical Substances of Unknown or Variable Composition, Complex Reaction Products and Biological Materials

Validated test method: An assay for which validation studies have been completed to determine the relevance (including accuracy) and reliability for a specific purpose. It is important to note that a validated test method may not have sufficient performance in terms of accuracy and reliability to be found acceptable for the proposed purpose (1).

Validation: The process by which the reliability and relevance of a particular approach, method, assay, process or assessment is established for a defined purpose (1).

¹² Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC (OJ L 304, 22.11.2007, p. 1).

VC (Vehicle control): The solvent that is used to dissolve test and control chemicals is tested solely as vehicle without dissolved chemical.

VM7: An immortalised adenocarcinoma cell that endogenously express estrogen receptor.

VM7Luc4E2: The VM7Luc4E2 cell line was derived from VM7 immortalised human-derived adenocarcinoma cells that endogenously express both forms of the estrogen receptor (ER α and ER β) and have been stably transfected with the plasmid pGudLuc7.ERE. This plasmid contains four copies of a synthetic oligonucleotide containing the estrogen response element upstream of the mouse mammary tumor viral (MMTV) promoter and the firefly luciferase gene.

Weak positive control: A weakly active substance selected from the reference chemicals list that is included in all tests to help ensure proper functioning of the assay.

Appendix 2

STABLY TRANSFECTED HUMAN ESTROGEN RECEPTOR- α TRANSACTIVATION ASSAY FOR DETECTION OF ESTROGENIC AGONIST AND ANTAGONIST ACTIVITY OF CHEMICALS USING THE HERA-HELA-9903 CELL LINE

INITIAL CONSIDERATIONS AND LIMITATIONS (SEE ALSO GENERAL INTRODUCTION)

1. This transactivation (TA) assay uses the hER α -HeLa-9903 cell line to detect estrogenic agonist activity mediated through human estrogen receptor alpha (hER α). The validation study of the Stably Transfected Transactivation (STTA) Assay by the Japanese Chemicals Evaluation and Research Institute (CERI) using the hER α -HeLa-9903 cell line to detect estrogenic agonist and antagonist activity mediated through human estrogen receptor alpha (hER α) demonstrated the relevance and reliability of the assay for its intended purpose (1).
2. This assay is specifically designed to detect hER α -mediated TA by measuring chemiluminescence as the endpoint. However, non-receptor-mediated luminescence signals have been reported at phytoestrogen concentrations higher than 1 μ M due to the over-activation of the luciferase reporter gene (2) (3). While the dose-response curve indicates that true activation of the ER system occurs at lower concentrations, luciferase expression obtained at high concentrations of phytoestrogens or similar compounds suspected of producing phytoestrogen-like over-activation of the luciferase reporter gene needs to be examined carefully in stably transfected ER TA assay systems (Appendix 1).
3. The sections “GENERAL INTRODUCTION” and “ER TA ASSAY COMPONENTS” should be read before using this assay for regulatory purposes. Definitions and abbreviations used in this TG are described in Appendix 2.1.

PRINCIPLE OF THE ASSAY (SEE ALSO GENERAL INTRODUCTION)

4. The assay is used to signal binding of the estrogen receptor with a ligand. Following ligand binding, the receptor-ligand complex translocates to the nucleus where it binds specific DNA response elements and transactivates a firefly luciferase reporter gene, resulting in increased cellular expression of luciferase enzyme. Luciferin is a substrate that is transformed by the luciferase enzyme to a bioluminescence product that can be quantitatively measured with a luminometer. Luciferase activity can be evaluated quickly and inexpensively with a number of commercially available test kits.
5. The test system utilises the hER α -HeLa-9903 cell line, which is derived from a human cervical tumor, with two stably inserted constructs: (i) the hER α expression construct

(encoding the full-length human receptor), and (ii) a firefly luciferase reporter construct bearing five tandem repeats of a vitellogenin Estrogen-Responsive Element (ERE) driven by a mouse metallothionein (MT) promoter TATA element. The mouse MT TATA gene construct has been shown to have the best performance, and so is commonly used. Consequently this hER α -HeLa-9903 cell line can measure the ability of a test chemical to induce hER α -mediated transactivation of luciferase gene expression.

6. In case of ER agonist assay, data interpretation is based upon whether or not the maximum response level induced by a test chemical equals or exceeds an agonist response equal to 10% of that induced by a maximally inducing (1 nM) concentration of the positive control (PC) 17 β -estradiol (E2) (i.e. the PC₁₀). In case of ER antagonist assay, data interpretation is based upon whether or not the response shows at least a 30% reduction in activity from the response induced by the spike in control (25 pM of E2) without cytotoxicity. Data analysis and interpretation are discussed in detail in paragraphs 34 - 48.

PROCEDURE

Cell Lines

7. The stably transfected hER α -HeLa-9903 cell line should be used for the assay. The cell line can be obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank¹³, upon signing a Material Transfer Agreement (MTA).
8. Only cells characterised as mycoplasma-free should be used in testing. RT-PCR (Real Time Polymerase Chain Reaction) is the method of choice for a sensitive detection of mycoplasma infection (4) (5) (6).

Stability of the cell line

9. To monitor the stability of the cell line, E2, 17 α -estradiol, 17 α -methyltestosterone and corticosterone should be used as the reference standards for agonist assay and a complete concentration-response curve in the test concentration range provided in Table 1 should be measured at least once each time the assay is performed, and the results should be in agreement with the results provided in Table 1.

¹³ JCRB Cell Bank : National Institute of Biomedical Innovation, 7-6-8 Asagi Saito, Ibaraki-shi, Osaka 567-0085, Japan Fax: +81-72-641-9812

10. In case of antagonist assay, complete concentration curves for two reference standards, tamoxifen and flutamide, should be measured simultaneously with each run. Correct qualitative classification as positive or negative for the two chemicals should be monitored.

Cell Culture and Plating Conditions

11. Cells should be maintained in Eagle's Minimum Essential Medium (EMEM) without phenol red, supplemented with 60 mg/l of antibiotic kanamycine and 10% dextran-coated-charcoal-treated fetal bovine serum (DCC-FBS), in a CO₂ incubator (5% CO₂) at 37±1°C. Upon reaching 75 -90% confluency, cells can be subcultured at 10 ml of 0.4 x 10⁵ – 1 x 10⁵ cells/ml for 100 mm cell culture dish. Cells should be suspended with 10% FBS-EMEM (which is the same as EMEM with DCC-FBS) and then plated into wells of a microplate at a density of 1 x 10⁴ cells/(100 µl x well). Next, the cells should be pre-incubated in a 5% CO₂ incubator at 37°±1°C for 3 hours before the chemical exposure. The plastic-ware should be free of estrogenic activity.
12. To maintain the integrity of the response, the cells should be grown for more than one passage from the frozen stock in the conditioned media and should not be cultured for more than 40 passages. For the hERα-HeLa-9903 cell line, this will be less than three months. However the performance of cells may be reduced if they are grown in inappropriate culture conditions.
13. The DCC-FBS can be prepared as described in Appendix 2.2, or obtained from commercial sources.

Acceptability criteria

Positive and negative reference standards for ER agonist assay

14. Prior to and during the study, the responsiveness of the test system should be verified using the appropriate concentrations of a strong estrogen: E2, a weak estrogen (17α-estradiol), a very weak agonist (17α-methyltestosterone), and a negative substance (corticosterone). Acceptable range values derived from the validation study (1) are given in Table 1. These 4 concurrent reference standards should be included with each experiment and the results should fall within the given acceptable limits. If this is not the case, the cause for the failure to meet the acceptability criteria should be determined (e.g. cell handling, and serum and antibiotics for quality and concentration) and the assay repeated. Once the acceptability criteria have been achieved, to ensure minimum variability of EC₅₀, PC₅₀ and PC₁₀ values, consistent use of materials for cell culturing is essential. The four concurrent reference standards, which should be included in each experiment (conducted under the same conditions including the materials, passage level of cells and technicians), can ensure the sensitivity of the assay because the PC₁₀s of the three positive reference standards should fall within the acceptable range, as should the PC₅₀s and EC₅₀s where they can be calculated (see Table 1).

Table 1: Acceptable range values of the four reference standards for the ER agonist assay

Name	logPC ₅₀	logPC ₁₀	logEC ₅₀	Hill slope	Test range
17β-estradiol (E2) CAS No: 50-28-2	-11.4~-10.1	<-11	-11.3~-10.1	0.7~1.5	10 ⁻¹⁴ ~10 ⁻⁸ M
17α-estradiol CAS No: 57-91-0	-9.6~-8.1	-10.7~-9.3	-9.6~-8.4	0.9~2.0	10 ⁻¹² ~10 ⁻⁶ M
Corticosterone CAS No: 50-22-6	—	—	—	—	10 ⁻¹⁰ ~10 ⁻⁴ M
17α-methyltestosterone CAS No: 58-18-4	-6.0~-5.1	-8.0~-6.2	—	—	10 ⁻¹¹ ~10 ⁻⁵ M

Positive and negative reference standards for ER antagonist assay

15. Prior to and during the study, the responsiveness of the test system should be verified using the appropriate concentrations of a positive substance (Tamoxifen), and a negative substance (Flutamide). Acceptable range values derived from the validation study (1) are given in Table 2. These two concurrent reference standards should be included with each experiment and the results should be judged correct as shown in the criteria. If this is not the case, the cause for the failure to meet the criteria should be determined (e.g. cell handling, and serum and antibiotics for quality and concentration) and the assay repeated. In addition, IC₅₀ values for a positive substance (Tamoxifen) should be calculated and the results should fall within the given acceptable limits. Once the acceptability criteria have been achieved, to ensure minimum variability of IC₅₀ values, consistent use of materials for cell culturing is essential. The two concurrent reference standards, which should be included in each experiment (conducted under the same conditions including the materials, passage level of cells and technicians), can ensure the sensitivity of the assay (see Table 2).

Table 2: Criteria and acceptable range values of the two reference standards for the ER antagonist assay

Name	Criteria	LogIC ₅₀	Test range
Tamoxifen CAS No: 10540-29-1	Positive: IC ₅₀ should be calculated	-5.942~-7.596	10 ⁻¹⁰ ~10 ⁻⁵ M
Flutamide CAS No: 13311-84-7	Negative: IC ₃₀ should not be calculated	-	10 ⁻¹⁰ ~10 ⁻⁵ M

Positive and Vehicle Controls

16. The positive control (PC) for ER agonist assay (1 nM of E2) and for ER antagonist assay (10μM TAM) should be tested at least in triplicate in each plate. The vehicle that is used to dissolve a test chemical should be tested as a vehicle control (VC) at least in triplicate in each plate. In addition to this VC, if the PC uses a different vehicle than the test chemical, another VC should be tested at least in triplicate on the same plate with the PC.

Quality criteria for ER agonist assay

17. The mean luciferase activity of the positive control (1 nM E2) should be at least 4-fold that of the mean VC on each plate. This criterion is established based on the reliability of the endpoint values from the validation study (historically between four- and 30-fold).
18. With respect to the quality control of the assay, the fold-induction corresponding to the PC₁₀ value of the concurrent PC (1 nM E2) should be greater than 1+2SD of the fold-induction value (=1) of the concurrent VC. For prioritisation purposes, the PC₁₀ value can be useful to simplify the data analysis required compared to a statistical analysis. Although a statistical analysis provides information on significance, such an analysis is not a quantitative parameter with respect to concentration-based potential, and so is less useful for prioritisation purposes.

Quality criteria for ER antagonist assay

19. The mean luciferase activity of the spike in control (25 pM E2) should be at least 4-fold that of the mean VC on each plate. This criterion is established based on the reliability of the endpoint values from the validation study.
20. With respect to the quality control of the assay, relative transcriptional activation (RTA) of 1 nM E2 should be greater than 100%, RTA of 1 µM 4-Hydroxytamoxifen (OHT) should be less than 40.6% and RTA of 100 µM Digitonin (Dig) should be less than 0%.

Demonstration of Laboratory Proficiency (see paragraph 14 and Tables 3 and 4 in « ER TA ASSAY COMPONENTS» of this test method).

Vehicle

21. Dimethyl sulfoxide (DMSO), or appropriate solvent, at the same concentration used for the different positive and negative controls and the test chemicals should be used as the concurrent VC. Test chemicals should be dissolved in a solvent that solubilises that test chemical and is miscible with the cell medium. Water, ethanol (95% to 100% purity) and DMSO are suitable vehicles. If DMSO is used, the level should not exceed 0.1% (v/v). For any vehicle, it should be demonstrated that the maximum volume used is not cytotoxic and does not interfere with assay performance.

Preparation of Test Chemicals

22. Generally, the test chemicals should be dissolved in DMSO or other suitable solvent, and serially diluted with the same solvent at a common ratio of 1:10 in order to prepare solutions for dilution with media.

Solubility and Cytotoxicity: Considerations for Range Finding.

23. A preliminary test should be carried out to determine the appropriate concentration range of chemical to be tested, and to ascertain whether the test chemical may have any solubility and cytotoxicity problems. Initially, chemicals are tested up to the maximum concentration of

1 µl/ml, 1 mg/ml, or 1 mM, whichever is the lowest. Based on the extent of cytotoxicity or lack of solubility observed in the preliminary test, the first definite run should test the chemical at log-serial dilutions starting at the maximum acceptable concentration (e.g. 1 mM, 100µM, 10µM, etc.) and the presence of cloudiness or precipitate or cytotoxicity noted. Concentrations in the second, and if necessary third run should be adjusted as appropriate to better characterise the concentration-response curve and to avoid concentrations which are found to be insoluble or to induce excessive cytotoxicity.

24. For ER agonists and antagonists, the presence of increasing levels of cytotoxicity can significantly alter or eliminate the typical sigmoidal response and should be considered when interpreting the data. Cytotoxicity testing methods that can provide information regarding 80% cell viability should be used, utilising an appropriate assay based upon laboratory experience.
25. Should the results of the cytotoxicity test show that the concentration of the test chemical has reduced the cell number by 20% or more, this concentration should be regarded as cytotoxic, and the concentrations at or above the cytotoxic concentration should be excluded from the evaluation.

Chemical Exposure and Assay Plate Organisation

26. The procedure for chemical dilutions (Steps-1 and 2) and exposure to cells (Step-3) can be conducted as follows:

Step-1: Each test chemical should be serially diluted in DMSO, or appropriate solvent, and added to the wells of a microtitre plate to achieve final serial concentrations as determined by the preliminary range finding test (typically in a series of, for example 1 mM, 100 µM, 10 µM, 1µM, 100 nM, 10 nM, 1 nM, 100 pM, and 10 pM (10^{-3} - 10^{-11} M)) for triplicate testing.

Step-2: Chemical dilution: First dilute 1.5 µl of the test chemical in the solvent to a volume of 500 µl of media.

Step-3: Chemical exposure of the cells: Add 50 µl of dilution with media (prepared in Step-2) to an assay well containing 10^4 cells/100 µl/well.

The recommended final volume of media required for each well is 150 µl. Test samples and reference standards can be assigned as shown in Table 3 and Table 4.

Table 3: Example of plate concentration assignment of the reference standards in the assay plate in ER agonist assay

Row	17 α -methyltestosterone			Corticosterone			17 α -estradiol			E2		
	1	2	3	4	5	6	7	8	9	10	11	12
A	conc 1 (10 µM)	→	→	100 µM	→	→	1 µM	→	→	10 nM	→	→
B	conc 2 (1 µM)	→	→	10 µM	→	→	100 nM	→	→	1 nM	→	→

C	conc 3 (100 nM)	→	→	1 μM	→	→	10 nM	→	→	100 pM	→	→
D	conc 4 (10 nM)	→	→	100 nM	→	→	1 nM	→	→	10 pM	→	→
E	conc 5 (1 nM)	→	→	10 nM	→	→	100 pM	→	→	1 pM	→	→
F	conc 6 (100 pM)	→	→	1 nM	→	→	10 pM	→	→	0.1 pM	→	→
G	conc 7 (10 pM)	→	→	100 pM	→	→	1 pM	→	→	0.01 pM	→	→
H	VC	→	→	→	→	→	PC	→	→	→	→	→

VC: Vehicle control (0.1% DMSO); PC: Positive control (1 nM E2)

27. The reference standards (E2, 17 α -estradiol, 17 α -methyl testosterone and corticosterone) should be tested in every run (Table 3). PC wells treated with 1 nM of E2 that can produce maximum induction of E2 and VC wells treated with DMSO (or appropriate solvent) alone should be included in each test assay plate (Table 4). If cells from different sources (e.g. different passage number, different lot, etc.) are used in the same experiment, the reference standards should be tested for each cell source.

Table 4: Example of plate concentration assignment of test and plate control chemicals in the assay plate in ER agonist assay

Row	Test Chemical 1			Test Chemical 2			Test Chemical 3			Test Chemical 4		
	1	2	3	4	5	6	7	8	9	10	11	12
A	conc 1 (10 μM)	→	→	1 mM	→	→	1 μM	→	→	10 nM	→	→
B	conc 2 (1 μM)	→	→	100 μM	→	→	100 nM	→	→	1 nM	→	→
C	conc 3 (100 nM)	→	→	10 μM	→	→	10 nM	→	→	100 pM	→	→
D	conc 4 (10 nM)	→	→	1 μM	→	→	1 nM	→	→	10 pM	→	→
E	conc 5 (1 nM)	→	→	100 nM	→	→	100 pM	→	→	1 pM	→	→
F	conc 6 (100 pM)	→	→	10 nM	→	→	10 pM	→	→	0.1 pM	→	→
G	conc 7 (10 pM)	→	→	1 nM	→	→	1 pM	→	→	0.01 pM	→	→
H	VC	→	→	→	→	→	PC	→	→	→	→	→

VC: Vehicle control (0.1% DMSO); PC: Positive control (1 nM E2)

Table 5: Example of plate concentration assignment of the reference standards in the assay plate in ER antagonist assay

Row	Tamoxifen			Flutamide			Test Chemical 1			Test Chemical 2		
	1	2	3	4	5	6	7	8	9	10	11	12
A	conc 1 (10 μM)	→	→	10 μM	→	→	10 μM	→	→	10 μM	→	→
B	conc 2 (1 μM)	→	→	1 μM	→	→	1 μM	→	→	1 μM	→	→
C	conc 3 (100 nM)	→	→	100 nM	→	→	100 nM	→	→	100 nM	→	→
D	conc 4 (10 nM)	→	→	10 nM	→	→	10 nM	→	→	10 nM	→	→
E	conc 5 (1 nM)	→	→	1 nM	→	→	1 nM	→	→	1 nM	→	→
F	conc 6 (100 pM)	→	→	100 pM	→	→	100 pM	→	→	100 pM	→	→
G	0.1% DMSO	→	→	→	→	→	1 μM OHT	→	→	100 μM Dig	→	→
H	VC	→	→	→	→	→	PC	→	→	→	→	→

VC: Vehicle control (0.1% DMSO), PC: Positive control (1 nM E2), OHT :4-Hydroxytamoxifen, Dig: Digitonin.

 = spiked with 25pM E2

28. To evaluate the antagonist activity of chemicals, assay wells located in rows from A to G should be spiked with 25pM E2. The reference standards (Tamoxifen and Flutamide) should be tested in every run. PC wells treated with 1 nM of E2 that can be used as quality control of hER α -HeLa-9903 cell line, VC wells treated with DMSO (or appropriate solvent), 0.1% DMSO wells treated with DMSO addition to the spiked E2 corresponding to “Spike-in-control”, wells treated with final concentration 1 μ M OHT and wells treated with 100 μ M Dig should be included in each test assay plate (Table 5). Subsequent assay plate should follow the same plate layout without reference standards wells (Table 6). If cells from different sources (e.g. different passage number, different lot, etc.) are used in the same experiment, the reference standards should be tested for each cell source.

Table 6: Example of plate concentration assignment of test and plate control chemicals in the assay plate in ER antagonist assay

Row	Test Chemical 1			Test Chemical 2			Test Chemical 3			Test Chemical 4		
	1	2	3	4	5	6	7	8	9	10	11	12
A	conc 1 (10 μ M)	→	→	10 μ M	→	→	10 μ M	→	→	10 μ M	→	→
B	conc 2 (1 μ M)	→	→	1 μ M	→	→	1 μ M	→	→	1 μ M	→	→
C	conc 3 (100 nM)	→	→	100 nM	→	→	100 nM	→	→	100 nM	→	→
D	conc 4 (10 nM)	→	→	10 nM	→	→	10 nM	→	→	10 nM	→	→
E	conc 5 (1 nM)	→	→	1 nM	→	→	1 nM	→	→	1 nM	→	→
F	conc 6 (100 pM)	→	→	100 pM	→	→	100 pM	→	→	100 pM	→	→
G	0.1% DMSO	→	→	→	→	→	1 μ M OHT	→	→	100 μ M Dig	→	→
H	VC	→	→	→	→	→	PC	→	→	→	→	→

VC: Vehicle control (0.1% DMSO), PC: Positive control (1 nM E2), OHT: 4-Hydroxytamoxifen, Dig: Digitonin.

 : Spiked with 25pM E2

29. The lack of edge effects should be confirmed, as appropriate, and if edge effects are suspected, the plate layout should be altered to avoid such effects. For example, a plate layout excluding the edge wells can be employed.

30. After adding the chemicals, the assay plates should be incubated in a 5% CO₂ incubator at 37±1°C for 20-24 hours to induce the reporter gene products.

31. Special considerations will need to be applied to those compounds that are highly volatile. In such cases, nearby control wells may generate false positives and this should be considered in light of expected and historical control values. In the few cases where volatility may be of concern, the use of “plate sealers” may help to effectively isolate individual wells during testing, and is therefore recommended in such cases.

32. Repeat definitive tests for the same chemical should be conducted on different days, to ensure independence.

Luciferase assay

33. A commercial luciferase assay reagent [e.g. Steady-Glo® Luciferase Assay System (Promega, E2510, or equivalent)] or a standard luciferase assay system (e.g. Promega, E1500, or equivalent) can be used for the assay, as long as the acceptability criteria are met. The assay reagents should be selected based on the sensitivity of the luminometer to be used. When using the standard luciferase assay system, Cell Culture Lysis Reagent (e.g. Promega, E1531, or equivalent) should be used before adding the substrate. The luciferase reagent should be applied following the manufacturers' instructions.

ANALYSIS OF DATA

ER agonist assay

34. In case of ER agonist assay, to obtain the relative transcriptional activity to PC (1 nM of E2), the luminescence signals from the same plate can be analysed according to the following steps (other equivalent mathematical processes are also acceptable):

Step 1. Calculate the mean value for the VC.

Step 2. Subtract the mean value of the VC from each well value to normalise the data.

Step 3. Calculate the mean for the normalised PC.

Step 4. Divide the normalised value of each well in the plate by the mean value of the normalised PC (PC=100%).

The final value of each well is the relative transcriptional activity for that well compared to the PC response.

Step 5. Calculate the mean value of the relative transcriptional activity for each concentration group of the test chemical. There are two dimensions to the response: the averaged transcriptional activity (response) and the concentration at which the response occurs (see following section).

EC₅₀, PC₅₀ and PC₁₀ induction considerations

35. The full concentration-response curve is required for the calculation of the EC₅₀, but this may not always be achievable or practical due to limitations of the test concentration range (for example due to cytotoxicity or solubility problems). However, as the EC₅₀ and maximum induction level (corresponding to the top value of the Hill-equation) are informative parameters, these parameters should be reported where possible. For the calculation of EC₅₀ and maximum induction level, appropriate statistical software should be used (e.g. Graphpad Prism statistical software). If the Hill's logistic equation is applicable to the concentration response data, the EC₅₀ should be calculated by the following equation (7):

$Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{\exp((\log \text{EC}_{50} - X) \times \text{Hill slope}))}$ Where:

X is the logarithm of concentration; and,

Y is the response and Y starts at the Bottom and goes to the Top in a sigmoid curve. Bottom is fixed at zero in the Hill's logistic equation.

36. For each test chemical, the following should be provided:

The RPC_{Max} which is the maximum level of response induced by a test chemical, expressed as a percentage of the response induced by 1 nM E2 on the same plate, as well as the PC_{Max} (concentration associated with the RPC_{Max}); and

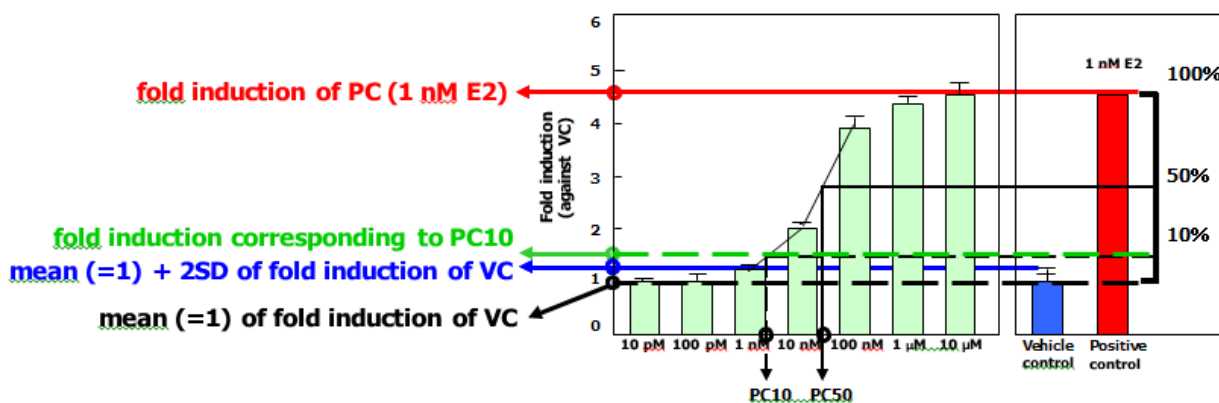
For positive chemicals, the concentrations that induce the PC_{10} and, if appropriate, the PC_{50} .

37. The PC_x value can be calculated by interpolating between 2 points on the X-Y coordinate, one immediately above and one immediately below a PC_x value. Where the data points lying immediately above and below the PC_x value have the coordinates (a,b) and (c,d) respectively, then the PC_x value may be calculated using the following equation:

$$\log[\text{PC}_x] = \log[c] + (x - d) / (d - b)$$

38. Descriptions of PC values are provided in Figure 1 below.

Figure 1: Example of how to derive PC-values. The PC (1 nM of E2) is included on each assay plate



ER antagonist assay

39. In case of ER antagonist assay, to obtain the relative transcriptional activity (RTA) to spike in control (25 pM of E2), the luminescence signals from the same plate can be analysed according to the following steps (other equivalent mathematical processes are also acceptable):

Step 1. Calculate the mean value for the VC.

Step 2. Subtract the mean value of the VC from each well value to normalise the data. Step 3. Calculate the mean for the normalised spike in control.

Step 4. Divide the normalised value of each well in the plate by the mean value of the normalised spike in control (spike in control=100%).

The final value of each well is the relative transcriptional activity for that well compared to the spike in control response.

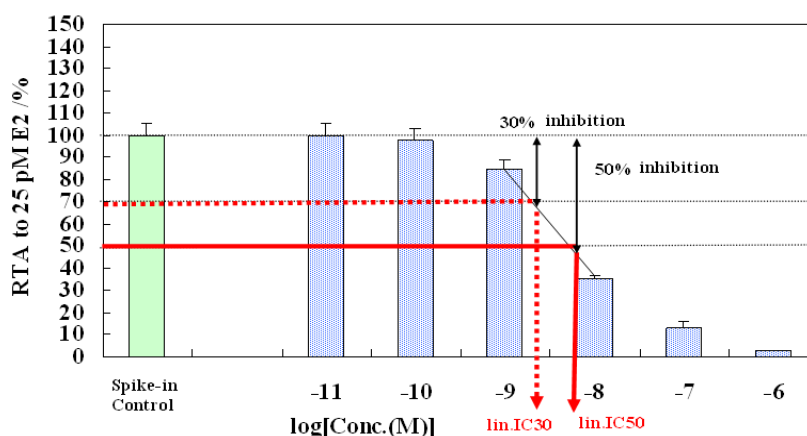
Step 5. Calculate the mean value of the relative transcriptional activity for each treatment.

IC₃₀ and IC₅₀ induction considerations

40. For positive chemicals, the concentrations that induce the IC₃₀ and, if appropriate, the IC₅₀ should be provided.
41. The IC_x value can be calculated by interpolating between 2 points on the X-Y coordinate, one immediately above and one immediately below a IC_x value. Where the data points lying immediately above and below the IC_x value have the coordinates (c,d) and (a,b) respectively, then the IC_x value may be calculated using the following equation:

$$\ln IC_x = a - (b - (100 - x)) (a - c) / (b - d)$$

Figure 2: Example of how to derive IC-values. The spike in control (25 pM of E2) is included on each assay plate



RTA: relative transcriptional activity

42. The results should be based on two (or three) independent runs. If two runs give comparable and therefore reproducible results, it is not necessary to conduct a third run. To be acceptable, the results should:

- Meet the acceptability criteria (see Acceptability criteria para 14-20),
- Be reproducible.

Data Interpretation Criteria

Table 7: Positive and negative decision criteria in ER agonist assay

Positive	If the RPC_{Max} is obtained that is equal to or exceeds 10% of the response of the positive control in at least two of two or two of three runs.
Negative	If the RPC_{Max} fails to achieve at least 10% of the response of the positive control in two of two or two of three runs.

Table 8: Positive and negative decision criteria in ER antagonist assay

Positive	If the IC_{30} is calculated in at least two of two or two of three runs.
Negative	If the IC_{30} fails to calculate in two of two or two of three runs.

43. Data interpretation criteria are shown in Tables 7 and 8. Positive results will be characterised by both the magnitude of the effect and the concentration at which the effect occurs. Expressing results as a concentration at which a 50% (PC_{50}) or 10% (PC_{10}) of PC values are reached for the agonist assay, and 50% (IC_{50}) or 30% (IC_{30}) of the spike-in control value is inhibited for the antagonist assay, accomplishes both of these goals. However, a test chemical is determined to be positive, if the maximum response induced by the test chemical

(RPCMax) is equal to or exceeds 10% of the response of the PC in at least two of two or two of three runs, while a test chemical is considered negative if the RPCMax fails to achieve at least 10% of the response of the positive control in two of two or two of three runs.

44. The calculations of PC₁₀, PC₅₀ and PC_{Max} in ER agonist assay and IC₃₀ and IC₅₀ in ER antagonist assay can be made by using a spreadsheet available with the Test Guideline on the OECD public website¹.
45. It should be sufficient to obtain PC₁₀ or PC₅₀ and IC₃₀ or IC₅₀ values at least twice. However, should the resulting base-line for data in the same concentration range show variability with an unacceptably high coefficient of variation (CV; %) the data may not be considered reliable and the source of the high variability should be identified. The CV of the raw data triplicates (i.e. luminescence intensity data) of the data points that are used for the calculation of PC₁₀ should be less than 20%.
46. Meeting the acceptability criteria indicates the assay system is operating properly, but it does not ensure that any particular run will produce accurate data. Duplicating the results of the first run is the best insurance that accurate data were produced.
47. In case of ER agonist assay, where more information is required in addition to the screening and prioritisation purposes of this TG for positive test chemicals, particularly for PC10-PC49 chemicals, as well as chemicals suspected to over-stimulate luciferase, it can be confirmed that the observed luciferase-activity is solely an ER α -specific response, using an ER α antagonist (see Appendix 2.1).

TEST REPORT

48. See paragraph 20 of “ER TA ASSAY COMPONENTS”.

¹ <http://www.oecd.org/env/testguidelines>

LITERATURE

- (1) OECD (2015). Report of the Inter-Laboratory Validation for Stably Transfected Transactivation Assay to detect Estrogenic and Anti-estrogenic Activity. Environment, Health and Safety Publications, Series on Testing and Assessment (No 225), Organisation for Economic Cooperation and Development, Paris.
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Appendix 2.1

FALSE POSITIVES: ASSESSMENT OF NON-RECEPTOR MEDIATED LUMINESCENCE SIGNALS

1. False positives in the ER agonist assay might be generated by non-ER-mediated activation of the luciferase gene, or direct activation of the gene product or unrelated fluorescence. Such effects are indicated by an incomplete or unusual dose-response curve. If such effects are suspected, the effect of an ER antagonist (e.g. 4- hydroxytamoxifen (OHT) at non-toxic concentration) on the response should be examined. The pure antagonist ICI 182780 may not be suitable for this purpose as a sufficient concentration of ICI 182780 may decrease the VC value, and this will affect the data analysis.
2. To ensure validity of this approach, the following needs to be tested in the same plate:
 - Agonistic activity of the unknown chemical with / without 10 μ M of OHT
 - VC (in triplicate)
 - OHT (in triplicate)
 - 1 nM of E2 (in triplicate) as agonist PC
 - 1 nM of E2 + OHT (in triplicate)

Data interpretation criteria

Note: All wells should be treated with the same concentration of the vehicle.

- If the agonistic activity of the unknown chemical is NOT affected by the treatment with ER antagonist, it is classified as “Negative”.
- If the agonistic activity of the unknown chemical is completely inhibited, apply the decision criteria.
- If the agonistic activity at the lowest concentration is equal to, or is exceeding, PC₁₀ response the unknown chemical is inhibited equal to or exceeding PC₁₀ response. The difference in the responses between the non-treated and treated wells with the ER antagonist is calculated and this difference should be considered as the true response and should be used for the calculation of the appropriate parameters to enable a classification decision to be made.

Data analysis

Check the performance standard.

Check the CV between wells treated under the same conditions.

1. Calculate the mean of the VC
2. Subtract the mean of VC from each well value **not** treated with OHT
3. Calculate the mean of OHT
4. Subtract the mean of the VC from each well value treated with OHT
5. Calculate the mean of the PC
6. Calculate the relative transcriptional activity of all other wells relative to the PC.

Appendix 2.2

PREPARATION OF SERUM TREATED WITH DEXTRAN COATED CHARCOAL (DCC)

1. The treatment of serum with dextran-coated charcoal (DCC) is a general method for removal of estrogenic compounds from serum that is added to cell medium, in order to exclude the biased response associated with residual estrogens in serum. 500 ml of fetal bovine serum (FBS) can be treated by this procedure.

Components

2. The following materials and equipment will be required:

Materials

Activated charcoal

Dextran

Magnesium chloride hexahydrate ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$)

Sucrose

1 M HEPES buffer solution (pH 7.4)

Ultrapure water produced from a filter system

Equipment

Autoclaved glass container (size should be adjusted as appropriate) General Laboratory Centrifuge (that can set temperature at 4°C)

Procedure

3. The following procedure is adjusted for the use of 50 ml centrifuge tubes:

[Day-1] Prepare dextran-coated charcoal suspension with 1 l of ultrapure water containing 1.5 mM of MgCl_2 , 0.25 M sucrose, 2.5 g of charcoal, 0.25 g dextran and 5 mM of HEPES and stir it at 4°C, overnight.

[Day-2] Dispense the suspension in 50 ml centrifuge tubes and centrifuge at 10000 rpm at 4°C for 10 minutes. Remove the supernatant and store half of the charcoal sediment at 4°C for the use on Day-3. Suspend the other half of the charcoal with FBS that has been gently thawed to avoid precipitation, and heat-inactivated at 56°C for 30 minutes, then transfer into an autoclaved glass container such as an Erlenmeyer flask. Stir this suspension gently at 4°C, overnight.

[Day-3] Dispense the suspension with FBS into centrifuge tubes for centrifugation at

10000 rpm at 4°C for 10 minutes. Collect FBS and transfer into the new charcoal sediment prepared and stored on Day-2. Suspend the charcoal sediment and stir this suspension gently in an autoclaved glass container at 4°C, overnight.

[Day-4] Dispense the suspension for centrifugation at 10000 rpm at 4°C for 10 minutes and sterilise the supernatant by filtration through 0.2 µm sterile filter. This DCC treated FBS should be stored at -20°C and can be used for up a year.

APPENDIX 3

VM7LUC ESTROGEN RECEPTOR TRANSACTIVATION ASSAY FOR IDENTIFYING ESTROGEN RECEPTOR AGONISTS AND ANTAGONISTS

INITIAL CONSIDERATIONS AND LIMITATIONS (SEE ALSO GENERAL INTRODUCTION)

1. This assay uses the VM7Luc4E2 cell line¹. It has been validated by the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) (1). The VM7Luc cell lines predominantly express endogenous ER α and a minor amount of endogenous ER β (2) (3) (4).
2. This assay is applicable to a wide range of substances, provided they can be dissolved in dimethyl sulfoxide (DMSO; CASRN 67-68-5), do not react with DMSO or the cell culture medium, and are not cytotoxic at the concentrations being tested. If use of DMSO is not possible, another vehicle such as ethanol or water may be used (see paragraph 12). The demonstrated performance of the VM7Luc ER TA (ant)agonist assay suggests that data generated with this assay may inform upon ER mediated mechanisms of action and could be considered for prioritisation of substances for further testing.
3. This assay is specifically designed to detect hER α and hER β -mediated TA by measuring chemiluminescence as the endpoint. Chemiluminescence use in bioassays is widespread because luminescence has a high signal-to-background ratio (10). However, the activity of firefly luciferase in cell-based assays can be confounded by substances that inhibit the luciferase enzyme, causing both apparent inhibition or increased luminescence due to protein stabilisation (10). In addition, in some luciferase-based ER reporter gene assays, non-receptor-mediated luminescence signals have been reported at phytoestrogen concentrations

¹ Before June 2016, this cell line was designated as BG1Luc cell line. BG-1 cells were originally described by Geisinger et al. (1998) (12) and were later characterized by researchers at the National Institute of Environmental Health Sciences (NIEHS) (13). Relatively recently, it was discovered that there exist two different variants of BG-1 cells being used by researchers, BG-1 Fr and BG-1 NIEHS. In-depth analysis, including DNA testing, of these two BG-1 variant cell lines carried out by Li and coworkers (2014) (14) showed that the BG-1 Fr was unique and that the BG-1 NIEHS, i.e. the original cell line used to develop the assay, was not the BG1 human ovarian carcinoma cell line, but was instead a variant of the MCF7 human breast cancer cell line. The cell line used in the assay, originally referred to as BG1Luc4E2 (15), will now be designated as VM7Luc4E2 (“V” = variant; “M7” = MCF7 cells). Likewise, the assay will now be designated as the VM7Luc ER TA. While this changes the origin of the cell line upon which the assay is based, it does not affect published validation studies nor the utility and application of this assay for screening of estrogenic/anti-estrogenic chemicals.

higher than 1 µM due to the over-activation of the luciferase reporter gene (9) (11). While the dose-response curve indicates that true activation of the ER system occurs at lower concentrations, luciferase expression obtained at high concentrations of phytoestrogens or similar compounds suspected of producing phytoestrogen-like over-activation of the luciferase reporter gene needs to be examined carefully in stably transfected ER TA assay systems (see Appendix 2).

4. The “GENERAL INTRODUCTION” and “ER TA ASSAY COMPONENTS” should be read before using this assay for regulatory purposes. Definitions and abbreviations used in this test method are described in Appendix 1.

PRINCIPLE OF THE ASSAY (SEE ALSO GENERAL INTRODUCTION)

5. The assay is used to indicate ER ligand binding, followed by translocation of the receptor-ligand complex to the nucleus. In the nucleus, the receptor-ligand complex binds to specific DNA response elements and transactivates the reporter gene (*luc*), resulting in the production of luciferase and the subsequent emission of light, which can be quantified using a luminometer. Luciferase activity can be quickly and inexpensively evaluated with a number of commercially available kits. The VM7Luc ER TA utilises an ER responsive human breast adenocarcinoma cell line, VM7, which has been stably transfected with a firefly *luc* reporter construct under control of four estrogen response elements placed upstream of the mouse mammary tumour virus promoter (MMTV), to detect substances with *in vitro* ER agonist or antagonist activity. This MMTV promoter exhibits only minor cross-reactivity with other steroid and non-steroid hormones (8). Criteria for data interpretation are described in detail in paragraph 41. Briefly, a positive response is identified by a concentration-response curve containing at least three points with non-overlapping error bars (mean $\pm$ SD), as well as a change in amplitude (normalised relative light unit [RLU]) of at least 20% of the maximal value for the reference standard (17 β -estradiol [E2; CASRN 50-28-2] for the agonist assay, raloxifene HCl [Ral; CASRN 84449-90-1]/E2 for the antagonist assay).

PROCEDURE

Cell Line

6. The stably transfected VM7Luc4E2 cell line should be used for the assay. The cell line is currently only available with a technical licensing agreement from the University of

California, Davis, California, USA¹, and from Xenobiotic Detection Systems Inc., Durham, North Carolina, USA².

Stability of the Cell Line

7. To maintain the stability and integrity of the cell line, the cells should be grown for more than one passage from the frozen stock in cell maintenance media (see paragraph 9). Cells should not be cultured for more than 30 passages. For the VM7Luc4E2 cell line, 30 passages will be approximately three months.

Cell Culture and Plating Conditions

8. Procedures specified in the Guidance on Good Cell Culture Practice (5) (6) should be followed to assure the quality of all materials and methods in order to maintain the integrity, validity, and reproducibility of any work conducted.
9. VM7Luc4E2 cells are maintained in RPMI 1640 medium supplemented with 0.9% Pen-Strep and 8.0% fetal bovine serum (FBS) in a dedicated tissue culture incubator at 37°C ± 1°C, 90% ± 5% humidity, and 5.0% ± 1% CO₂/air.
10. Upon reaching ~80% confluence, VM7Luc4E2 cells are subcultured and conditioned to an estrogen-free environment for 48 hours prior to plating the cells in 96-well plates for exposure to test chemicals and analysis of estrogen dependent induction of luciferase activity. The estrogen-free medium (EFM) contains Dulbecco's Modification of Eagle's Medium (DMEM) without phenol red, supplemented with 4.5% charcoal/dextran-treated FBS, 1.9% L-glutamine, and 0.9% Pen-Strep. All plasticware should be free of estrogenic activity [see detailed protocol (7)].

Acceptability Criteria

11. Acceptance or rejection of a test is based on the evaluation of reference standard and control results from each experiment conducted on a 96-well plate. Each reference standard is tested in multiple concentrations and there are multiple samples of each reference and control concentration. Results are compared to quality controls (QC) for these parameters that were derived from the agonist and antagonist historical databases generated by each laboratory

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during the demonstration of proficiency. The historical databases are updated with reference standard and control values on a continuous basis. Changes in equipment or laboratory conditions may necessitate generation of updated historical databases.

Agonist Test

Range Finder Test

- Induction: Plate induction should be measured by dividing the average highest E2 reference standard relative light unit (RLU) value by the average DMSO control RLU value. Five-fold induction is usually achieved, but for purpose of acceptance, induction should be greater than or equal to four-fold.
- DMSO control results: Solvent control RLU values should be within 2.5 times the standard deviation of the historical solvent control mean RLU value.
- An experiment that fails either acceptance criterion should be discarded and repeated.

Comprehensive Test

It includes acceptability criteria from the agonist range finder test and the following:

- Reference standard results: The E2 reference standard concentration-response curve should be sigmoidal in shape and have at least three values within the linear portion of the concentration-response curve.
- Positive control results: Methoxychlor control RLU values should be greater than the DMSO mean plus three times the standard deviation from the DMSO mean.
- An experiment that fails any single acceptance criterion should be discarded and repeated.

Antagonist Test

Range Finder Test

- Reduction: Plate reduction is measured by dividing the average highest Ral/E2 reference standard RLU value by the average DMSO control RLU value. Five-fold reduction is usually achieved, but for the purposes of acceptance, reduction should be greater than or equal to three-fold.
- E2 control results: E2 control RLU values should be within 2.5 times the standard deviation of the historical E2 control mean RLU value.
- DMSO control results: DMSO control RLU values should be within 2.5 times the standard deviation of the historical solvent control mean RLU value.
- An experiment that fails any single acceptance criterion will be discarded and repeated.

Comprehensive Test

It includes acceptance criteria from the antagonist range finder test and the following:

- Reference standard results: The Ral/E2 reference standard concentration-response curve should be sigmoidal in shape and have at least three values within the linear portion of the concentration-response curve.
- Positive control results: Tamoxifen/E2 control RLU values should be less than the E2 control mean minus three times the standard deviation from the E2 control mean.
- An experiment that fails any single acceptance criterion will be discarded and repeated.

Reference Standards, Positive, and Vehicle Controls

Vehicle Control (Agonist and Antagonist Assays)

12. The vehicle that is used to dissolve the test chemicals should be tested as a vehicle control. The vehicle used during the validation of the VM7Luc ER TA assay was 1% (v/v) dimethylsulfoxide (DMSO, CASRN 67-68-5) (see paragraph 24). If a vehicle other than DMSO is used, all reference standards, controls, and test chemicals should be tested in the same vehicle, if appropriate.

Reference Standard (Agonist Range Finder)

13. The reference standard is E2 (CASRN 50-28-2). For range finder testing, the reference standard is comprised of a serial dilution of four concentrations of E2 (1.84×10^{-10} , 4.59×10^{-11} , 1.15×10^{-11} and 2.87×10^{-12} M), with each concentration tested in duplicate wells.

Reference Standard (Agonist Comprehensive)

14. E2 for comprehensive testing is comprised of a 1:2 serial dilution consisting of 11 concentrations (ranging from 3.67×10^{-10} to 3.59×10^{-13} M) of E2 in duplicate wells.

Reference Standard (Antagonist Range Finder)

15. The reference standard is a combination of Ral (CASRN 84449-90-1) and E2 (CASRN 50-28-2). Ral/E2 for range finder testing is comprised of a serial dilution of three concentrations of Ral (3.06×10^{-9} , 7.67×10^{-10} , and 1.92×10^{-10} M) plus a fixed concentration (9.18×10^{-11} M) of E2 in duplicate wells.

Reference Standard (Antagonist Comprehensive)

16. Ral/E2 for comprehensive testing is comprised of a 1:2 serial dilution of Ral (ranging from 2.45×10^{-8} to 9.57×10^{-11} M) plus a fixed concentration (9.18×10^{-11} M) of E2 consisting of nine concentrations of Ral/E2 in duplicate wells.

Weak Positive Control (Agonist)

17. The weak positive control is 9.06×10^{-6} M *p,p'*-methoxychlor (methoxychlor; CASRN 72-43-5) in EFM.

Weak Positive Control (Antagonist)

18. The weak positive control consists of tamoxifen (CASRN 10540-29-1) 3.36×10^{-6} M with 9.18×10^{-11} M E2 in EFM.

E2 Control (Antagonist Assay Only)

19. The E2 control is 9.18×10^{-11} M E2 in EFM and used as a base line negative control.

Fold-Induction (Agonist)

20. The induction of luciferase activity of the reference standard (E2) is measured by dividing the average highest E2 reference standard RLU value by the average DMSO control RLU value, and the result should be greater than four-fold.

Fold-Reduction (Antagonist)

21. The mean luciferase activity of the reference standard (Ral/E2) is measured by dividing the average highest Ral/E2 reference standard RLU value by the average DMSO control RLU value and should be greater than three-fold.

Demonstration of Laboratory Proficiency (see paragraph 14 and Tables 3 and 4 in “ER TA ASSAY COMPONENTS” of this test method)

Vehicle

22. Test chemicals should be dissolved in a solvent that solubilises the test chemical and is miscible with the cell medium. Water, ethanol (95% to 100% purity) and DMSO are suitable vehicles. If DMSO is used, the level should not exceed 1% (v/v). For any vehicle, it should be demonstrated that the maximum volume used is not cytotoxic and does not interfere with the assay performance. Reference standards and controls are dissolved in 100% solvent and then diluted down to appropriate concentrations in EFM.

Preparation of Test chemicals

23. The test chemicals are dissolved in 100% DMSO (or appropriate solvent), and then diluted down to appropriate concentrations in EFM. All test chemicals should be allowed to equilibrate to room temperature before being dissolved and diluted. Test chemical solutions should be prepared fresh for each experiment. Solutions should not have noticeable precipitate or cloudiness. Reference standard and control stocks may be prepared in bulk; however, final reference standard, control dilutions and test chemicals should be freshly prepared for each experiment and used within 24 hours of preparation.

Solubility and Cytotoxicity: Considerations for Range Finding

24. Range finder testing consists of seven point - 1:10 serial dilutions run in duplicate. Initially, test chemicals are tested up to the maximum concentration of 1 mg/ml (~1 mM) for agonist testing and 20 µg/ml (~10 µM) for antagonist testing. Range finder experiments are used to determine the following:

- Test chemical starting concentrations to be used during comprehensive testing
 - Test chemical dilutions (1:2 or 1:5) to be used during comprehensive testing
25. An assessment of cell viability/cytotoxicity is included in the agonist and antagonist assay protocols (7) and is incorporated into range finder and comprehensive testing. The cytotoxicity method that was used to assess cell viability during the validation of the VM7Luc ER TA (1) was a scaled qualitative visual observation method; however, a quantitative method for the determination of cytotoxicity can be used (see protocol (7)). Data from test chemical concentrations that cause more than 20% reduction in viability cannot be used.

Test chemical Exposure and Assay Plate Organisation

26. Cells are counted and plated into 96-well tissue culture plates (2×10^5 cells per well) in EFM and incubated for 24 hours to allow the cells to attach to the plate. The EFM is removed and replaced with test and reference chemicals in EFM and incubated for 19-24 hours. Special considerations will need to be applied to those substances that are highly volatile since nearby control wells may generate false positive results. In such cases, “plate sealers” may help to effectively isolate individual wells during testing, and are therefore recommended.

Range Finder Tests

27. Range finder testing uses all wells of the 96-well plate to test up to six test chemicals as seven point 1:10 serial dilutions in duplicate (see [Figures 1 and 2](#)).
- *Agonist* range finder testing uses four concentrations of E2 in duplicate as the reference standard and four replicate wells for the DMSO control.
 - *Antagonist* range finder testing uses three concentrations of Ral/E2 with 9.18×10^{-11} M E2 in duplicate as the reference standard, with three replicate wells for the E2 and DMSO controls.

Figure 1: Agonist Range Finder Test 96-well Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	TC1-1	TC1-1	TC2-1	TC2-1	TC3-1	TC3-1	TC4-1	TC4-1	TC5-1	TC5-1	TC6-1	TC6-1
B	TC1-2	TC1-2	TC2-2	TC2-2	TC3-2	TC3-2	TC4-2	TC4-2	TC5-2	TC5-2	TC6-2	TC6-2
C	TC1-3	TC1-3	TC2-3	TC2-3	TC3-3	TC3-3	TC4-3	TC4-3	TC5-3	TC5-3	TC6-3	TC6-3
D	TC1-4	TC1-4	TC2-4	TC2-4	TC3-4	TC3-4	TC4-4	TC4-4	TC5-4	TC5-4	TC6-4	TC6-4
E	TC1-5	TC1-5	TC2-5	TC2-5	TC3-5	TC3-5	TC4-5	TC4-5	TC5-5	TC5-5	TC6-5	TC6-5
F	TC1-6	TC1-6	TC2-6	TC2-6	TC3-6	TC3-6	TC4-6	TC4-6	TC5-6	TC5-6	TC6-6	TC6-6
G	TC1-7	TC1-7	TC2-7	TC2-7	TC3-7	TC3-7	TC4-7	TC4-7	TC5-7	TC5-7	TC6-7	TC6-7
H	E2-1	E2-2	E2-3	E2-4	VC	VC	VC	VC	E2-1	E2-2	E2-3	E2-4

Abbreviations: E2-1 to E2-4 = concentrations of the E2 reference standard (from high to low); TC1-1 to TC1-7 = concentrations (from high to low) of test chemical 1 (TC1); TC2-1 to TC2-7 = concentrations (from high to low) of test chemical 2 (TC2); TC3-1 to TC3-7 = concentrations (from high to low) of test chemical 3 (TC3); TC4-1 to TC4-7 = concentrations (from high to low) of test chemical 4 (TC4); TC5-1 to TC5-7 = concentrations (from high to low) of test chemical 5 (TC5); TC6-1 to TC6-7 = concentrations (from high to low) of test chemical 6 (TC6); VC = vehicle control (DMSO [1% v/v EFM.]).

Figure 2: Antagonist Range Finder Test 96-well Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	TC1-1	TC1-1	TC2-1	TC2-1	TC3-1	TC3-1	TC4-1	TC4-1	TC5-1	TC5-1	TC6-1	TC6-1
B	TC1-2	TC1-2	TC2-2	TC2-2	TC3-2	TC3-2	TC4-2	TC4-2	TC5-2	TC5-2	TC6-2	TC6-2
C	TC1-3	TC1-3	TC2-3	TC2-3	TC3-3	TC3-3	TC4-3	TC4-3	TC5-3	TC5-3	TC6-3	TC6-3
D	TC1-4	TC1-4	TC2-4	TC2-4	TC3-4	TC3-4	TC4-4	TC4-4	TC5-4	TC5-4	TC6-4	TC6-4
E	TC1-5	TC1-5	TC2-5	TC2-5	TC3-5	TC3-5	TC4-5	TC4-5	TC5-5	TC5-5	TC6-5	TC6-5
F	TC1-6	TC1-6	TC2-6	TC2-6	TC3-6	TC3-6	TC4-6	TC4-6	TC5-6	TC5-6	TC6-6	TC6-6
G	TC1-7	TC1-7	TC2-7	TC2-7	TC3-7	TC3-7	TC4-7	TC4-7	TC5-7	TC5-7	TC6-7	TC6-7
H	Ral-1	Ral-2	Ral-3	VC	VC	VC	E2	E2	E2	Ral-1	Ral-2	Ral-3

Abbreviations: E2 = E2 control; Ral-1 to Ral-3 = concentrations of the Raloxifene/E2 reference standard (from high to low); TC1-1 to TC1-7 = concentrations (from high to low) of test chemical 1 (TC1); TC2-1 to TC2-7 = concentrations (from high to low) of test chemical 2 (TC2); TC3-1 to TC3-7 = concentrations (from high to low) of test chemical 3 (TC3); TC4-1 to TC4-7 = concentrations (from high to low) of test chemical 4 (TC4); TC5-1 to TC5-7 = concentrations (from high to low) of test chemical 5 (TC5); TC6-1 to TC6-7 = concentrations (from high to low) of test chemical 6 (TC6); VC = vehicle control (DMSO [1% v/v EFM.]).

Note: All test chemicals are tested in the presence of 9.18×10^{-11} M E2.

28. The recommended final volume of media required for each well is 200 μ l. Only use test plates in which the cells in all wells give a viability of 80% and above.

29. Determination of starting concentrations for comprehensive **agonist** testing is described in depth in the agonist protocol (7). Briefly, the following criteria are used:

- If there are no points on the test chemical concentration curve that are greater than the mean plus three times the standard deviation of the DMSO control, comprehensive testing will be conducted using an 11-point 1:2 serial dilution starting at the maximum soluble concentration.
- If there are points on the test chemical concentration curve that are greater than the mean plus three times the standard deviation of the DMSO control, the starting concentration to be used for the 11-point dilution scheme in comprehensive testing should be one log higher than the concentration giving the highest adjusted RLU value in the range finder. The 11-point dilution scheme will be based on either 1:2 or 1:5 dilutions according to the following criteria:

An 11-point 1:2 serial dilution should be used if the resulting concentration range will encompass the full range of responses based on the concentration response curve generated in the range finder test. Otherwise, use a 1:5 dilution.

- If a test chemical exhibits a biphasic concentration response curve in the range finder test, both phases should also be resolved in comprehensive testing.

30. Determination of starting concentrations for comprehensive **antagonist** testing is described in depth in the antagonist protocol (7). Briefly, the following criteria are used:

- If there are no points on the test chemical concentration curve that are less than the mean minus three times the standard deviation of the E2 control, comprehensive testing will be conducted using an 11-point 1:2 serial dilution starting at the maximum soluble concentration.
- If there are points on the test chemical concentration curve that are less than the mean minus three times the standard deviation of the E2 control, the starting concentration to be used for the 11-point dilution scheme in comprehensive testing should be one of the following:
 - The concentration giving the lowest adjusted RLU value in the range finder
 - The maximum soluble concentration (See antagonist protocol (7), Figure 14-2)
 - The lowest cytotoxic concentration (See antagonist protocol (7), Figure 14-3 for a related example).
- The 11-point dilution scheme will be based on either a 1:2 or 1:5 serial or dilution according to the following criteria:

An 11-point 1:2 serial dilution should be used if the resulting concentration range will encompass the full range of responses based on the concentration response curve generated in the range finder test. Otherwise a 1:5 dilution should be used.

Comprehensive Tests

31. Comprehensive testing consists of 11-point serial dilutions (either 1:2 or 1:5 serial dilutions based on the starting concentration for comprehensive testing criteria) with each concentration tested in triplicate wells of the 96-well plate (see [Figures 3 and 4](#)).
- *Agonist* comprehensive testing uses 11 concentrations of E2 in duplicate as the reference standard. Four replicate wells for the DMSO control and four replicate wells for the methoxychlor control (9.06×10^{-6} M) are included on each plate.
 - *Antagonist* comprehensive testing uses nine concentrations of Ral/E2 with 9.18×10^{-11} M E2 in duplicate as the reference standard, with four replicate wells for the E2 9.18×10^{-11} M control, four replicate wells for DMSO controls, and four replicate wells for tamoxifen 3.36×10^{-6} M.

Figure 3: Agonist Comprehensive Test 96-well Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	TC1-1	TC1-2	TC1-3	TC1-4	TC1-5	TC1-6	TC1-7	TC1-8	TC1-9	TC1-10	TC1-11	VC
B	TC1-1	TC1-2	TC1-3	TC1-4	TC1-5	TC1-6	TC1-7	TC1-8	TC1-9	TC1-10	TC1-11	VC
C	TC1-1	TC1-2	TC1-3	TC1-4	TC1-5	TC1-6	TC1-7	TC1-8	TC1-9	TC1-10	TC1-11	VC
D	TC2-1	TC2-2	TC2-3	TC2-4	TC2-5	TC2-6	TC2-7	TC2-8	TC2-9	TC2-10	TC2-11	VC
E	TC2-1	TC2-2	TC2-3	TC2-4	TC2-5	TC2-6	TC2-7	TC2-8	TC2-9	TC2-10	TC2-11	Meth
F	TC2-1	TC2-2	TC2-3	TC2-4	TC2-5	TC2-6	TC2-7	TC2-8	TC2-9	TC2-10	TC2-11	Meth
G	E2-1	E2-2	E2-3	E2-4	E2-5	E2-6	E2-7	E2-8	E2-9	E2-10	E2-11	Meth
H	E2-1	E2-2	E2-3	E2-4	E2-5	E2-6	E2-7	E2-8	E2-9	E2-10	E2-11	Meth

Abbreviations: TC1-1 to TC1-11 = concentrations (from high to low) of test chemical 1; TC2-1 to TC2-11 = concentrations (from high to low) of test chemical 2; E2-1 to E2-11 = concentrations of the E2 reference standard (from high to low); Meth = p,p' methoxychlor weak positive control; VC = DMSO (1% v/v) EFM vehicle control

Figure 4: Antagonist Comprehensive Test 96-well Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	TC1-1	TC1-2	TC1-3	TC1-4	TC1-5	TC1-6	TC1-7	TC1-8	TC1-9	TC1-10	TC1-11	VC
B	TC1-1	TC1-2	TC1-3	TC1-4	TC1-5	TC1-6	TC1-7	TC1-8	TC1-9	TC1-10	TC1-11	VC
C	TC1-1	TC1-2	TC1-3	TC1-4	TC1-5	TC1-6	TC1-7	TC1-8	TC1-9	TC1-10	TC1-11	VC
D	TC2-1	TC2-2	TC2-3	TC2-4	TC2-5	TC2-6	TC2-7	TC2-8	TC2-9	TC2-10	TC2-11	VC
E	TC2-1	TC2-2	TC2-3	TC2-4	TC2-5	TC2-6	TC2-7	TC2-8	TC2-9	TC2-10	TC2-11	Tam
F	TC2-1	TC2-2	TC2-3	TC2-4	TC2-5	TC2-6	TC2-7	TC2-8	TC2-9	TC2-10	TC2-11	Tam
G	Ral-1	Ral-2	Ral-3	Ral-4	Ral-5	Ral-6	Ral-7	Ral-8	Ral-9	E2	E2	Tam
H	Ral-1	Ral-2	Ral-3	Ral-4	Ral-5	Ral-6	Ral-7	Ral-8	Ral-9	E2	E2	Tam

Abbreviations: E2 = E2 control; Ral-1 to Ral-9 = concentrations of the Raloxifene/E2 reference standard (from high to low); Tam = Tamoxifen/E2 weak positive control; TC1-1 to TC1-11 = concentrations (from high to low) of test chemical 1 (TC1); TC2-1 to TC2-11 = concentrations (from high to low) of test chemical 2 (TC2); VC = vehicle control (DMSO [1% v/v EFM.]).

Note: As noted, all reference and test wells contain a fixed concentration of E2 (9.18×10^{-11} M)

32. Repeat comprehensive tests for the same chemical should be conducted on different days, to ensure independence. At least two comprehensive tests should be conducted. If the results of the tests contradict each other (e.g. one test is positive, the other negative), or if one of the tests is inadequate, a third additional test should be conducted.

Measure of Luminescence

33. Luminescence is measured in the range of 300 to 650 nm, using an injecting luminometer and with software that controls the injection volume and measurement interval (7). Light emission from each well is expressed as RLU per well.

ANALYSIS OF DATA

EC₅₀ /IC₅₀ determination

34. The EC₅₀ value (half maximal effective concentration of a test chemical [agonists]) and the IC₅₀ value (half maximal inhibitory concentration of a test chemical [antagonists]) are determined from the concentration-response data. For test chemicals that are positive at one or more concentrations, the concentration of test chemical that causes a half-maximal response (IC₅₀ or EC₅₀) is calculated using a Hill function analysis or an appropriate alternative. The Hill function is a four-parameter logistic mathematical model relating the test chemical concentration to the response (typically following a sigmoidal curve) using the equation below:

$$Y = Bottom + \frac{(Top - Bottom)}{1 + 10^{(lgEC_{50} - X)Hill\ Slope}}$$

Where:

Y = response (i.e. RLUs);

X = the logarithm of concentration;

Bottom = the minimum response;

Top = the maximum response;

lg EC₅₀ (or lg IC₅₀) = the logarithm of X as the response midway between Top and Bottom;

Hillslope = the steepness of the curve.

The model calculates the best fit for the Top, Bottom, Hillslope, and IC₅₀ and EC₅₀ parameters. For the calculation of EC₅₀ and IC₅₀ values, appropriate statistical software should be used (e.g. Graphpad Prism^R statistical software).

Determination of Outliers

35. Good statistical judgment could be facilitated by including (but not limited to) the Q-test (see agonist and antagonist protocols (7) for determining “unusable” wells that will be excluded from the data analysis.
36. For E2 reference standard replicates (sample size of two), any adjusted RLU value for a replicate at a given concentration of E2 is considered an outlier if its value is more than 20% above or below the adjusted RLU value for that concentration in the historical database.

Collection and Adjustment of Luminometer Data for Range Finder Testing

37. Raw data from the luminometer should be transferred to a spreadsheet template designed for the assay. It should be determined whether there are outlier data points that need to be removed. (See Test Acceptance Criteria for parameters that are determined in the analyses.) The following calculations should be performed:

Agonist

- Step 1 Calculate the mean value for the DMSO vehicle control (VC).
- Step 2 Subtract the mean value of the DMSO VC from each well value to normalise the data.
- Step 3 Calculate the mean fold induction for the reference standard (E2).
- Step 4 Calculate the mean EC₅₀ value for the test chemicals.

Antagonist

- Step 1 Calculate the mean value for the DMSO VC.
- Step 2 Subtract the mean value of the DMSO VC from each well value to normalise the data.
- Step 3 Calculate the mean fold reduction for the reference standard (Ral/E2).
- Step 4 Calculate the mean value for the E2 reference standard.
- Step 5 Calculate the mean IC₅₀ value for the test chemicals.

Collection and Adjustment of Luminometer Data for Comprehensive Testing

38. Raw data from the luminometer should be transferred to a spreadsheet template designed for the assay. It should be determined whether there are outlier data points that need to be removed. (See Test Acceptance Criteria for parameters that are determined in the analyses.) The following calculations are performed:

Agonist

- Step 1 Calculate the mean value for the DMSO VC.
- Step 2 Subtract the mean value of the DMSO VC from each well value to normalise the data.
- Step 3 Calculate the mean fold induction for the reference standard (E2).
- Step 4 Calculate the mean EC₅₀ value for E2 and the test chemicals.
- Step 5 Calculate the mean adjusted RLU value for methoxychlor.

Antagonist

- Step 1 Calculate the mean value for the DMSO VC.
- Step 2 Subtract the mean value of the DMSO VC from each well value to normalise the data.
- Step 3 Calculate the mean fold induction for the reference standard (Ral/E2).
- Step 4 Calculate the mean IC₅₀ value for Ral/E2 and the test chemicals.
- Step 5 Calculate the mean adjusted RLU value for tamoxifen.
- Step 6 Calculate the mean value for the E2 reference standard.

Data Interpretation Criteria

39. The VM7Luc ER TA is intended as part of a weight of evidence approach to help prioritise substances for ED testing *in vivo*. Part of this prioritisation procedure will be the classification of the test chemical as positive or negative for either ER agonist or antagonist activity. The positive and negative decision criteria used in the VM7Luc ER TA validation study are described in Table 1.

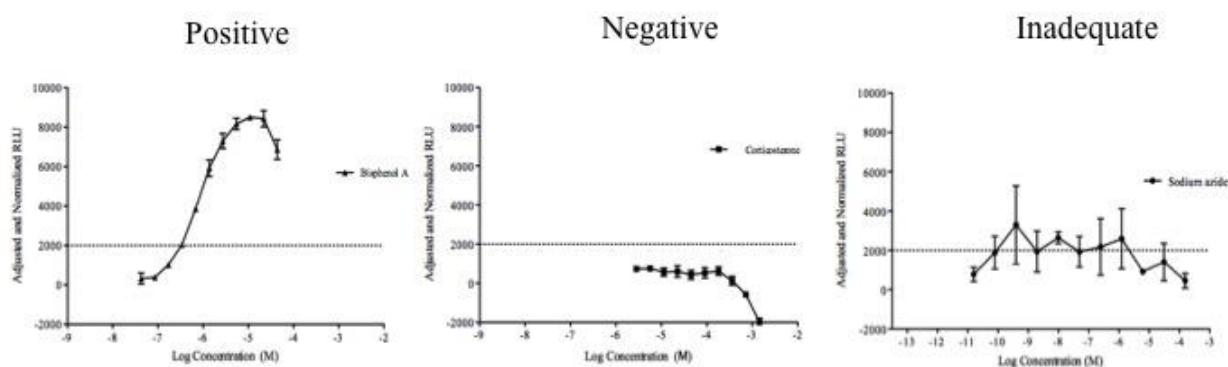
Table 1: Positive and Negative Decision Criteria

AGONIST ACTIVITY	
Positive	- All test chemicals classified as <i>positive for</i> ER agonist activity should have a concentration–response curve consisting of a baseline, followed by a positive slope, and concluding in a plateau or peak. In some cases, only two of these characteristics (baseline–slope or slope–peak) may be defined. - The line defining the positive slope should contain at least three points with non-overlapping error bars (mean $\pm$ SD). Points forming the baseline are excluded, but the linear portion of the curve may include the peak or first point of the plateau. - A positive classification requires a response amplitude, the difference between baseline and peak, of at least 20% of the maximal value for the reference standard, E2 (i.e. 2000 RLUs or more when the maximal response value of the reference standards [E2] is adjusted to 10,000 RLUs). - If possible, an EC₅₀ value should be calculated for each positive test chemical.
Negative	The average adjusted RLU for a given concentration is at or below the mean DMSO control RLU value plus three times the standard deviation of the DMSO RLU.
Inadequate	Data that cannot be interpreted as valid for showing either the presence or absence of activity because of major qualitative or quantitative limitations are considered inadequate and cannot be used to determine whether the test chemical is positive or negative. Chemicals should be retested.
ANTAGONIST ACTIVITY	

Positive	- Test chemical data produce a concentration-response curve consisting of a baseline, which is followed by a negative slope. - The line defining the negative slope should contain at least three points with non-overlapping error bars; points forming the baseline are excluded but the linear portion of the curve may include the first point of the plateau. - There should be at least a 20% reduction in activity from the maximal value for the reference standard, Ral/E2 (i.e. 8000 RLU or less when the maximal response value of the reference standard [Ral/E2] is adjusted to 10,000 RLUs). - The highest non-cytotoxic concentrations of the test chemical should be less than or equal to 1×10^{-5} M. - If possible, an IC_{50} value should be calculated for each positive test chemical.
Negative	All data points are above the EC_{80} value (80% of the E2 response, or 8000 RLUs), at concentrations less than 1.0×10^{-5} M.
Inadequate	Data that cannot be interpreted as valid for showing either the presence or absence of activity because of major qualitative or quantitative limitations are considered inadequate and cannot be used to determine whether the test chemical is positive or negative. Chemical should be retested.

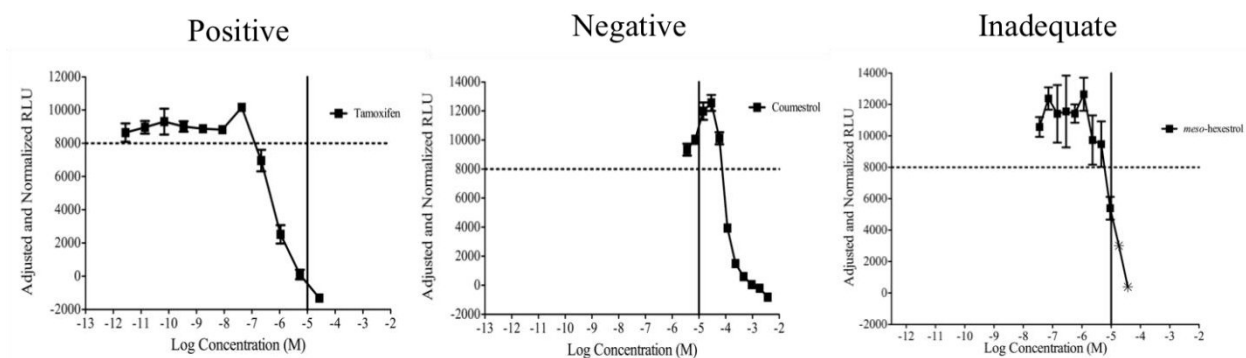
40. Positive results will be characterised by both the magnitude of the effect and the concentration at which the effect occurs, where possible. Examples of positive, negative and inadequate data are shown in [Figures 5 and 6](#).

Figure 5: Agonist Examples: Positive, Negative and Inadequate Data



Dashed line indicates 20% of E2 response, 2000 adjusted and normalised RLUs.

Figure 6: Antagonist Examples: Positive, Negative, and Inadequate Data



Dashed line indicates 80% of Ral/E2 response, 8000 adjusted and normalised RLUs.

Solid line indicates 1.00×10^{-5} M. For a response to be considered positive, it should be below the 8000 RLU line, and at concentrations less than 1.00×10^{-5} M.

Asterisked concentrations in the meso-hexestrol graph indicate viability scores of "2" or greater.

The test results for meso-hexestrol are considered inadequate data because the only response that is below 8,000 RLU occurs at 1.00×10^{-5} M.

41. The calculations of EC_{50} and IC_{50} can be made using a four-parameter Hill Function (see agonist protocol and antagonist protocol for more details (7)). Meeting the acceptability criteria indicates the system is operating properly, but it does not ensure that any particular run will produce accurate data. Duplicating the results of the first run is the best assurance that accurate data were produced (see paragraph 19 of "ER TA ASSAY COMPONENTS").

TEST REPORT

42. See paragraph 20 of "ER TA ASSAY COMPONENTS".

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APPENDIX 4

STABLY TRANSFECTED HUMAN ESTROGEN RECEPTOR-A TRANSACTIVATION ASSAY FOR DETECTION OF ESTROGENIC AGONIST AND ANTAGONIST ACTIVITY OF CHEMICALS USING THE ERA CALUX CELL LINE

INITIAL CONSIDERATIONS AND LIMITATIONS (SEE ALSO GENERAL INTRODUCTION)

1. The ER α CALUX transactivation assay uses the human U2OS cell line to detect estrogenic agonist and antagonist activity mediated through human estrogen receptor alpha (hER α). The validation study of the stably transfected ER α CALUX bioassay by BioDetection Systems BV (Amsterdam, the Netherlands) demonstrated the relevance and reliability of the assay for its intended purpose (1). The ER α CALUX cell line expresses stably transfected human ER α only (2) (3).
2. This assay is specifically designed to detect hER α -mediated transactivation by measuring bioluminescence as the endpoint. The use of bioluminescence is commonly used in bioassays because of the high signal-to-noise ratio (4).
3. Phytoestrogen concentrations higher than 1 μ M have been reported to over-activate the luciferase reporter gene, resulting in non-receptor-mediated luminescence (5) (6) (7). Therefore, higher concentrations of phytoestrogens or other similar compounds that can over-activate the luciferase expression, have to be examined carefully in stably transfected ER transactivation assays (see Appendix 2).
4. The “GENERAL INTRODUCTION” and “ER TA ASSAY COMPONENTS” should be read before using this assay for regulatory purposes. Definitions and abbreviations used in this test method are described in Appendix 1.

PRINCIPLE OF THE ASSAY (SEE ALSO GENERAL INTRODUCTION)

5. The bioassay is used to assess ER ligand binding and subsequent translocation of the receptor-ligand complex to the nucleus. In the nucleus, the receptor-ligand complex binds specific DNA response elements and transactivates a firefly luciferase reporter gene, resulting in increased cellular expression of the luciferase enzyme. Following the addition of the luciferase substrate luciferine, the luciferine is transformed into a bioluminescent product. The light produced can easily be detected and quantified using a luminometer.
6. The test system utilises stably transfected ER α CALUX cells. ER α CALUX cells originated from the human osteoblastic osteosarcoma U2OS cell line. Human U2OS cells were stably transfected with 3xHRE-TATA-Luc and pSG5-neo-hER α using the calcium phosphate co-

precipitation method. The U2OS cell line was selected as the best candidate to serve as the estrogen- (and other steroid hormone) responsive reporter cell line, based on the observation that the U2OS cell line showed little or no endogenous receptor activity. The absence of endogenous receptors was assessed using luciferase reporter plasmids only, showing no activity when receptor ligands were added. Furthermore, this cell line supported strong hormone-mediated responses when cognate receptors were transiently introduced (2) (3) (8).

7. Testing chemicals for estrogenic or anti-estrogenic activity using the ER α CALUX cell line include a prescreen run and comprehensive runs. During the prescreen run, the solubility, cytotoxicity and a refined concentration-range of test chemicals for comprehensive testing are determined. During the comprehensive runs, the refined concentration-ranges of test chemicals are tested in the ER α CALUX bioassays followed by the classification of the test chemicals for agonism or antagonism.
8. Criteria for data interpretation are described in detail in paragraph 59. Briefly, a test chemical is considered positive for agonism in case at least two consecutive concentrations of the test chemical show a response that is equal or higher than 10% of the maximum response of the reference standard 17 β -estradiol (PC₁₀). A test chemical is considered positive for antagonism in case at least two consecutive concentrations of the test chemical show a response that is equal or lower than 80% of the maximum response of the reference standard tamoxifen (PC₈₀).

PROCEDURE

Cell lines

9. The stably transfected U2OS ER α CALUX cell line should be used for the assay. The cell line can be obtained from BioDetection Systems BV, Amsterdam, the Netherlands with a technical licensing agreement.
10. Only mycoplasma free cell cultures should be used. Cell batches used should either be certified negative for mycoplasma contamination, or a mycoplasma test should be performed before use. RT-PCR (Real Time Polymerase Chain Reaction) should be used for sensitive detection of mycoplasma infection (9).

Stability of the cell line

11. To maintain the stability and integrity of the CALUX cells, the cells should be stored in liquid nitrogen (-80°C). Following thawing of cells to start a new culture, cells should be sub-cultured at least twice before being used to assess the estrogenic agonist and antagonist activity of chemicals. Cells should not be sub-cultured for more than 30 passages.

12. To monitor the stability of the cell line over time, the responsiveness of the agonistic and antagonistic test system should be verified by evaluating the EC₅₀ or IC₅₀ of the reference standard. In addition, the relative induction of the positive control sample (PC) and the negative control sample (NC) should be monitored. The results should be in agreement with the acceptance criteria for the agonistic (Table 3C) or antagonistic ERα CALUX bioassay (Table 4C). The reference standards, positive and negative controls are given in Table 1 and Table 2 for the agonistic and antagonistic mode respectively.

Cell Culture and plating conditions

13. The U2OS cells should be cultured in growth medium (DMEM/F12 (1:1) medium with phenol red as pH indicator, supplemented with fetal bovine serum (7.5%), non-essential amino acids (1%), 10 Units/ml of penicillin, streptomycin and geneticin (G-418) as selection marker). Cells should be placed in a CO₂ incubator (5% CO₂) at 37°C and 100% humidity. When cells reach an 85-95% confluency, cells should either be subcultured or prepared for seeding in 96-well microtiter plates. In case of the latter, cells should be resuspended at 1x10⁵ cells/ml in estrogen free assay medium (DMEM/F12 (1:1) medium without phenol red, supplemented with Dextran-Coated Charcoal treated fetal bovine serum (5% v/v), non-essential amino acids (1% v/v), 10 Units/ml of penicillin and streptomycin) and plated into the wells of the 96-well microtiterplates (100 µl of homogenised cell suspension). Cells should be pre-incubated in a CO₂ incubator (5% CO₂, 37°C, 100% humidity) for 24 hours prior to exposure. Plastic ware should be estrogen free.

Acceptability criteria

14. Agonistic and antagonistic activities of the test chemical(s) are tested in test series. A test series consists of a maximum of 6 microtiter plates. Each test series contains at least 1 full series of dilutions of a reference standard, a positive control sample, a negative control sample and solvent controls. Figures 1 and 2 give the plate setup for agonistic and antagonistic tests series.
15. Each dilution of the reference standards, test chemicals, all solvent controls, and positive and negative controls should be analysed in triplicate. Each of the triplicate analyses should fulfil the requirements given in Table 3A and Table 4A.
16. A complete series of dilutions of the reference standard (17β-estradiol for agonism; tamoxifen for antagonism) is measured on the first plate in each test series. To be able to compare the analysis results of the remaining 5 microtiter plates with the first microtiter plate containing the complete concentration-response curve of the reference standard, all plates should contain 3 control samples: solvent control, the highest concentration of the reference standard tested, and the approximate EC₅₀ (agonism) or IC₅₀ (antagonism) concentration of the reference standard. The ratio of the average control samples on the first plate and the remaining

5 plates should fulfil the requirements as given in Table 3C (agonism) or Table 4C (antagonism).

17. For each of the microtiter plates within a test series, the z-factor is calculated (10). The z-factor should be calculated using the responses at the highest and lowest concentration of the reference standard. A microtiter plate is considered valid in case it fulfils the requirements as stated in Table 3C (agonism) or Table 4C (antagonism).
18. The reference standard should demonstrate a sigmoidal dose-response curve. The EC₅₀ or IC₅₀ derived from the response of the series of dilutions of the reference standard, should fulfil the requirements as indicated in Table 3C (agonism) or Table 4C (antagonism).
19. Each test series should contain a positive control and negative control sample. The calculated relative induction of both the positive and negative control sample should fulfil the requirements as indicated in Table 3C (agonism) or Table 4C (antagonism).
20. During all measurements, the induction factor of the highest concentration of the reference standard should be measured by dividing the average highest 17β-estradiol reference standard relative light unit (RLU) response by the average reference solvent control RLU response. This induction factor should fulfil the minimum requirements for the fold induction as indicated in Table 3C (agonism) or Table 4C (antagonism).
21. Only microtiter plates that fulfil all above mentioned acceptance criteria are considered valid and can be used to evaluate the response of test chemicals.
22. The acceptance criteria are applicable to both prescreen and comprehensive runs.

Table 1: Concentrations of reference standard, positive control (PC) and negative control (NC) for the agonistic CALUX bioassay

	Substance	CAS RN	Test range (M)
Reference standard	17β-estradiol	50-28-2	1*10 ⁻¹³ - 1*10 ⁻¹⁰
Positive control (PC)	17α-methyltestosterone	58-18-4	3*10 ⁻⁶
Negative control (NC)	corticosterone	50-22-6	1*10 ⁻⁸

Table 2: Concentrations of reference standard, positive control (PC) and negative control (NC) for the antagonistic CALUX bioassay

	Substance	CAS RN	Test range (M)
Reference standard	tamoxifen	10540-29-1	3*10 ⁻⁹ - 1*10 ⁻⁵

Positive control (PC)	4-hydroxytamoxifen	68047-06-3	1×10^{-9}
Negative control (NC)	resveratrol	501-36-0	1×10^{-5}

Table 3: Acceptance criteria for the agonistic ER α CALUX bioassay

A - individual samples on a plate		Criterion
1	Maximum %SD of triplicate wells (for NC, PC, each dilution of the test chemical and the reference standard, except C0)	< 15%
2	Maximum %SD of triplicate wells (for reference standard and test chemical solvent controls (C0, SC))	< 30%
3	Maximum LDH leakage, as a measure of cytotoxicity.	< 120%
B - within a single microtiter plate		
4	Ratio of the reference standard solvent control (C0; plate 1) and test chemical solvent control (SC; plates 2 to x)	0.5 to 2.0
5	Ratio of the appr. EC50 and highest reference standard concentrations on plate 1 and the appr. EC50 and highest reference standard concentrations on plates 2 to x (C4, C8)	0.70 to 1.30
6	Z-factor for each plate	>0.6
C - within a single series of analyses (all plates within one series)		
7	Sigmoidal curve of reference standard	Yes (17β-estradiol)
8	EC50 range reference standard 17β-estradiol	4*10 ⁻¹² – 4*10 ⁻¹¹ M
9	Minimum fold induction of the highest 17β-estradiol concentration, with respect to the reference standard solvent control.	5
10	Relative induction (%) PC.	> 30%
11	Relative induction (%) NC	<10%

Appr.: approximative; PC: positive control; NC: negative control; SC: test chemical solvent control; C0: reference standard solvent control; SD: standard deviation; LDH: lactate dehydrogenase

Table 4: Acceptance criteria for the antagonistic ER α CALUX bioassay

A - individual samples on a plate			Criterion
1	Maximum %SD of triplicate wells (for NC, PC, each dilution of the test		< 15%

	chemical and the reference standard, solvent control (C0))	
2	Maximum %SD of triplicate wells (for vehicle control (VC) and highest reference standard concentration (C8))	< 30%
3	Maximum LDH leakage, as a measure of cytotoxicity.	< 120%
B - within a single microtiter plate		
4	Ratio of the reference standard solvent control (C0; plate 1) and test chemical solvent control (SC; plates 2 to x)	0.70 to 1.30
5	Ratio of the appr. IC50 reference standard concentrations on plate 1 and the appr. IC50 reference standard concentrations on plates 2 to x (C4)	0.70 to 1.30
6	Ratio of the highest reference standard concentrations on plate 1 and the highest reference standard concentrations on plates 2 to x (C8)	0.50 to 2.0
7	Z-factor for each plate	>0.6
C - within a single series of analyses (all plates within one series)		
8	Sigmoidal curve of reference standard	Yes (Tamoxifen)
9	IC50 range reference standard (Tamoxifen)	$1 \cdot 10^{-8}$ - $1 \cdot 10^{-7}$ M
10	Minimum fold induction of the reference standard solvent control, with respect to the highest Tamoxifen concentration.	2.5
11	Relative induction (%) PC.	<70%
12	Relative induction (%) NC	>85%

Appr.: approximative; PC: positive control; NC: negative control; VC: vehicle control (solvent control without fixed concentration of agonist reference standard); SC: test chemical solvent control; C0: reference standard solvent control; SD: standard deviation; LDH: lactate dehydrogenase

Solvent/vehicle control, reference standards, positive controls, negative controls

23. For both the prescreen run and comprehensive runs, the same solvent/vehicle control, reference standards, positive controls and negative controls should be used. In addition, the concentration of reference standards, positive controls and negative controls should be the same.

Solvent control

24. The solvent used to dissolve the test chemicals should be tested as a solvent control. Dimethylsulfoxide (DMSO, 1% (v/v); CASRN 67-68-5) was used as vehicle during the validation of the ER α CALUX bioassay. If a solvent other than DMSO is used, all reference standards, controls, and test chemicals should be tested in the same vehicle. Please note that the solvent control for antagonistic studies contains a fixed concentration of the agonist reference standard 17 β -estradiol (approximately EC₅₀ concentration). To test the solvent used for antagonistic studies, a vehicle control should be prepared and tested.

Vehicle control (antagonism)

25. For testing antagonism, the assay medium is supplemented with a fixed concentration of the agonist reference standard 17 β -estradiol (approximately EC₅₀ concentration). To test the solvent used to dissolve the test chemicals for antagonism, an assay medium without a fixed concentration of the agonist reference standard 17 β -estradiol should be prepared. This control sample is indicated as the vehicle control. Dimethylsulfoxide (DMSO, 1% (v/v); CASRN 67-68-5) was used as vehicle during the validation of the ER α CALUX bioassay. If a solvent other than DMSO is used, all reference standards, controls, and test chemicals should be tested in the same vehicle.

Reference standards

26. The agonistic reference standard is 17 β -estradiol (Table 1). The reference standards comprise a series of dilutions of eight concentrations of 17 β -estradiol (1×10^{-13} , 3×10^{-13} , 1×10^{-12} , 3×10^{-12} , 6×10^{-12} , 1×10^{-11} , 3×10^{-11} , 1×10^{-10} M).
27. The antagonistic reference standard is tamoxifen (Table 2). The reference standards comprise a series of dilutions of eight concentrations of tamoxifen (3×10^{-9} , 1×10^{-8} , 3×10^{-8} , 1×10^{-7} , 3×10^{-7} , 1×10^{-6} , 3×10^{-6} , 1×10^{-5} M). Each of the concentrations of the antagonistic reference standard is co-incubated with a fixed concentration of the agonistic reference standard 17 β -estradiol (3×10^{-12} M).

Positive control

28. The positive control for agonistic studies is 17 α -methyltestosterone (Table 1).

29. The positive control for antagonistic studies is 4-hydroxytamoxifen (Table 2). The antagonistic positive control is co-incubated with a fixed concentration of the agonistic reference standard 17 β -estradiol (3×10^{-12} M).

Negative control

30. The negative control for agonistic studies is corticosterone (Table 1).
31. The negative control for antagonistic studies is resveratrol (Table 2). The antagonistic negative control is co-incubated with a fixed concentration of the agonistic reference standard 17 β -estradiol (3×10^{-12} M).

Demonstration of laboratory proficiency (see paragraph 14 and Tables 3 and 4 in «ER TA ASSAY COMPONENTS» of this test method)

Vehicle

32. The solvent used to dissolve test chemicals should solubilise the test chemical completely and should be miscible with the cell medium. DMSO, water and ethanol (95% to 100% purity) are suitable solvents. In case DMSO is used as solvent, the maximum concentration of DMSO during incubation should not exceed 1% (v/v). Prior to use, the solvent should be tested for absence of cytotoxicity and interference with the assays performance.

Preparation of reference standards, positive controls, negative controls and test chemicals

33. Reference standards, positive controls, negative controls and test chemicals are dissolved in 100% DMSO (or an appropriate solvent). Appropriate (serial) dilutions should then be prepared in the same solvent. Before being dissolved, all substances should be allowed to equilibrate to room temperature. Freshly prepared stock solutions of reference standards, positive controls, negative controls and test chemicals should not have noticeable precipitate or cloudiness. Reference standard and control stocks may be prepared in bulk. Stock solutions of test chemicals should be prepared fresh before each experiment. Final dilutions of reference standards, positive controls, negative controls and test chemicals should be prepared for each experiment fresh and used within 24 hours of preparation.

Solubility, cytotoxicity and range finding.

34. During the prescreen run, the solubility of the test chemicals in the solvent of choice is determined. A maximum stock concentration of 0.1 M is prepared. In case this concentration shows solubility problems, lower stock solutions should be prepared until test chemicals are fully solubilised. During the prescreen run, 1:10 serial dilutions of test chemical are tested. The maximum assay concentration for agonist or antagonist testing is 1 mM. Following prescreening, an appropriate refined concentration range for test chemicals is derived that should be tested during the comprehensive runs. The dilutions used for comprehensive testing should be 1x, 3x, 10x, 30x, 100x, 300x, 1000x and 3000x.

35. Cytotoxicity testing is included in the agonist and antagonist assay protocol (11). Cytotoxicity testing is incorporated in both the prescreen run and comprehensive runs. The method used to assess cytotoxicity during the validation of the ER α CALUX bioassay was the lactate dehydrogenase (LDH) leakage test in combination with qualitative visual inspection of cells (see Appendix 4.1) following exposure to test chemicals. However, other quantitative methods for the determination of cytotoxicity (e.g. tetrazolium-based colorimetric (MTT) assay or cytotoxicity CALUX bioassay) can be used. In general, test chemical concentrations that show more than 20% reduction of cell viability are considered cytotoxic and therefore cannot be used for data evaluation. With respect to the LDH leakage assay, the concentration of the test chemical is regarded cytotoxic when the percentage LDH leakage is higher than 120%.

Test chemical exposure and assay plate organisation

36. Following trypsination of a confluent flask of cultured cells, cells are re-suspended at 1×10^5 cells/ml in estrogen free assay medium. Hundred μ l of re-suspended cells are plated in the inner-wells of a 96-well microtiter plate. The outer wells are filled with 200 μ l of Phosphate Buffered Saline (PBS) (see Figures 1 and 2). The plated cells are pre-incubated for 24 hours in a CO₂ incubator (5% CO₂, 37°C, 100% humidity).
37. After pre-incubation, the plates are inspected for visual cytotoxicity (see Appendix 4.1), contamination and confluence. Only plates that show no visual cytotoxicity, contamination and have a minimum of 85% confluence are used for testing. The medium from the inner wells is carefully removed and replaced by 200 μ l of estrogen free assay medium containing appropriate dilutions series of reference standards, test chemicals, positive controls, negative controls and solvent controls (Table 5: agonist studies; Table 6: antagonist studies). All reference standards, test chemicals, positive controls, negative controls and solvent controls are tested in triplicate. In Figure 1, the plate layout for agonist testing is given. In Figure 2, the plate layout for antagonist testing is given. The plate layout for prescreen testing and comprehensive testing is identical. For antagonist testing, all inner-wells, except for the vehicle control wells (VC), also contain a fixed concentration of agonist reference standard 17 β -estradiol (3×10^{-12} M). Note that reference standards C8 and C4 should be added to each TC plate.
38. Following exposure of the cells to all chemicals, the 96-well microtiter plates should be incubated for another 24 hours in a CO₂ incubator (5% CO₂, 37°C, 100% humidity).

Figure 1: Plate layout of the 96-well microtiter plates for prescreening and assessment of agonistic effect.

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		C0	C1	C2	C3	C4	C5	C6	C7	C8	PC	

C		C0	C1	C2	C3	C4	C5	C6	C7	C8	PC	
D		C0	C1	C2	C3	C4	C5	C6	C7	C8	PC	
E		SC	TC1-1	TC1-2	TC1-3	TC1-4	TC1-5	TC1-6	TC1-7	TC1-8	NC	
F		SC	TC1-1	TC1-2	TC1-3	TC1-4	TC1-5	TC1-6	TC1-7	TC1-8	NC	
G		SC	TC1-1	TC1-2	TC1-3	TC1-4	TC1-5	TC1-6	TC1-7	TC1-8	NC	
H												

Subsequent plates

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		SC	TC2-1	TC2-2	TC2-3	TC2-4	TC2-5	TC2-6	TC2-7	TC2-8	C8 (max)	
C		SC	TC2-1	TC2-2	TC2-3	TC2-4	TC2-5	TC2-6	TC2-7	TC2-8	C8 (max)	
D		SC	TC2-1	TC2-2	TC2-3	TC2-4	TC2-5	TC2-6	TC2-7	TC2-8	C8 (max)	
E		SC	TCx-1	TCx-2	TCx-3	TCx-4	TCx-5	TCx-6	TCx-7	TCx-8	C4 (EC50)	
F		SC	TCx-1	TCx-2	TCx-3	TCx-4	TCx-5	TCx-6	TCx-7	TCx-8	C4 (EC50)	
G		SC	TCx-1	TCx-2	TCx-3	TCx-4	TCx-5	TCx-6	TCx-7	TCx-8	C4 (EC50)	
H												

C0 = reference standard solvent.

C(1-8) = series of dilutions (1-8, low-to-high concentrations) of reference standard.

PC = positive control.

NC = negative control.

TCx-(1-8) = dilutions (1-8, low-to-high concentrations) of test chemical for the prescreen run and assessment of agonistic effect of test chemical x.

SC = solvent control of the test chemical (optimally the same solvent as in C0, but possibly from another batch).

Grey cells: = Outer wells, filled up with 200 µl of PBS.

Figure 2: Plate layout of the 96-well microtiter plates for antagonistic prescreening and assessment of antagonistic effect.

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		C0	C1	C2	C3	C4	C5	C6	C7	C8	VC	
C		C0	C1	C2	C3	C4	C5	C6	C7	C8	VC	
D		C0	C1	C2	C3	C4	C5	C6	C7	C8	VC	
E		NC	TC1-1	TC1-2	TC1-3	TC	TC1-5	TC1-6	TC1-7	TC1-8	PC	
F		NC	TC1-1	TC1-2	TC1-3	TC	TC1-5	TC1-6	TC1-7	TC1-8	PC	
G		NC	TC1-1	TC1-2	TC1-3	TC	TC1-5	TC1-6	TC1-7	TC1-8	PC	
H												

Subsequent plates

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		SC	TC2-1	TC2-2	TC2-3	TC2-4	TC2-5	TC2-6	TC2-7	TC2-8	C8 (max)	
C		SC	TC2-1	TC2-2	TC2-3	TC2-4	TC2-5	TC2-6	TC2-7	TC2-8	C8 (max)	
D		SC	TC2-1	TC2-2	TC2-3	TC2-4	TC2-5	TC2-6	TC2-7	TC2-8	C8 (max)	
E		C4 (IC50)	TCx-1	TCx-2	TCx-3	TCx-4	TCx-5	TCx-6	TCx-7	TCx-8	C8 (max)	
F		C4 (IC50)	TCx-1	TCx-2	TCx-3	TCx-4	TCx-5	TCx-6	TCx-7	TCx-8	C8 (max)	
G		C4 (IC50)	TCx-1	TCx-2	TCx-3	TCx-4	TCx-5	TCx-6	TCx-7	TCx-8	C8 (max)	
H												

- C0 = reference standard solvent.
- C(1-8) = series of dilutions (1-8, low-to-high concentrations) of reference standard.
- NC = negative control.
- PC = positive control.
- TCx-(1-8) = dilutions (1-8, low-to-high concentrations) of test chemical for the prescreen run and assessment of agonistic effect of test chemical x.
- SC = solvent control of the test chemical (optimally the same solvent as in C0, but possibly from another batch).
- VC = vehicle control (solvent control without fixed concentration of agonist reference standard 17 β -estradiol).
- Grey cells: = Outer wells, filled up with 200 μ l of PBS.
- Note: all inner-wells, except for the vehicle control wells (VC), also contain a fixed concentration of agonist reference standard 17 β -estradiol (3.0*10⁻¹² M)

Measurement of luminescence

39. The measurement of luminescence is described in detail in the agonist and antagonist assay protocol (10). The medium from the wells should be removed and the cells should be lysed following 24 hours of incubation in order to open up the cell membrane and allow measurement of luciferase activity.
40. For measuring the luminescence, this procedure requires a luminometer equipped with 2 injectors. The luciferase reaction is started by injection of the substrate luciferin. The reaction is stopped by addition of 0.2 M NaOH. The reaction is stopped to prevent carry over of luminescence from one well to the other.
41. Light emitted from each well is expressed as Relative Light Units (RLUs) per well.

Prescreen run

42. The prescreen analysis results are used to determine a refined concentration-range of test chemicals for comprehensive testing. Evaluation of prescreen analysis results and the determination of the refined concentration-range of test chemicals for comprehensive testing, is described in depth in the agonist and antagonist assay protocol (10). Here, a brief summary of the procedures for determining the concentration range of test chemicals for agonist and antagonist testing, is given. See Tables 5 and 6 for guidance of serial dilution design.

Selection of concentrations for assessment of agonistic effects

43. During the prescreen run, test chemicals should be tested using the series of dilutions as indicated in Tables 5 (agonism) and 6 (antagonism). All concentrations should be tested in triplicate wells according to the plate layout as indicated in Figure 1 (agonism) or 2 (antagonism).
44. Only analysis results that fulfil the acceptance criteria (Table 3) are considered valid and can be used to evaluate the response of test chemicals. In case one or more microtiter plates in an

analysis series fail to fulfil the acceptance criteria, the respective microtiterplates should be re-analysed. In case the first plate containing the complete series of dilutions of the reference standard fails the acceptance criteria, the complete test series (6 plates) have to be re-analysed.

45. Initial concentration ranges of test chemicals should be adjusted and the prescreen run should be repeated in case:
- cytotoxicity is observed. The prescreen procedure should be repeated with lower non-cytotoxic concentrations of the test chemical.
 - the prescreen of the test chemical does not show a full dose-response curve because the concentrations tested generate maximum induction. The prescreen run should be repeated using lower concentrations of the test chemical.
46. When a valid dose-related response is observed, the (lowest) concentration at which maximum induction is observed and does not show cytotoxicity, should be selected. The highest concentration of the test chemical to be tested in the comprehensive runs, should be 3-times this selected concentration.
47. A complete refined dilution series of the test chemical should be prepared with dilutions steps as indicated in Table 5, starting with the highest concentration as determined above.
48. A test chemical that does not elicit any agonistic effect, should be tested in the comprehensive runs starting with the highest, non-cytotoxic concentration identified during prescreening.

Selection of concentrations for assessment of antagonistic effects

49. Only analysis results that fulfil the acceptance criteria (Table 4) are considered valid and can be used to evaluate the response of test chemicals. In case one or more microtiter plates in an analysis series fail to fulfil the acceptance criteria, the respective microtiterplates should be re-analysed. In case the first plate containing the complete series of dilutions of the reference standard fails the acceptance criteria, the complete test series (6 plates) have to be re-analysed.
50. Initial concentration ranges of test chemicals should be adjusted and the prescreen run should be repeated in case:
- cytotoxicity is observed. The prescreen procedure should be repeated with lower non-cytotoxic concentrations of the test chemical.
 - the prescreen of the test chemical does not show a full dose-response curve because the concentrations tested generate maximum inhibition. The prescreen should be repeated using lower concentrations of the test chemical.

51. When a valid dose-related response is found, the (lowest) concentration at which maximum inhibition is observed and does not show cytotoxicity, should be selected. The highest concentration of the test chemical to be tested in the comprehensive runs, should be 3-times this selected concentration.
52. A complete refined dilution series of the test chemical should be prepared with the dilutions steps as indicated in Table 6, starting with the highest concentration as determined above.
53. Test chemicals that do not elicit any antagonistic effects, should be tested in the comprehensive runs starting with the highest, non-cytotoxic concentration tested during prescreening.

Comprehensive runs

54. Following the selection of the refined concentration ranges, test chemicals should be tested comprehensively using the series of dilutions as indicated in Tables 5 (agonism) and 6 (antagonism). All concentrations should be tested in triplicate wells according to the plate layout as indicated in Figure 1 (agonism) or 2 (antagonism).
55. Only analysis results that fulfil the acceptance criteria (Table 3 and 4) are considered valid and can be used to evaluate the response of test chemicals. In case one or more microtiter plates in an analysis series fail to fulfil the acceptance criteria, the respective microtiterplates should be re-analysed. In case the first plate containing the complete series of dilutions of the reference standard fails the acceptance criteria, the complete test series (6 plates) have to be re-analysed.

Table 5: Concentration and dilutions of reference standards, controls and test chemicals used for agonist testing

Reference 17 β -estradiol conc. (M)		TCx - prescreen run dilution		TCx - comprehensive run dilution		Controls conc. (M)	
C0	0	TCx-1	10,000,000 x	TCx-1	3,000 x	PC	3*10 ⁻⁶
C1	1*10 ⁻¹³	TCx-2	1,000,000 x	TCx-2	1,000 x	NC	1*10 ⁻⁸
C2	3*10 ⁻¹³	TCx-3	100,000 x	TCx-3	300 x	C0	0
C3	1*10 ⁻¹²	TCx-4	10,000 x	TCx-4	100 x	SC	0
C4	3*10 ⁻¹²	TCx-5	1,000 x	TCx-5	30 x		
C5	6*10 ⁻¹²	TCx-6	100 x	TCx-6	10 x		
C6	1*10 ⁻¹¹	TCx-7	10 x	TCx-7	3 x		
C7	3*10 ⁻¹¹	TCx-8	1 x	TCx-8	1 x		
C8	1*10 ⁻¹⁰						

TCx - test chemical x

PC - positive control (17 α -methyltestosterone)

NC - negative control (corticosterone)

C0 - reference standard solvent control

SC - test chemical solvent control

Table 6: Concentration and dilutions of reference standards, controls and test chemicals used for antagonist testing

Reference tamoxifen conc. (M)		TCx - prescreen run dilution		TCx - comprehensive run dilution		Controls conc. (M)	
C0	0	TCx-1	10,000,000 x	TCx-1	3,000 x	PC	1*10 ⁻⁹
C1	3*10 ⁻⁹	TCx-2	1,000,000 x	TCx-2	1,000 x	NC	1*10 ⁻⁵
C2	1*10 ⁻⁸	TCx-3	100,000 x	TCx-3	300 x	C0	0
C3	3*10 ⁻⁸	TCx-4	10,000 x	TCx-4	100 x	SC	0
C4	1*10 ⁻⁷	TCx-5	1,000 x	TCx-5	30 x	Supplemented agonist conc. (M)	3*10 ⁻¹²
C5	3*10 ⁻⁷	TCx-6	100 x	TCx-6	10 x		
C6	1*10 ⁻⁶	TCx-7	10 x	TCx-7	3 x		
C7	3*10 ⁻⁶	TCx-8	1 x	TCx-8	1 x	17β- estradiol	
C8	1*10 ⁻⁵						

TCx - test chemical x

PC - positive control (4-hydroxytamoxifen)

NC - negative control (resveratrol)

C0 - reference standard solvent control

SC - test chemical solvent control

VC - vehicle control (does not contain fixed concentration of the agonistic reference standard 17β-estradiol (3.0*10⁻¹² M))

Collection of data and data analysis

56. Following the prescreen and comprehensive runs, the EC₁₀, EC₅₀, PC₁₀, PC₅₀ and maximum induction (TCX_{max}) of a test chemical should be determined for agonistic testing. For antagonistic testing, the IC₂₀, IC₅₀, PC₈₀, PC₅₀ and minimum induction (TCX_{min}) should be calculated. In Figure 3 (agonism) and 4 (antagonism), a graphical representation of these parameters is given. The required parameters are calculated based on the relative induction of each test chemical (relative to the maximum induction of the reference standard (=100%)). Non-linear regression (variable slope, 4 parameters) should be used for evaluation of data according to the following equation:

$$Y = Bottom + \frac{(Top - Bottom)}{(1 + 10^{((lgEC_{50} - X) * Hill Slope)})}$$

Where:

X = Log of dose or concentration

Y = Response (relative induction (%))

Top = Maximum induction (%)

Bottom	=	Minimum induction (%)
LogEC ₅₀	=	Log of concentration at which 50% of maximum response is observed
HillSlope	=	Slope factor or Hill slope

57. Raw data from the luminometer, expressed as Relative Light Units (RLUs), should be transferred to the data analysis spreadsheet designed for the prescreen and comprehensive runs. Raw data should meet the acceptance criteria as indicated in Table 3A and 3B (agonism) or 4A and 4B (antagonism). In case the raw data meet the acceptance criteria, the following calculation steps are performed to determine the required parameters:

Agonism

- Subtract the average RLU of the reference standard solvent control from each of the raw analysis data of the reference standards.
- Subtract the average RLU for the test chemical solvent control from each of the raw analysis data of the test chemicals.
- Calculate the relative induction of each concentration of the reference standard. Set the induction of the highest concentration of the reference standard at 100%.
- Calculate the relative induction of each concentration of test chemical compared to the highest concentration of the reference standard as 100%.
- Evaluate the analysis results following non-linear regression (variable slope, 4 parameters).
- Determine the EC₅₀ and EC₁₀ of the reference standard.
- Determine the EC₅₀ and EC₁₀ of the test chemicals.
- Determine the maximum relative induction of the test chemical (TC_{max}).
- Determine the PC₁₀ and PC₅₀ of the test chemicals.

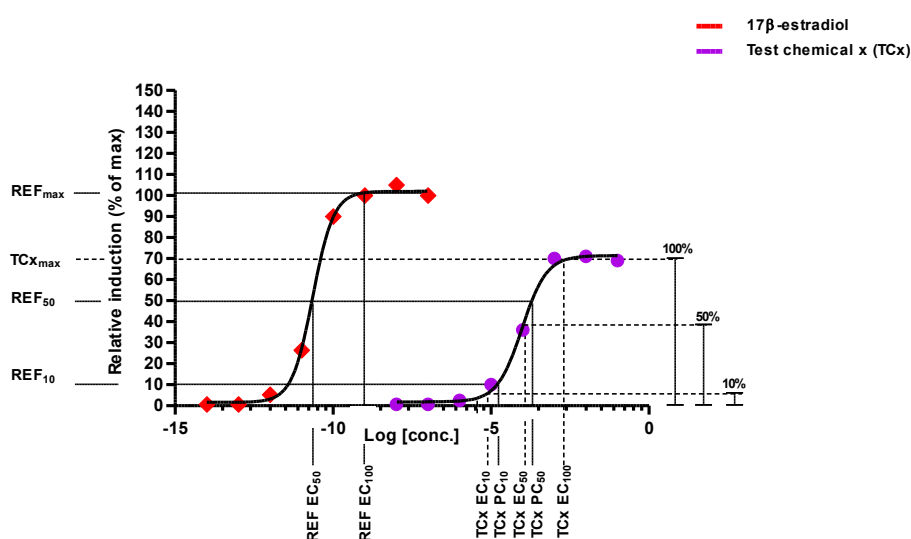
For test chemicals, a full dose-response curve may not always be achieved due to e.g. cytotoxicity or solubility problems. Hence, the EC₅₀, EC₁₀ and PC₅₀ cannot be determined. In such case, only the PC₁₀ and TC_{max} can be determined.

Antagonism

- Subtract the average RLU of the highest reference standard concentration from each of the raw analysis data of the reference standard s.
- Subtract the average RLU of the highest reference standard concentration from each of the raw analysis data of the test chemicals.
- Calculate the relative induction of each concentration of the reference standard. Set the induction of the lowest concentration of the reference standard at 100%.

- Calculate the relative induction of each concentration of test chemical compared to the lowest concentration of the reference standard as 100%.
- Evaluate the analysis results following non-linear regression (variable slope, 4 parameters).
- Determine the IC_{50} and IC_{20} of the reference standard.
- Determine the IC_{50} and IC_{20} of the test chemicals.
- Determine the minimum relative induction of the test chemical (TC_{min}).
- Determine the PC_{80} and PC_{50} of the test chemicals.

Figure 3: Overview of parameters determined in the agonist assay



EC_{10} = concentration of a substance at which 10% of its maximum response is observed.

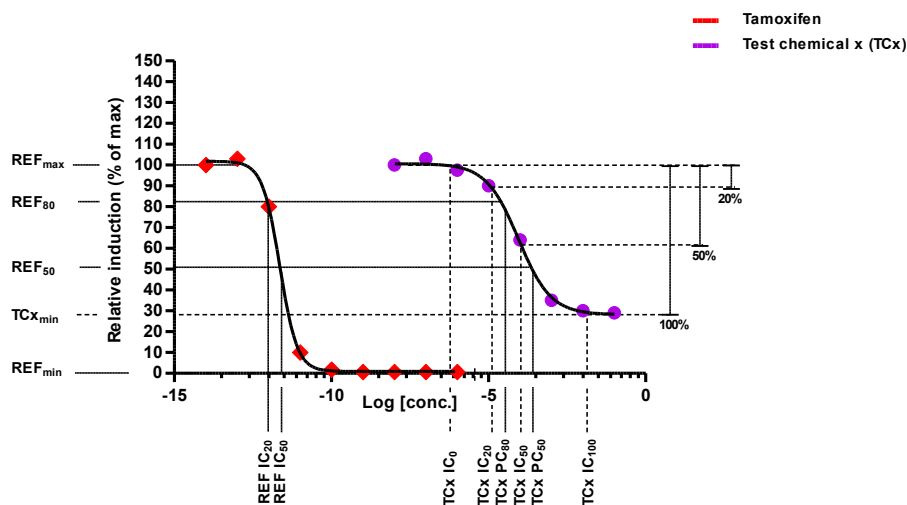
EC_{50} = concentration of a substance at which 50% of its maximum response is observed.

PC_{10} = concentration of a test chemical at which its response is equal to the EC_{10} of the reference standard.

PC_{50} = concentration of a test chemical at which its response is equal to the EC_{50} of the reference standard.

$TC_{x_{max}}$ = maximum relative induction of test chemical.

Figure 4: Overview of parameters determined in the antagonist assay



IC₂₀ = concentration of a substance at which 80% of its maximum response is observed (20% inhibition).

IC₅₀ = concentration of a substance at which 50% of its maximum response is observed (50% inhibition).

PC₈₀ = concentration of a test chemical at which its response is equal to the IC₂₀ of the reference standard.

PC₅₀ = concentration of a test chemical at which its response is equal to the IC₅₀ of the reference standard.

TC_{xmin} = minimum relative induction of test chemical.

For test chemicals, a full dose-response curve may not always be achieved due to e.g. cytotoxicity or solubility problems. Hence, the IC₅₀, IC₂₀ and PC₅₀ cannot be determined. In such case, only the PC₂₀ and TC_{min} can be determined.

58. The results should be based on two (or three) independent runs. If two runs give comparable and therefore reproducible results, it is not necessary to conduct a third run. To be acceptable, the results should:

- Meet the acceptability criteria (see Acceptability criteria paragraphs 14-22),
- Be reproducible.

Data interpretation criteria

59. For the interpretation of data and the decision whether a test chemical is considered positive or negative, the following criteria are to be used:

Agonism

For each comprehensive run, a test chemical is considered **positive** in case:

- 1 The TC_{max} is equal or exceeds 10% of the maximum response of the reference standard (REF_{10}).
- 2 At least 2 consecutive concentrations of the test chemical are equal to or exceed the REF_{10} .

For each comprehensive run, a test chemical is considered **negative** in case:

- 1 The TC_{max} does not exceed 10% of the maximum response of the reference standard (REF_{10}).
- 2 Less than 2 concentrations of the test chemical are equal to or exceed the REF_{10} .

Antagonism

For each comprehensive run, a test chemical is considered **positive** in case:

- 1 The TC_{min} is equal or lower than 80% of the maximum response of the reference standard ($REF_{80} = 20\%$ inhibition).
- 2 At least 2 consecutive concentrations of the test chemical are equal to or lower than the REF_{80} .

For each comprehensive run, a test chemical is considered **negative** in case:

- 1 The TC_{min} exceeds 80% of the maximum response of the reference standard ($REF_{80} = 20\%$ inhibition).
- 2 Less than 2 concentrations of the test chemical are equal to or lower than the REF_{80} .

60. To characterise the potency of the positive response of a test chemical, the magnitude of the effect (agonism: TC_{max} ; antagonism: TC_{min}) and the concentration at which the effect occurs (agonism: EC_{10} , EC_{50} , PC_{10} , PC_{50} ; antagonism: IC_{20} , IC_{50} , PC_{80} , PC_{50}) should be reported.

TEST REPORT

61. See paragraph 20 of “ER TA ASSAY COMPONENTS”

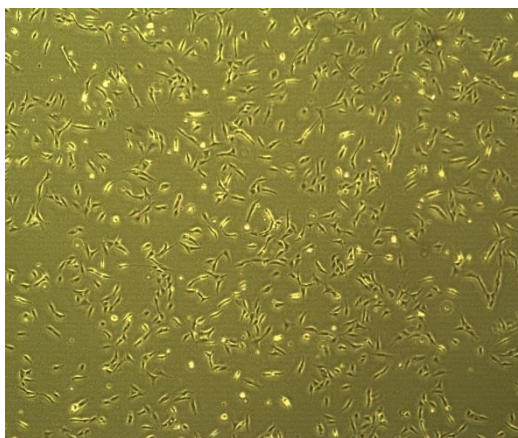
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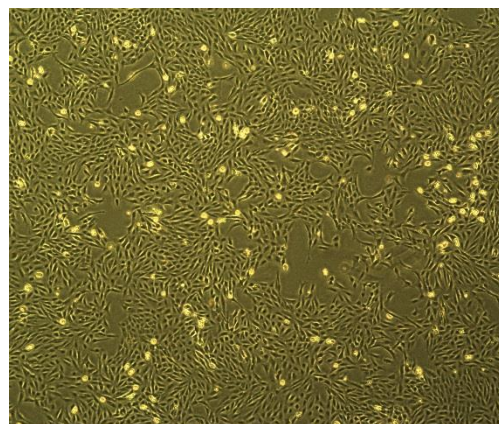
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Appendix 4.1: Visual inspection of cell viability

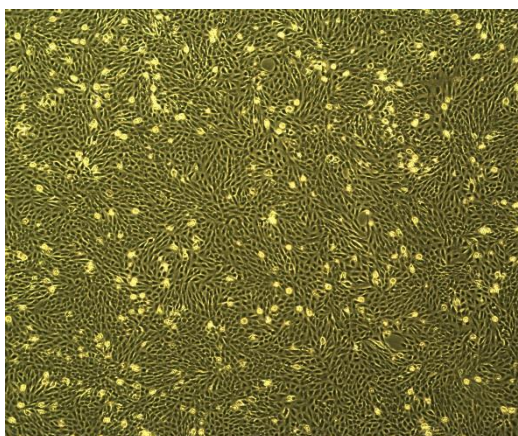


<5% confluency. Cells have just been seeded.
100% cell viability.
Classification: “no cytotoxicity”



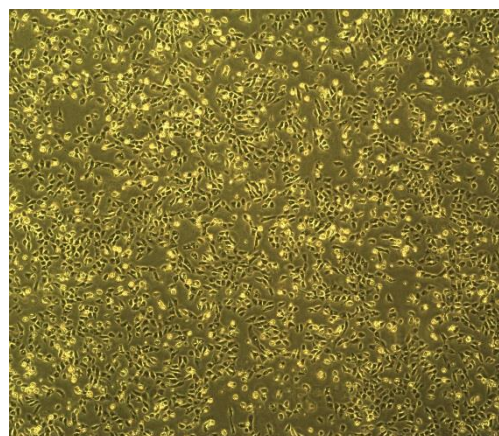
> 85% confluency. At this stage, cells are
exposed to test chemicals. > 95% cell viability.

Classification: “no cytotoxicity”

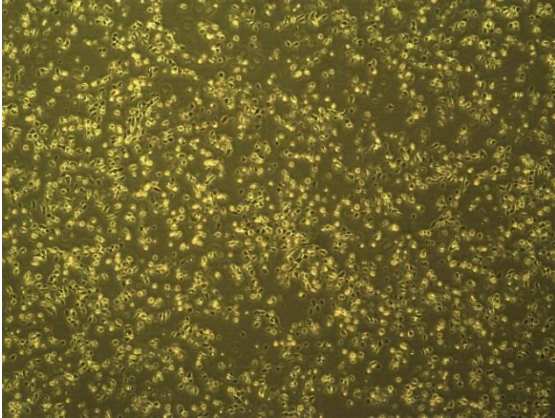


> 95% confluency. Cells are densely packed and
start to overgrow. > 95% cell viability.

Classification: “no cytotoxicity”



< 25% cell viability. Cells become detached and
contact between cells decreases. Cells are
rounded. Classification: “cytotoxicity”



< 5% cell viability. Cells are fully detached and contact between cells is broken. Cells are rounded. Classification: "cytotoxicity"

B.67 *IN VITRO* MAMMALIAN CELL GENE MUTATION TESTS USING THE THYMIDINE KINASE GENE

INTRODUCTION

1. This test method (TM) is equivalent to the OECD test guideline 490 (2016). Test methods are periodically reviewed and revised in the light of scientific progress, regulatory needs and animal welfare. The mouse lymphoma assay (MLA) and TK6 test using the thymidine kinase (TK) locus were originally contained in test method B.17. Subsequently, the MLA Expert Workgroup of the International Workshop for Genotoxicity Testing (IWGT) has developed internationally harmonised recommendations for assay acceptance criteria and data interpretation for the MLA (1)(2)(3)(4)(5), and these recommendations are incorporated into this new test method B.67. This test method is written for the MLA and, because it also utilises the TK locus, the TK6 test. While the MLA has been widely used for regulatory purposes, the TK6 has been used much less frequently. It should be noted that in spite of the similarity between the endpoints the two cell lines are not interchangeable and regulatory programs may validly express a preference for one over the other for a particular regulatory use. For instance, the validation of the MLA demonstrated its appropriateness for detecting not only gene mutation, but also, the ability of a test chemical to induce structural chromosomal damage. This test method is part of a series of test methods on genetic toxicology. A document that provides succinct information on genetic toxicology testing and an overview of the recent changes that were made to genetic toxicity OECD test guidelines has been developed by OECD (6).
2. The purpose of the *in vitro* mammalian cell gene mutation tests is to detect gene mutations induced by chemicals. The cell lines used in these tests measure forward mutations in reporter genes, specifically the endogenous thymidine kinase gene (*TK* for human cells and *Tk* for rodent cells, collectively referred to as *TK* in this test method). This test method is intended for use with two cell lines: the L5178Y TK^{+/-}-3.7.2C mouse lymphoma cell line (generally called L5178Y) and the TK6 human lymphoblastoid cell line (generally called TK6). Although the two cell lines vary because of their origin, cell growth, p53-status, etc., the *TK* gene mutation tests can be conducted in a similar way in both cell types as described in this test method.
3. The autosomal and heterozygous nature of the thymidine kinase gene enables the detection of viable colonies whose cells are deficient in the enzyme thymidine kinase following mutation from *TK*^{+/-} to *TK*^{-/-}. This deficiency can result from genetic events affecting the *TK* gene including both gene mutations (point mutations, frame-shift mutations, small deletions, etc.) and chromosomal events (large deletions,

chromosome rearrangements and mitotic recombination). The latter events are expressed as loss of heterozygosity, which is a common genetic change of tumor suppressor genes in human tumorigenesis. Theoretically, loss of the entire chromosome carrying the *TK* gene resulting from spindle impairment and/or mitotic non-disjunction can be detected in the MLA. Indeed, a combination of cytogenetic and molecular analysis clearly shows that some MLA TK mutants are the result of nondisjunction. However, the weight of evidence shows that the *TK* gene mutation tests cannot reliably detect aneuploids when applying standard cytotoxicity criteria (as described in this test method) and therefore, it is not appropriate to use these tests to detect aneuploids (7)(8)(9).

4. In the *TK* gene mutation tests, two distinct phenotypic classes of *TK* mutants are generated; the normal growing mutants that grow at the same rate as the TK heterozygous cells, and slow growing mutants which grow with prolonged doubling times. The normal growing and slow growing mutants are recognised as large colony and small colony mutants in the MLA and as early appearing colony and late appearing colony mutants in the TK6. The molecular and cytogenetic nature of both large and small colony MLA mutants has been explored in detail (8)(10)(11)(12)(13). The molecular and cytogenetic nature of the early appearing and late appearing TK6 mutants has also been extensively investigated (14)(15)(16)(17). Slow growing mutants for both cell types have suffered genetic damage that involves putative growth regulating gene(s) near the *TK* locus which results in prolonged doubling times and the formation of late appearing or small colonies (18). The induction of slow growing mutants has been associated with chemicals that induce gross structural changes at the chromosomal level. Cells whose damage does not involve the putative growth regulating gene(s) near the TK locus grow at rates similar to the parental cells and become normal growing mutants. The induction of primarily normal growing mutants is associated with chemicals primarily acting as point mutagens. Consequently it is essential to count both slow growing and normal growing mutants in order to recover all of the mutants and to provide some insight into the type(s) of damage (mutagens vs. clastogens) induced by the test chemical (10)(12)(18)(19).
5. The test method is organised so as to provide general information that applies to both MLA and TK6 and specialised guidance for the individual tests.
6. Definitions used are provided in Appendix 1.

INITIAL CONSIDERATIONS AND LIMITATIONS

7. Tests conducted *in vitro* generally require the use of an exogenous source of metabolic activation. The exogenous metabolic activation system does not entirely mimic *in vivo* conditions.

8. Care should be taken to avoid conditions that could lead to artefactual positive results (i.e. possible interaction with the test system) not caused by interaction between the test chemical and the genetic material of the cell; such conditions include changes in pH or osmolality, interaction with the medium components (20)(21), or excessive levels of cytotoxicity (22)(23)(24). Cytotoxicity exceeding the recommended top cytotoxicity levels as defined in paragraph 28 is considered excessive for the MLA and TK6. In addition, it should be noted that test chemicals that are thymidine analogues, or behave like thymidine analogues can increase the mutant frequency by selective growth of the spontaneous background mutants during cell treatment and require additional test methods for adequate evaluation (25).
9. For manufactured nanomaterials, specific adaptations of this test method may be needed but are not described in this test method.
10. Before using the test method for testing a mixture to generate data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed, when there is a regulatory requirement for testing the mixture.
11. Mutant cells deficient in thymidine kinase enzyme activity because of a mutation $TK^{+/-}$ to $TK^{-/-}$ are resistant to the cytostatic effects of the pyrimidine analogue trifluorothymidine (TFT). The TK proficient cells are sensitive to TFT, which causes the inhibition of cellular metabolism and halts further cell division. Thus, mutant cells are able to proliferate in the presence of TFT and form visible colonies, whereas cells containing the TK enzyme are not.

PRINCIPLE OF THE TEST

12. Cells in suspension are exposed to the test chemical, both with and without an exogenous source of metabolic activation (see paragraph 19), for a suitable period of time (see paragraph 33), and then sub-cultured to determine cytotoxicity and to allow phenotypic expression prior to mutant selection. Cytotoxicity is determined by relative total growth (RTG—see paragraph 25) for the MLA and by relative survival (RS—see paragraph 26) for TK6. The treated cultures are maintained in growth medium for a sufficient period of time, characteristic of each cell type (see paragraph 37), to allow near-optimal phenotypic expression of induced mutations. Following phenotypic expression, mutant frequency is determined by seeding known numbers of cells in medium containing the selective agent to detect mutant colonies, and in medium without selective agent to determine the cloning efficiency (viability). After a suitable incubation time, colonies are counted. Mutant frequency is calculated based on the number of mutant colonies corrected by the cloning efficiency at the time of mutant selection.

DESCRIPTION OF THE METHOD

Preparations

Cells

13. For MLA: Because the MLA was developed and characterised using the $TK^{+/-}$ -3.7.2C subline of L5178Y cells, this specific subline has to be used for the MLA. The L5178Y cell line was derived from a methylcholanthrene-induced thymic lymphoma from a DBA-2 mouse (26). Clive and co-workers treated L5178Y cells (designated by Clive as $TK^{+/+}$ -3) with ethylmethane sulfonate and isolated a $TK^{-/-}$ (designated as $TK^{-/-}$ -3.7) clone using bromodeoxyuridine as the selective agent. From the $TK^{-/-}$ clone a spontaneous $TK^{+/-}$ clone (designated as $TK^{+/-}$ -3.7.2.) and a subclone (designated as $TK^{+/-}$ -3.7.2C) were isolated and characterised for use in the MLA (27). The karyotype for the cell line has been published (28)(29)(30)(31). The modal chromosome number is 40. There is one metacentric chromosome (t12;13) that should be counted as one chromosome. The mouse *TK* locus is located on the distal end of chromosome 11. The L5178Y $TK^{+/-}$ -3.7.2C cell line has mutations in both p53 alleles and produces mutant-p53 protein (32) (33). The p53 status of the $TK^{+/-}$ -3.7.2C cell line is likely responsible for the ability of the test to detect large-scale damage (17).
14. For TK6: The TK6 is a human lymphoblastoid cell line. The parent cell line is an Epstein-Barr virus-transformed cell line, WI-L2, which was originally derived from a 5-year-old male with hereditary spherocytosis. The first isolated clone, HH4, was mutagenised with ICR191 and a *TK* heterozygous cell line, TK6, was generated (34). TK6 cells are nearly diploid and the representative karyotype is 47, XY, 13+, t(14; 20), t(3; 21) (35). The human *TK* locus is located on the long arm of chromosome 17. The TK6 is a p53-competent cell line, because it has a wild-type p53 sequence in both alleles and expresses only wild-type p53 protein (36).
15. For both the MLA and the TK6, when first establishing or replenishing a master stock, it is advisable for the testing laboratory to assure the absence of *Mycoplasma* contamination, karyotype the cells or paint the chromosomes harboring the *TK* locus, and to check population doubling times. The normal cell cycle time for the cells used in the testing laboratory should be established and should be consistent with published cell characteristics (16)(19)(37). This master stock should be stored at -150° C or below and used to prepare all working cell stocks.
16. Either prior to establishing a large number of cryopreserved working stocks or just prior to use in an experiment, the culture may need to be cleansed of pre-existing mutant cells [unless the solvent control mutant frequency (MF) is already within the acceptable range—see Table 2 for the MLA)]. This is accomplished using methotrexate (aminopterin) to select against TK-deficient cells and adding thymidine, hypoxanthine and glycine (L5178Y) or 2'-deoxycytidine (TK6) to the culture to

ensure optimal growth of the TK-competent cells (19)(38)(39), and (40) for TK6). General advice on good practice for the maintenance of cell cultures as well as specific advice for L5178Y and TK6 cells can be found in (19)(31)(37)(39)(41). For laboratories requiring master cell stocks to initiate either the MLA or TK6 or to obtain new master cell stocks, a cell repository of well characterised cells is available (37).

Media and culture conditions

17. For both tests, appropriate culture medium and incubation conditions (e.g. culture vessels, humidified atmosphere of 5% CO₂, incubation temperature of 37°C) should be used for maintaining cultures. Cell cultures should always be maintained under conditions that ensure that they are growing in log phase. It is particularly important to choose media and culture conditions that ensure optimal growth of cells during the expression period and cloning for both mutant and non-mutant cells. For the MLA and the TK6, it is also important that the culture conditions ensure optimal growth of both the large colony/early appearing and the small colony/late appearing TK mutants. More culture details, including the need to properly heat inactivate horse serum if RPMI medium is used during mutant selection can be found in (19)(31)(38)(39)(40)(42).

Preparation of cultures

18. Cells are propagated from stock cultures, seeded in culture medium at a density such that the suspension cultures will continue to grow exponentially through the treatment and expression periods.

Metabolic activation

19. Exogenous metabolising systems should be used when employing L5178Y and TK6 cells because they have inadequate endogenous metabolic capacity. The most commonly used system that is recommended by default unless otherwise justified, is a co-factor-supplemented post-mitochondrial fraction (S9) prepared from the livers of rodents (generally rats) treated with enzyme-inducing agents such as Aroclor 1254 (43)(44)(45) or a combination of phenobarbital and β -naphthoflavone (46)(47)(48)(49)(50)(51). The latter combination does not conflict with the Stockholm Convention on Persistent Organic Pollutants (52) and has been shown to be as effective as Aroclor 1254 for inducing mixed-function oxidases (45)(46)(47)(48)(49). The S9 fraction typically is used at concentrations ranging from 1-2% but may be increased to 10% (v/v) in the final test medium. The choice of type and concentration of exogenous metabolic activation system or metabolic inducer employed may be influenced by the class of test chemicals.

Test chemical preparations

20. Solid test chemicals should be prepared in appropriate solvents and diluted, if appropriate, prior to treatment of the cells (see paragraph 21). Liquid test chemicals may be added directly to the test system and/or diluted prior to treatment of the test system. Gaseous or volatile test chemicals should be tested by appropriate modifications to the standard protocols, such as treatment in sealed culture vessels (53)(54)(55). Preparations of the test chemical should be made just prior to treatment unless stability data demonstrate the acceptability of storage.

TEST CONDITIONS

Solvents

21. The solvent should be chosen to optimise the solubility of the test chemical without adversely impacting the conduct of the test, e.g. changing cell growth, affecting the integrity of the test chemical, reacting with culture vessels, impairing the metabolic activation system. It is recommended that, wherever possible, the use of an aqueous solvent (or culture medium) should be considered first. Well established solvents are water or dimethyl sulfoxide. Generally organic solvents should not exceed 1% (v/v) and aqueous solvents (saline or water) should not exceed 10% (v/v) in the final treatment medium. If other than well-established solvents are used (e.g. ethanol or acetone), their use should be supported by data indicating their compatibility with the test chemicals, the test system and their lack of genetic toxicity at the concentration used. In the absence of that supporting data, it is important to add untreated controls (see Appendix 1, Definitions) to demonstrate that no deleterious or mutagenic effects are induced by the chosen solvent.

MEASURING CYTOTOXICITY AND CHOOSING TREATMENT CONCENTRATIONS

22. When determining the highest test chemical concentration, concentrations that have the capability of producing artefactual positive responses, such as those producing excessive cytotoxicity (see paragraph 28), precipitation (see paragraph 29) in the culture medium, or marked changes in pH or osmolality (see paragraph 8), should be avoided. If the test chemical causes a marked change in the pH of the medium at the time of addition, the pH might be adjusted by buffering the final treatment medium so as to avoid artefactual positive results and to maintain appropriate culture conditions.
23. Concentration selection is based on cytotoxicity and other considerations (see paragraphs 27-30). While the evaluation of cytotoxicity in an initial test may be useful to better define the concentrations to be used in the main experiment, an initial test is not required. Even if an initial cytotoxicity evaluation is performed, the measurement of cytotoxicity for each culture is still required in the main experiment. If a range

finding experiment is conducted, it should cover a wide range of concentrations and can either be terminated at day 1 after treatment or carried through the 2 day expression and to mutant selection (should it appear that the concentrations used are appropriate).

24. Cytotoxicity should be determined for each individual test culture and control culture: methods for MLA (2) and the TK6 (15) are defined by internationally agreed practice.
25. For both the agar and microwell versions of the MLA: Cytotoxicity should be evaluated using relative total growth (RTG) which was originally defined by Clive and Spector in 1975 (2). This measure includes the relative suspension growth (RSG: test culture vs. solvent control) during the cell treatment, the expression time and the relative cloning efficiency (RCE: test culture vs. solvent control) at the time that mutants are selected (2). It should be noted that the RSG includes any cell loss occurring in the test culture during treatment (See Appendix 2 for formulae).
26. For TK6: Cytotoxicity should be evaluated using relative survival (RS) i.e. cloning efficiency of cells plated immediately after treatment, adjusted for any cell loss during treatment, based on cell count as compared to the negative control (assigned a survival of 100%) (See Appendix 2 for the formula).
27. At least four test concentrations (not including the solvent and positive controls) that meet the acceptability criteria (appropriate cytotoxicity, number of cells, etc) should be evaluated. While the use of duplicate cultures is advisable, either replicate or single treated cultures may be used at each concentration tested. The results obtained for replicate cultures at a given concentration should be reported separately but can be pooled for the data analysis (55). For test chemicals demonstrating little or no cytotoxicity, concentration intervals of approximately 2 to 3 fold will usually be appropriate. Where cytotoxicity occurs, concentrations should be selected to cover the cytotoxicity range from that producing cytotoxicity as described in paragraph 28 and including concentrations at which there is moderate and little or no cytotoxicity. Many test chemicals exhibit steep concentration response curves and in order to cover the whole range of cytotoxicity or to study the concentration response in detail, it may be necessary to use more closely spaced concentrations and more than four concentrations, in particular in situations where a repeat experiment is required (see paragraph 70). The use of more than 4 concentrations may be particularly important when using single cultures.
28. If the maximum concentration is based on cytotoxicity, the highest concentration should aim to achieve between 20 and 10% RTG for the MLA, and between 20 and 10% RS for the TK6 (paragraph 67).

29. For poorly soluble test chemicals that are not cytotoxic at concentrations below the lowest insoluble concentration, the highest concentration analysed should produce turbidity or a precipitate visible by eye or with the aid of an inverted microscope at the end of the treatment with the test chemical. Even if cytotoxicity occurs above the lowest insoluble concentration, it is advisable to test at only one concentration producing turbidity or with a visible precipitate because artifactual effects may result from the precipitate. Because the MLA and TK6 use suspension cultures, particular care should be taken to assure that the precipitate does not interfere with the conduct of the test. The determination of solubility in the culture medium prior to the experiment may also be useful.
30. If no precipitate or limiting cytotoxicity is observed, the highest test concentration should correspond to 10 mM, 2 mg/ml or 2 µl/ml, whichever is the lowest (57)(58). When the test chemical is not of defined composition e.g. substance of unknown or variable composition, complex reaction products or biological materials [i.e. Chemical Substances of unknown or Variable Composition (UVCBs)], environmental extracts etc., the top concentration, may need to be higher (e.g. 5 mg/ml), in the absence of sufficient cytotoxicity, to increase the concentration of each of the components. It should be noted however that these requirements may differ for human pharmaceuticals (59).

Controls

31. Concurrent negative controls (see paragraph 21), consisting of the solvent alone in the treatment medium and handled in the same way as the treatment cultures, should be included for every experimental condition.
32. Concurrent positive controls are needed to demonstrate the ability of the laboratory to identify mutagens under the conditions of the test protocol used, the effectiveness of the exogenous metabolic activation system (when applicable), and to demonstrate adequate detection of both small/late appearing and large/early appearing *TK* mutants. Examples of positive controls are given in the table 1 below. Alternative positive control substances can be used, if justified. Because *in vitro* mammalian cell tests for genetic toxicity are sufficiently standardised for short-term treatments (3-4 hours) done concurrently with and without metabolic activation using the same treatment duration, the use of positive controls may be confined to a mutagen requiring metabolic activation. In this case, this single positive control response will demonstrate both the activity of the metabolic activation system and the responsiveness of the test system. If used, long term treatment (i.e. 24 hours without S9) should however have its own positive control, as the treatment duration will differ from the test using metabolic activation. Each positive control should be used at one or more concentrations expected to give reproducible and detectable increases over

background in order to demonstrate the sensitivity of the test system, and the response should not be compromised by cytotoxicity exceeding the limits specified in this TM (see paragraph 28).

Table 1: Reference substances recommended for assessing laboratory proficiency and for selection of positive controls

Category	Substance	CASRN
1. Mutagens active without metabolic activation		
Methyl methanesulphonate	66-27-3	
Mitomycin C	50-07-7	
4-Nitroquinoline-N-Oxide	56-57-5	
2. Mutagens requiring metabolic activation		
Benzo(a)pyrene	50-32-8	
Cyclophosphamide (monohydrate)	50-18-0 (6055-19-2)	
7,12-Dimethylbenzanthracene	57-97-6	
3-Methylcholanthrene	56-49-5	

PROCEDURE

Treatment with test chemical

33. Proliferating cells are treated with the test chemical in the presence and absence of a metabolic activation system. Exposure should be for a suitable period of time (usually 3 to 4 hours is adequate). It should be noted however that these requirements may differ for human pharmaceuticals (59). For MLA, in cases where the short-term treatment yields negative results, and there is information suggesting the need for longer treatment [e.g. nucleoside analogs, poorly soluble chemicals, (5)(59)], consideration should be given to conducting the test with longer treatment, i.e. 24 hours without S9.
34. The minimum number of cells used for each test (control and treated) culture at each stage in the test should be based on the spontaneous mutant frequency. A general guide is to treat and passage sufficient cells in each experimental culture so as to maintain at least 10 but ideally 100 spontaneous mutants in all phases of the test (treatment, phenotypic expression and mutant selection) (56).
35. For MLA the recommended acceptable spontaneous mutant frequency is between $35-140 \times 10^{-6}$ (agar version) and $50-170 \times 10^{-6}$ (microwell version) (see Table 2). To have at least 10 and ideally 100 spontaneous mutants surviving treatment for each test culture, it is necessary to treat at least 6×10^6 cells. Treating this number of cells, and

maintaining sufficient cells during expression and cloning for mutant selection, provides for a sufficient number of spontaneous mutants (10 or more) during all phases of the experiment, even for the cultures treated at concentrations that result in 90% cytotoxicity (as measured by an RTG of 10%) (19)(38)(39).

36. For the TK6, the spontaneous mutant frequency is generally between 2 and 10×10^{-6} . To have at least 10 spontaneous mutants surviving treatment for each culture, it is necessary to treat at least 20×10^6 cells. Treating this number of cells provides for a sufficient number of spontaneous mutants (10 or more) even for the cultures treated at concentrations that cause 90% cytotoxicity during treatment (10% RS). In addition a sufficient number of cells must be cultured during the expression period and plated for mutant selection (60).

Phenotypic expression time and measurement of cytotoxicity and mutant frequency

37. At the end of the treatment period, cells are cultured for a defined time to allow near optimal phenotypic expression of newly induced mutants; specific to each cell line. For the MLA, the phenotypic expression period is 2 days. For the TK6, the phenotypic expression period is 3-4 days. If a 24 hr treatment is used, the expression period begins after the end of treatment.
38. During the phenotypic expression period, cells are enumerated on a daily basis. For the MLA the daily cell counts are used to calculate the daily suspension growth (SG). Following the 2 day expression period, cells are suspended in medium with and without selective agent for the determination of the numbers of mutants (selection plates) and for cloning efficiency (viability plates), respectively. For MLA there are two equally acceptable methods for mutant selection cloning; one using soft agar and the other using liquid medium in 96-well plates (19) (38) (39). Cloning in the TK6 is conducted using liquid media and 96-well plates (16).
39. Trifluorothymidine (TFT) is the only recommended selective agent for *TK* mutants (61).
40. For the MLA, agar plates and microwell plates are counted after 10-12 days incubation. For the TK6, colonies in microwell plates are scored after 10-14 days for the early appearing mutants. In order to recover the slow growing (late appearing) TK6 mutants, it is necessary to re-feed the cells with growth medium and TFT after counting the early appearing mutants and then to incubate the plates for an additional 7-10 days (62). See paragraphs 42 & 44 for a discussion concerning the enumeration of the slow and normal growth *TK* mutants.
41. The appropriate calculations for the two tests including the two methods (agar and microwell) for the MLA are in Appendix 2. For the agar method of the MLA, colonies are counted and the number of mutant colonies adjusted by the cloning efficiency to

calculate a MF. For the microwell version of the MLA and the TK6, cloning efficiency both for the selection and cloning efficiency plates is determined according to the Poisson distribution (63). The MF is calculated from these two cloning efficiencies.

Mutant Colony characterisation

42. For the MLA, if the test chemical is positive (see paragraphs 62-63), colony characterisation by colony sizing or growth should be performed on at least one of the test cultures (generally the highest acceptable positive concentration) and on the negative and positive controls. If the test chemical is negative (see paragraph 64), mutant colony characterisation should be performed on the negative and positive controls. For the microwell method of the MLA, small colony mutants are defined as those covering less than 25% of the well's diameter and large colony mutants as those that cover more than 25% of the well's diameter. For the agar method, an automatic colony counter is used to enumerate the mutant colonies and for colony sizing. Approaches to colony sizing are detailed in the literature (19)(38)(40). Colony characterisation on the negative and positive control is needed to demonstrate that the studies are adequately conducted.
43. The test chemical cannot be determined to be negative if the both the large and small colony mutants are not adequately detected in the positive control. Colony characterisation can be used to provide general information concerning the ability of the test chemical to cause point mutations and/or chromosomal events (paragraph 4).
44. TK6: Normal growing and slow growing mutants are differentiated by a difference in incubation time (see paragraph 40). For the TK6 generally both the early and late appearing mutants are scored for all of the cultures including the negative and positive controls. Colony characterisation of the negative and positive control is needed to demonstrate that the studies are adequately conducted. The test chemical cannot be determined to be negative if both the early appearing and late appearing mutants are not adequately detected in the positive control. Colony characterisation can be used to provide general information concerning the ability of the test chemical to cause point mutations and/or chromosomal events (paragraph 4).

Proficiency of the laboratory

45. In order to demonstrate sufficient experience with the test prior to using it for routine testing, the laboratory should have performed a series of experiments with reference positive substances acting via different mechanisms (at least one active with and one active without metabolic activation selected from the substances listed in Table 1) and various negative controls (including untreated cultures and various solvents/vehicles). These positive and negative control responses should be consistent with the literature.

This requirement is not applicable to laboratories that have experience, i.e. that have an historical data base available as defined in paragraphs 47-50. For the MLA the values obtained for both positive and negative controls should be consistent with the IWGT recommendations (see Table 2).

46. A selection of positive control substances (see Table 1) should be investigated with short and long treatments (if using long treatments) in the absence of metabolic activation, and also with short treatment in the presence of metabolic activation, in order to demonstrate proficiency to detect mutagenic chemicals, to determine the effectiveness of the metabolic activation system and to demonstrate the appropriateness of the cell growth conditions during treatment, phenotypic expression and mutant selection and of the scoring procedures. A range of concentrations of the selected substances should be chosen so as to give reproducible and concentration-related increases above the background in order to demonstrate the sensitivity and dynamic range of the test system.

Historical control data

47. The laboratory should establish:
 - A historical positive control range and distribution,
 - A historical negative (untreated, solvent) control range and distribution.
48. When first acquiring data for an historical negative control distribution, concurrent negative controls should be consistent with published negative control data. As more experimental data are added to the control distribution, concurrent negative controls should ideally be within the 95% control limits of that distribution (64)(65).
49. The laboratory's historical negative control database should initially be built with a minimum of 10 experiments but would preferably consist of at least 20 experiments conducted under comparable experimental conditions. Laboratories should use quality control methods, such as control charts (e.g. C-charts or X-bar charts (65), to identify how variable their positive and negative control data are, and to show that the methodology is 'under control' in their laboratory (66). Further details and recommendations on how to build and use the historical data can be found in the literature (64).
50. Negative control data should consist of mutant frequencies from single or preferably replicate cultures as described in paragraph 27. Concurrent negative controls should ideally be within the 95% control limits of the distribution of the laboratory's historical negative control database. Where negative control data fall outside the 95% control limit they may be acceptable for inclusion in the historical control distribution as long as these data are not extreme outliers, there is evidence that the test system is

‘under control’ (see paragraph 49) and there is evidence of no technical or human failure.

51. Any changes to the experimental protocol should be considered in terms of the consistency of the data with the laboratory’s existing historical control databases. Any major inconsistencies should result in the establishment of a new historical control database.

DATA AND REPORTING

Presentation of the results

52. The presentation of data for both the MLA and TK6 should include, for both treated and control cultures, data required for the calculation of cytotoxicity (RTG or RS, respectively) and mutant frequencies, as described below.
53. For MLA, individual culture data should be provided for RSG, RTG, the cloning efficiency at the time of mutant selection and the number of mutant colonies (for agar version) or number of empty wells (for microwell version). MF should be expressed as number of mutant cells per million surviving cells. If the response is positive, small and large colony MFs (and/or percentage of the total MF) should be given for at least one concentration of the test chemical (generally the highest positive concentration) and the negative and positive controls. In the case of a negative response, the small and large colony MF should be given for the negative control and the positive control.
54. For TK6, individual culture data should be provided for RS, the cloning efficiency at the time of mutant selection and the number of empty wells for early appearing and late appearing mutants. MF should be expressed as number of mutant cells per number of surviving cells, and should include the total MF as well as the MF (and/or percentage of the total MF) of the early and late appearing mutants.

Acceptability Criteria

55. For both the MLA and the TK6 the following criteria should be met before determining the overall results for a specific test chemical:
 - Two experimental conditions (short treatment with and without metabolic activation - see paragraph 33) were conducted unless one resulted in positive results.
 - Adequate number of cells and concentrations should be analysable (see paragraphs 27, 34-36).
 - The criteria for the selection of top concentration are consistent with those described in paragraphs 28-30.

Acceptability criteria for negative and positive controls

56. The IWGT Expert MLA Workgroup analysis of an extensive amount of MLA data resulted in international consensus for specific acceptability criteria for the MLA (1)(2)(3)(4)(5). Therefore, this test method provides specific recommendations for determining the acceptability of negative and positive controls and for evaluating individual substance results in the MLA. The TK6 has a much smaller database and has not undergone evaluation by a workgroup.
57. For MLA, every experiment should be evaluated as to whether the untreated/solvent control meets the IWGT MLA Workgroup acceptance criteria ((4) and Table 2, below) for the: (1) MF (note that the IWGT acceptable MFs are different for the agar and microwell versions of the MLA), (2) cloning efficiency (CE) at the time of mutant selection and (3) suspension growth (SG) for the solvent control (see Appendix 2 for formulae).

Table 2: Acceptability criteria for the MLA

Parameter	Soft Agar Method	Microwell Method
Mutant Frequency	35 – 140 x 10 ⁻⁶	50 – 170 x10 ⁻⁶
Cloning Efficiency	65 – 120%	65 – 120%
Suspension Growth	8 – 32 fold (3-4 hour treatment) 32 – 180 fold (24 hour treatment, if conducted)	8 – 32 fold (3-4 hour treatment) 32 – 180 fold (24 hour treatment, if conducted)

58. For MLA, every test should also be evaluated as to whether the positive control(s) meets at least one of the following two acceptance criteria developed by the IWGT workgroup:
- The positive control should demonstrate an absolute increase in total MF, that is, an increase above the spontaneous background MF [an induced MF (IMF)] of at least 300 x 10⁻⁶. At least 40% of the IMF should be reflected in the small colony MF.
 - The positive control has an increase in the small colony MF of at least 150 x 10⁻⁶ above that seen in the concurrent untreated/solvent control (a small colony IMF of 150 x 10⁻⁶).
59. For the TK6, a test will be acceptable if the concurrent negative control is considered acceptable for addition to the laboratory historical negative control database as described in paragraphs 48-49. In addition, the concurrent positive controls (see paragraph 32) should induce responses that are compatible with those generated in the historical positive control data base and produce a statistically significant increase compared with the concurrent negative control.

60. For both tests, the upper limit of cytotoxicity observed in the positive control culture should be the same as of the experimental cultures. That is, the RTG/RS should not be less than 10%. It is sufficient to use a single concentration (or one of the concentrations of the positive control cultures if more than one concentration is used) to demonstrate that the acceptance criteria for the positive control have been satisfied. Further, the MF of the positive control must be within the acceptable range established for the laboratory.

Evaluation and interpretation of results

61. For the MLA, significant work on biological relevance and criteria for a positive response has been conducted by The Mouse Lymphoma Expert Workgroup of the IWGT (4). Therefore, this test method provides specific recommendations for the interpretation of test chemical results from the MLA (see paragraphs 62-64). The TK6 has a much smaller database and has not undergone evaluation by a workgroup. Therefore, the recommendations for the interpretation of data for the TK6 are given in more general terms (see paragraphs 65-66). Additional recommendations apply to both tests (see paragraphs 67-71).

MLA

62. An approach for defining positive and negative responses is recommended to assure that the increased MF is biologically relevant. In place of statistical analysis generally used for other tests, it relies on the use of a predefined induced mutant frequency (i.e. increase in MF above concurrent control), designated the Global Evaluation Factor (GEF), which is based on the analysis of the distribution of the negative control MF data from participating laboratories (4). For the agar version of the MLA the GEF is 90×10^{-6} and for the microwell version of the MLA the GEF is 126×10^{-6} .
63. Providing that all acceptability criteria are fulfilled, a test chemical is considered to be clearly positive if, in any of the experimental conditions examined (see paragraph 33), the increase in MF above the concurrent background exceeds the GEF and the increase is concentration related (e.g. using a trend test). The test chemical is then considered able to induce mutation in this test system.
64. Providing that all acceptability criteria are fulfilled, a test chemical is considered to be clearly negative if, in all experimental conditions examined (see paragraph 33) there is no concentration related response or, if there is an increase in MF, it does not exceed the GEF. The test chemical is then considered unable to induce mutations in this test system.

TK6

65. Providing that all acceptability criteria are fulfilled, a test chemical is considered to be clearly positive if, in any of the experimental conditions examined (see paragraph 33):

- at least one of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control
- the increase is concentration-related when evaluated with an appropriate trend test (see paragraph 33)
- any of the results are outside the distribution of the historical negative control data (e.g. Poisson-based 95% control limit; see paragraph 48).

When all of these criteria are met, the test chemical is then considered able to induce mutation in this test system. Recommendations for the most appropriate statistical methods can be found in the literature (66)(67).

66. Providing that all acceptability criteria are fulfilled, a test chemical is considered clearly negative if, in all experimental conditions examined (see paragraph 33):

- none of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control,
- there is no concentration-related increase when evaluated with an appropriate trend test
- all results are inside the distribution of the historical negative control data (e.g. Poisson-based 95% control limit; see paragraph 48).

The test chemical is then considered unable to induce mutations in this test system.

For both the MLA and TK6:

67. If the maximum concentration is based on cytotoxicity, the highest concentration should aim to achieve between 20 and 10% RTG/RS. The consensus is that care should be taken when interpreting positive results only found between 20 and 10% RTG/RS and a result would not be considered positive if the increase in MF occurred only at or below 10% RTG/RS (if evaluated) (2)(59).

68. There are some circumstances under which additional information may assist in determining that a test chemical is not mutagenic when there is no culture showing an RTG value between 10-20 % RTG/RS. These situations are outlined as follows: (1) There is no evidence of mutagenicity (e.g. no dose response, no mutant frequencies above those seen in the concurrent negative control or historical background ranges, etc.) in a series of data points within 100% to 20% RTG/RS and there is at least one data point between 20 and 25% RTG/RS. (2) There is no evidence of mutagenicity (e.g. no dose response, no mutant frequencies above those seen in the concurrent negative control or historical background ranges, etc.) in a series of data points between 100% to 25% RTG/RS and there is also a negative data point slightly below 10% RTG/RS. In both of these situations the test chemical can be concluded to be negative.

69. There is no requirement for verification of a clearly positive or negative response.
70. In cases when the response is neither clearly negative nor clearly positive as described above and/or in order to assist in establishing the biological relevance of a result the data should be evaluated by expert judgement and/or further investigations. Performing a repeat experiment possibly using modified experimental conditions [e.g. concentration spacing to increase the probability of attaining data points within the 10-20% RTG/RS range, using other metabolic activation conditions (i.e. S9 concentration or S9 origin) and duration of treatment] could be useful.
71. In rare cases, even after further investigations, the data set will preclude making a conclusion of positive or negative results. Therefore the test chemical response should be concluded to be equivocal (interpreted as equally likely to be positive or negative).

TEST REPORT

72. The test report should include the following information:

Test chemical:

- source, lot number, limit date for use, if available;
- stability of the test chemical itself, if known;
- solubility and stability of the test chemical in solvent, if known;
- measurement of pH, osmolality, and precipitate in the culture medium to which the test chemical was added, as appropriate.

Mono-constituent substance:

- physical appearance, water solubility, and additional relevant physicochemical properties;
- chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc.

Multi-constituent substance, UVCBs and mixtures:

- characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents.

Solvent:

- justification for choice of solvent;
- percentage of solvent in the final culture medium.

Cells:

For Laboratory master cultures:

- type and source of cells, and history in the testing laboratory;
- karyotype features and/or modal number of chromosomes;
- methods for maintenance of cell cultures;
- absence of mycoplasma;
- cell doubling times.

Test conditions:

- rationale for selection of concentrations and number of cell cultures; including e.g. cytotoxicity data and solubility limitations;
- composition of media, CO₂ concentration, humidity level;
- concentration of test chemical expressed as final concentration in the culture medium (e.g. µg or mg/ml or mM of culture medium);
- concentration (and/or volume) of solvent and test chemical added in the culture medium;
- incubation temperature;
- incubation time;
- duration of treatment;
- cell density during treatment;
- type and composition of metabolic activation system (source of S9, method of preparation of the S9 mix, the concentration or volume of S9 mix and S9 in the final culture medium, quality controls of S9);
- positive and negative control substances, final concentrations for each conditions of treatment;
- length of expression period (including number of cells seeded, and subcultures and feeding schedules, if appropriate);
- identity of the selective agent and its concentration;
- for the MLA, the version used (agar or microwell) should be indicated
- criteria for acceptability of the tests;
- methods used to enumerate numbers of viable and mutant cells;
- methods used for the measurements of cytotoxicity;

- any supplementary information relevant to cytotoxicity and method used;
- duration of incubation times after plating;
- definition of colonies of which size and type are considered (including criteria for "small" and "large" colonies, as appropriate);
- criteria for considering studies as positive, negative or equivocal;
- methods used to determine pH, osmolality, if performed and precipitation if relevant.

Results:

- number of cells treated and number of cells sub-cultured for each culture;
- toxicity parameters (RTG for MLA and RS for TK6);
- signs of precipitation and time of the determination;
- number of cells plated in selective and non-selective medium;
- number of colonies in non-selective medium and number of resistant colonies in selective medium and related mutant frequencies;
- colony sizing for the negative and positive controls and if the test chemical is positive, at least one concentration, and related mutant frequencies;
- concentration-response relationship, where possible;
- concurrent negative (solvent) and positive control data (concentrations and solvents);
- historical negative (solvent) and positive control data (concentrations and solvents) with ranges, means and standard deviations; number of tests upon which the historical controls are based;
- statistical analyses (for individual cultures and pooled replicates if appropriate), and p-values if any; and for the MLA, the GEF evaluation.

Discussion of the results

Conclusion

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Appendix 1

DEFINITIONS

Aneugen: Any chemical or process that, by interacting with the components of the mitotic and meiotic cell division cycle, leads to aneuploidy in cells or organisms.

Aneuploidy: Any deviation from the normal diploid (or haploid) number of chromosomes by a single chromosome or more than one, but not by entire set(s) of chromosomes (polyploidy).

Base-pair-substitution mutagens: Chemicals that cause substitution of base pairs in the DNA.

Chemical: A substance or a mixture.

Cloning efficiency: The percentage of cells plated at a low density that are able to grow into a colony that can be counted.

Clastogen: Any chemical or process which causes structural chromosomal aberrations in populations of cells or organisms.

Cytotoxicity: For the assays covered in this test method, cytotoxicity is identified as a reduction in relative total growth (RTG) or relative survival (RS) for the MLA and TK6, respectively.

Forward mutation: A gene mutation from the parental type to the mutant form which gives rise to an alteration or a loss of the enzymatic activity or the function of the encoded protein.

Frameshift mutagens: Chemicals which cause the addition or deletion of single or multiple base pairs in the DNA molecule.

Genotoxic: A general term encompassing all types of DNA or chromosomal damage, including DNA breakage, adducts, rearrangements, mutations, chromosome aberrations, and aneuploidy. Not all types of genotoxic effects result in mutations or stable chromosomal damage.

Mitotic recombination: During mitosis, recombination between homologous chromatids possibly resulting in the induction of DNA double strand breaks or in a loss of heterozygosity.

Mutagenic: Produces a heritable change of DNA base-pair sequences(s) in genes or of the structure of chromosomes (chromosome aberrations).

Mutant frequency (MF): The number of mutant cells observed divided by the number of viable cells.

Phenotypic expression time: The time after treatment during which the genetic alteration is fixed within the genome and any pre-existing gene products are depleted to the point that the phenotypic trait is altered.

Relative survival (RS): RS is used as the measure of treatment-related cytotoxicity in the TK6. It is the relative cloning efficiency (CE) of cells plated immediately after the cell treatment adjusted by any loss of cells during treatment as compared with the cloning efficiency of the negative control.

Relative suspension growth (RSG): For the MLA, the relative total two day suspension growth of the test culture compared to the total two-day suspension growth of the negative/solvent control (Clive and Spector, 1975). The RSG should include the relative growth of the test culture compared to the negative/solvent control during the treatment period.

Relative total growth (RTG): RTG is used as the measure of treatment-related cytotoxicity in the MLA. It is a measure of relative (to the vehicle control) growth of test cultures during the treatment, two-day expression and mutant selection cloning phases of the test. The RSG of each test culture is multiplied by the relative cloning efficiency of the test culture at the time of mutant selection and expressed relative to the cloning efficiency of the negative/solvent control (Clive and Spector, 1975).

S9 liver fractions: Supernatant of liver homogenate after 9000g centrifugation, i.e. raw liver extract

S9 mix: Mix of the liver S9 fraction and cofactors necessary for metabolic enzyme activity.

Suspension growth (SG): The fold-increase in the number of cells over the course of the treatment and expression phases of the MLA. The SG is calculated by multiplying the fold-increase on day 1 by the fold-increase on day 2 for the short (3 or 4 hr) treatment. If a 24 hr treatment is used the SG is the fold-increase during the 24 hr treatment multiplied by the fold increases on expression days 1 and 2.

Solvent control: General term to define the control cultures receiving the solvent alone used to dissolve the test chemical.

Test chemical: Any substance or mixture tested using this test method.

Untreated controls: Untreated controls are cultures that receive no treatment (i.e. neither test chemical nor solvent) but are processed the same way as the cultures receiving the test chemical.

Appendix 2

FORMULAS

Cytotoxicity

For both versions (agar and microwell) of the MLA

Cytotoxicity is defined as the Relative Total Growth (RTG) which includes the Relative Suspension Growth (RSG) during the 2 day expression period and the Relative Cloning Efficiency (RCE) obtained at the time of mutant selection. RTG, RSG and RCE are all expressed as a percentage.

Calculation of RSG: Suspension Growth one (SG₁) is the growth rate between day 0 and day 1 (cell concentration at day 1 / cell concentration at day 0) and Suspension Growth two (SG₂) is the growth rate between day 1 and day 2 (cell concentration at day 2 / cell concentration at day 1). The RSG is the total SG (SG₁ x SG₂) for the treated culture compared to the untreated/solvent control. That is: $RSG = [SG_{1(test)} \times SG_{2(test)}] / [SG_{1(control)} \times SG_{2(control)}]$ The SG₁ should be calculated from the initial cell concentration used at the beginning of cell treatment. This accounts for any differential cytotoxicity that occurs in the test culture(s) during the cell treatment.

RCE is the relative cloning efficiency of the test culture compared to the relative cloning efficiency of the untreated/solvent control obtained at the time of mutant selection.

Relative Total Growth (RTG): $RTG = RSG \times RCE$

TK6

Relative Survival (RS):

Cytotoxicity is evaluated by relative survival, i.e. cloning efficiency (CE) of cells plated immediately after treatment adjusted by any loss of cells during treatment as compared with cloning efficiency in the negative controls (assigned a survival of 100%). The adjustment for cell loss during treatment can be calculated as:

$$\text{Adjusted CE} = \text{CE} \times \frac{\text{Number of cells at the end of treatment}}{\text{Number of cells at the beginning of treatment}}$$

The RS for a culture treated by a test chemical is calculated as:

$$RS = \frac{\text{Adjusted CE in the treated culture}}{\text{Adjusted CE in the solvent control}} \times 100$$

Mutant frequency for both the MLA and TK6

Mutant frequency (MF) is the cloning efficiency of mutant colonies in selective medium (CE_M) adjusted by the cloning efficiency in non-selective medium at the time of mutant selection (CE_V). That is, $MF = CE_M / CE_V$. The calculation of these two cloning efficiencies is described below for the agar and microwell cloning methods.

MLA Agar Version: In the soft agar version of the MLA, the number of colonies on the mutant selection plate (C_M) and number of colonies on the unselected or cloning efficiency (viable count) plate (C_V) are obtained by directly counting the clones. When 600 cells are plated for cloning efficiency (CE) for the mutant selection (CE_M) plates and the unselected or cloning efficiency (viable count) plates (CE_V) and 3×10^6 cells are used for mutant selection,

$$CE_M = C_M / (3 \times 10^6) = (C_M / 3) \times 10^{-6}$$

$$CE_V = C_V / 600$$

MLA and TK6 Microwell Version: In the microwell version of the MLA, C_M and C_V are determined as the product of the total number of microwells (TW) and the probable number of colonies per well (P) on microwell plates.

$$C_M = P_M \times TW_M$$

$$C_V = P_V \times TW_V$$

From the zero term of the Poisson distribution (Furth et al., 1981), the P is given by

$$P = -\ln(EW / TW)$$

Where, EW is empty wells and TW is total wells. Therefore,

$$CE_M = C_M / T_M = (P_M \times TW_M) / T_M$$

$$CE_V = C_V / T_V = (P_V \times TW_V) / T_V$$

For the microwell version of the MLA, small and large colony mutant frequencies will be calculated in an identical manner, using the relevant number of empty wells for small and large colonies.

For TK6, small and large colony mutant frequencies are based on the early appearing and late appearing mutants.

B.68 SHORT TIME EXPOSURE *IN VITRO* TEST METHOD FOR IDENTIFYING i) CHEMICALS INDUCING SERIOUS EYE DAMAGE AND ii) CHEMICALS NOT REQUIRING CLASSIFICATION FOR EYE IRRITATION OR SERIOUS EYE DAMAGE

INTRODUCTION

1. This test method (TM) is equivalent to OECD test guideline (TG) 491 (2017). The Short Time Exposure (STE) test method is an *in vitro* method that can be used under certain circumstances and with specific limitations for hazard classification and labelling of chemicals (substances and mixtures) that induce serious eye damage as well as those that do not require classification for either serious eye damage or eye irritation, as defined by the United Nations (UN) Globally Harmonized System of Classification and Labelling of Chemicals (GHS) (1) and the European Union (EU) Regulation 1272/2008 on Classification, Labelling and Packaging of Substances and Mixtures (CLP)¹.
2. For many years, the eye hazard potential of chemicals has been evaluated primarily using an *in vivo* rabbit eye test (TM B.5 (8), equivalent to OECD TG 405). It is generally accepted that, in the foreseeable future, no single *in vitro* alternative test will be able to fully replace the *in vivo* rabbit eye test to predict across the full range of serious eye damage/eye irritation responses for different chemical classes. However, strategic combinations of alternative test methods used in a (tiered) testing strategy may well be able to fully replace the rabbit eye test (2). The top-down approach is designed for the testing of chemicals that can be expected, based on existing information, to have a high irritancy potential or induce serious eye damage. Conversely, the bottom-up approach is designed for the testing of chemicals that can be expected, based on existing information, not to cause sufficient eye irritation to require a classification. While the STE test method is not considered to be a complete replacement for the *in vivo* rabbit eye test, it is suitable for use as part of a tiered testing strategy for regulatory classification and labelling, such as the top-down/bottom-up approach, to identify without further testing (i) chemicals inducing serious eye damage (UN GHS/CLP Category 1) and (ii) chemicals (excluding highly volatile substances and all solid chemicals other than surfactants) that do not require

¹ Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006, OJ L 353/1, 31.12.2008

classification for eye irritation or serious eye damage (UN GHS/CLP No Category) (1)(2). However, a chemical that is neither predicted to cause serious eye damage (UN GHS/CLP Category 1) nor UN GHS/CLP No Category (does not induce either serious eye damage or eye irritation) by the STE test method would require additional testing to establish a definitive classification. Furthermore, the appropriate regulatory authorities should be consulted before using the STE in a bottom-up approach under classification schemes other than the UN GHS/CLP. The choice of the most appropriate test method and the use of this test method should be seen in the context of the OECD Guidance Document on an Integrated Approaches on Testing and Assessment for Serious Eye Damage and Eye irritation (14).

3. The purpose of this test method is to describe the procedures used to evaluate the eye hazard potential of a test chemical based on its ability to induce cytotoxicity in the Short Time Exposure Test method. The cytotoxic effect of chemicals on corneal epithelial cells is an important mode of action (MOA) leading to corneal epithelium damage and eye irritation. Cell viability in the STE test method is assessed by the quantitative measurement, after extraction from cells, of blue formazan salt produced by the living cells by enzymatic conversion of the vital dye MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), also known as Thiazolyl Blue Tetrazolium Bromide (3). The obtained cell viability is compared to the solvent control (relative viability) and used to estimate the potential eye hazard of the test chemical. A test chemical is classified as UN GHS/CLP Category 1 when both the 5% and 0.05% concentrations result in a cell viability smaller than or equal to ($\leq$) 70%. Conversely, a chemical is predicted as UN GHS/CLP No Category when both 5% and 0.05% concentrations result in a cell viability higher than ($>$) 70%.
4. The term “test chemical” is used in this test method to refer to what is tested and is not related to the applicability of the STE test method to the testing of substances and/or mixtures. Definitions are provided in the Appendix.

INITIAL CONSIDERATIONS AND LIMITATIONS

5. This test method is based on a protocol developed by Kao Corporation (4), which was the subject of two different validation studies: one by the Validation Committee of the Japanese Society for Alternative to Animal Experiments (JSAAE) (5) and another by the Japanese Center for the Validation of Alternative Methods (JaCVAM) (6). A peer review was conducted by NICEATM/ICCVAM based on the validation study reports and background review documents on the test method (7).
6. When used to identify chemicals (substances and mixtures) inducing serious eye damage (UN GHS/CLP Category 1 (1), data obtained with the STE test method on 125 chemicals (including both substances and mixtures), showed an overall accuracy

of 83% (104/125), a false positive rate of 1% (1/86), and a false negative rate of 51% (20/39) as compared to the *in vivo* rabbit eye test (7). The false negative rate obtained is not critical in the present context, since all test chemicals that induce a cell viability of $\leq 70\%$ at a 5% concentration and $> 70\%$ at 0.05% concentration would be subsequently tested with other adequately validated *in vitro* test methods or, as a last option, in the *in vivo* rabbit eye test, depending on regulatory requirements and in accordance with the sequential testing strategy and weight-of-evidence approaches currently recommended (1) (8). Mainly mono-constituent substances were tested, although a limited amount of data also exist on the testing of mixtures. The test method is nevertheless technically applicable to the testing of multi-constituent substances and mixtures. However, before use of this test method on a mixture for generating data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed when there is a regulatory requirement for testing of the mixture. The STE test method showed no other specific shortcomings when used to identify test chemicals as UN GHS/CLP Category 1. Investigators could consider using this test method on test chemicals, whereby cell viability $\leq 70\%$ at both 5% and 0.05% concentration should be accepted as indicative of a response inducing serious eye damage that should be classified as UN GHS/CLP Category 1 without further testing.

7. When used to identify chemicals (substances and mixtures) not requiring classification for eye irritation and serious eye damage (i.e. UN GHS/CLP No Category), data obtained with the STE test method on 130 chemicals (including both substances and mixtures), showed an overall accuracy of 85% (110/130), a false negative rate of 12% (9/73), and a false positive rate of 19% (11/57) as compared to the *in vivo* rabbit eye test (7). If highly volatile substances and solid substances other than surfactants are excluded from the dataset, the overall accuracy improves to 90% (92/102), the false negative rate to 2% (1/54), and the false positive to 19% (9/48) (7). As a consequence, the potential shortcomings of the STE test method when used to identify test chemicals not requiring classification for eye irritation and serious eye damage (UN GHS/CLP No Category) are a high false negative rate for i) highly volatile substances with a vapor pressure over 6 kPa and ii) Solid chemicals (substances and mixtures) other than surfactants and mixtures composed only of surfactants. Such chemicals are excluded from the applicability domain of the STE test method (7).
8. In addition to the chemicals mentioned in paragraphs 6 and 7, the STE test method generated dataset also contains in-house data on 40 mixtures, which when compared to the *in vivo* Draize eye test, showed an accuracy of 88% (35/40), a false positive rate of 50% (5/10), and a false negative rate of 0% (0/30) for predicting mixtures that do not require classification under the UN GHS/CLP classification systems (9). The STE

test method can therefore be applied to identify mixtures as UN GHS/CLP No Category in a bottom-up approach with the exception of solid mixtures other than those composed only of surfactants as an extension of its limitation to solid substances. Furthermore, mixtures containing substances with vapour pressure higher than 6kPa should be evaluated with care to avoid potential under-predictions, and should be justified on a case-by-case basis.

9. The STE test method cannot be used for the identification of test chemicals as UN GHS/CLP Category 2, or UN GHS Category 2A (eye irritation) or 2B (mild eye irritation), due to the considerable number of UN GHS/CLP Category 1 chemicals under-predicted as Category 2, 2A, or 2B and UN GHS/CLP No Category chemicals over-predicted as Category 2, 2A, or 2B (7). For this purpose, further testing with another suitable method may be required.
10. The STE test method is suitable for test chemicals that are dissolved or uniformly suspended for at least 5 minutes in physiological saline, 5% dimethyl sulfoxide (DMSO) in saline, or mineral oil. The STE test method is not suitable for test chemicals that are insoluble or cannot be uniformly suspended for at least 5 minutes in physiological saline, 5% DMSO in saline, or mineral oil. The use of mineral oil in the STE test method is possible because of the short-time exposure. Therefore, the STE test method is suitable for predicting the eye hazard potential of water-insoluble test chemicals (*e.g.*, long-chain fatty alcohols or ketones) provided that they are miscible in at least one of the three above proposed solvents (4).
11. The term "test chemical" is used in this test method to refer to what is being tested¹¹ and is not related to the applicability of the STE test method to the testing of substances and/or mixtures.

PRINCIPLE OF THE TEST

12. The STE test method is a cytotoxicity-based *in vitro* assay that is performed on a confluent monolayer of Statens Seruminstitut Rabbit Cornea (SIRC) cells, cultured on a 96-well polycarbonate microplate (4). After five-minute exposure to a test chemical, the cytotoxicity is quantitatively measured as the relative viability of SIRC cells using the MTT assay (4). Decreased cell viability is used to predict potential adverse effects leading to ocular damage.

¹ In June 2013, the Joint Meeting agreed that where possible, a more consistent use of the term "test chemical" describing what is being tested should now be applied in new and updated test methods.

13. It has been reported that 80% of a solution dropped into the eye of a rabbit is excreted through the conjunctival sac within three to four minutes, while greater than 80% of a solution dropped into the human eye is excreted within one to two minutes (10). The STE test method attempts to approximate these exposure times and makes use of cytotoxicity as an endpoint to assess the extent of damage to SIRC cells following a five-minute exposure to the test chemical.

DEMONSTRATION OF PROFICIENCY

14. Prior to routine use of the STE test method described in this test method, laboratories should demonstrate technical proficiency by correctly classifying the eleven substances recommended in Table 1. These substances were selected to represent the full range of responses for serious eye damage or eye irritation based on results of *in vivo* rabbit eye tests (TG 405) and the UN GHS/CLP classification system (1). Other selection criteria included that the substances should be commercially available, that high-quality *in vivo* reference data should be available, and that high-quality *in vitro* data from the STE test method should be available (3). In situations where a listed substance is unavailable or where justifiable, another substance for which adequate *in vivo* and *in vitro* reference data are available could be used provided that the same criteria as described here are used.

Table 1: List of Proficiency Substances

Substance	CASRN	Chemical class ¹	Physical state	<i>In Vivo</i> UN GHS/CLP Cat. ²	Solvent in STE test	STE UN GHS/CLP Cat.
Benzalkonium chloride (10%, aqueous)	8001-54-5	Onium compound	Liquid	Category 1	Saline	Category 1
Triton X-100 (100%)	9002-93-1	Ether	Liquid	Category 1	Saline	Category 1
Acid Red 92	18472-87-2	Heterocyclic compound; Bromine compound; Chlorine compound	Solid	Category 1	Saline	Category 1
Sodium hydroxide	1310-73-2	Alkali; Inorganic chemical	Solid	Category 1 ³	Saline	Category 1
Butyrolactone	96-48-0	Lactone; Heterocyclic compound	Liquid	Category 2A (Category 2 in CLP)	Saline	No prediction can be made
1-Octanol	111-87-5	Alcohol	Liquid	Category 2A/B ⁴ (Category 2 in CLP)	Mineral Oil	No prediction can be

						made
Cyclopentanol	96-41-3	Alcohol; Hydrocarbon, cyclic	Liquid	Category 2A/B ⁵ (Category 2 in CLP)	Saline	No prediction can be made
2-Ethoxyethyl acetate	111-15-9	Alcohol; Ether	Liquid	No Category	Saline	No Category
Dodecane	112-40-3	Hydrocarbon, acyclic	Liquid	No Category	Mineral Oil	No Category
Methyl isobutyl ketone	108-10-1	Ketone	Liquid	No Category	Mineral Oil	No Category
1,1- Dimethylguanidine sulfate	598-65-2	Amidine; Sulfur compound	Solid	No Category	Saline	No Category

Abbreviations: CAS RN = Chemical Abstracts Service Registry Number

¹Chemical classes were assigned using information obtained from previous NICEATM publications and if not available, using the National Library of Medicine's Medical Subject Headings (MeSH[®]) (via ChemIDplus[®] [National Library of Medicine], available at <http://chem.sis.nlm.nih.gov/chemidplus/>) and structure determinations made by NICEATM.

²Based on results from the *in vivo* rabbit eye test (OECD TG 405) and using the UN GHS/CLP (1).

³Classification as Cat.1 is based on skin corrosive potential of 100% sodium hydroxide (listed as a proficiency chemical with skin corrosive potential in OECD TG 435) and the criterion for UN GHS/CLP category 1 (1).

⁴Classification as 2A or 2B depends on the interpretation of the UN GHS criterion for distinguishing between these two categories, i.e., 2 out of 6 vs 4 out of 6 animals with effects at day 7 necessary to generate a Category 2A classification. The *in vivo* dataset included 2 studies with 3 animals each. In one study two out of three animals showed effects at day 7 warranting a Cat. 2A classification (11), whereas in the second study all endpoints in all three animals recovered to a score of zero by day 7 warranting a Cat. 2B classification (12).

⁵Classification as 2A or 2B depends on the interpretation of the UN GHS criterion for distinguishing between these two categories, i.e., 1 out of 3 vs 2 out of 3 animals with effects at day 7 necessary to generate a Category 2A classification. The *in vivo* study included 3 animals. All endpoints apart from corneal opacity and conjunctivae redness in one animal recovered to a score of zero by day 7 or earlier. The one animal that did not fully recover by day 7 had a corneal opacity score of 1 and a conjunctivae redness of 1 (at day 7) that fully recovered at day 14 (11).

PROCEDURE

Preparation of the Cellular Monolayer

15. The rabbit cornea cell line, SIRC should be used for performing the STE test method. It is recommended that SIRC cells are obtained from a well-qualified cell bank, such as American Type Culture Collection CCL60.
16. SIRC cells are cultured at 37°C under 5% CO₂ and humidified atmosphere in a culture flask containing a culture medium comprising Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50–100 units/ml penicillin and 50–100 µg/ml streptomycin. Cells that have become confluent in the culture flask should be separated using trypsin-

ethylenediaminetetraacetic acid solution, with or without the use of a cell scraper. Cells are propagated (e.g. 2 to 3 passages) in a culture flask before being employed for routine testing, and should undergo no more than 25 passages from thawing.

17. Cells ready to be used for the STE test are then prepared at the appropriate density and seeded into 96-well plates. The recommended cell seeding density is 6.0×10^3 cells per well when cells are used four days after seeding, or 3.0×10^3 cells per well when cells are used five days after seeding, at a culture volume of 200 μ l. Cells used for the STE test that are seeded in a culture medium at the appropriate density will reach a confluence of more than 80% at the time of testing, *i.e.*, four or five days after seeding.

Application of the Test Chemicals and Control Substances

18. The first choice of solvent for dissolving or suspending test chemicals is physiological saline. If the test chemical demonstrates low solubility or cannot be dissolved or suspended uniformly for at least five minutes in saline, 5% DMSO (CAS#67-68-5) in saline is used as a second choice solvent. For test chemicals that cannot be dissolved or suspended uniformly for at least five minutes in either saline or 5% DMSO in saline, mineral oil (CAS#8042-47-5) is used as a third choice solvent.
19. Test chemicals are dissolved or suspended uniformly in the selected solvent at 5% (w/w) concentration and further diluted by serial 10-fold dilution to 0.5% and 0.05% concentration. Each test chemical is to be tested at both 5% and 0.05% concentrations. Cells cultured in the 96-well plate are exposed to 200 μ l/well of either a 5% or a 0.05% concentration of the test chemical solution (or suspension), for five minutes at room temperature. Test chemicals (mono-constituent substances or multi-constituent substances or mixtures) are considered as neat substances and diluted or suspended according to the method, regardless of their purity.
20. The culture medium described in paragraph 16 is used as a medium control in each plate of each repetition. Furthermore, cells are to be exposed also to solvent control samples in each plate of each repetition. The solvents listed in paragraph 18 have been confirmed to have no adverse effects on the viability of SIRC cells.
21. In the STE test method, 0.01% Sodium lauryl sulfate (SLS) in saline is to be used as a positive control in each plate of each repetition. In order to calculate cell viability of the positive control, each plate of each repetition has to also include a saline solvent control.
22. A blank is necessary to determine compensation for optical density and should be performed on wells containing only phosphate buffered saline, but no calcium and magnesium (PBS-) or cells.

23. Each sample (test chemical at 5% and 0.05%, medium control, solvent control, and positive control) should be tested in triplicate in each repetition by exposing the cells to 200 µl of the appropriate test or control chemical for five minutes at room temperature.
24. Benchmark substances are useful for evaluating the ocular irritancy potential of unknown chemicals of a specific chemical or product class, or for evaluating the relative irritancy potential of an ocular irritant within a specific range of irritant responses.

Cell Viability Measurement

25. After exposure, cells are washed twice with 200 µl of PBS and 200 µl of MTT solution (0.5 mg MTT/ml of culture medium) is added. After a two-hour reaction time in an incubator (37°C, 5% CO₂), the MTT solution is decanted, MTT formazan is extracted by adding 200 µl of 0.04 N hydrochloric acid-isopropanol for 60 minutes in the dark at room temperature, and the absorbance of the MTT formazan solution is measured at 570 nm with a plate reader. Interference of test chemicals with the MTT assay (by colorants or direct MTT reducers) only occurs if significant amount of test chemical is retained in the test system following rinsing after exposure which is the case for 3D Reconstructed human cornea or Reconstructed human epidermis tissues but is not relevant for the 2D cell cultures used for the STE test method.

Interpretation of Results and Prediction Model

26. The optical density (OD) values obtained for each test chemical are then used to calculate cell viability relative to the solvent control, which is set at 100%. The relative cell viability is expressed as a percentage and obtained by dividing the OD of test chemical by the OD of the solvent control after subtracting the OD of blank from both values.

$$\text{Cell viability (\%)} = \frac{(OD_{570} \text{ of test chemical}) - (OD_{570} \text{ of blank})}{(OD_{570} \text{ of solvent control}) - (OD_{570} \text{ of blank})} \times 100$$

Similarly, the relative cell viability of each solvent control is expressed as a percentage and obtained by dividing the OD of each solvent control by the OD of the medium control after subtracting the OD of blank from both values.

27. Three independent repetitions, each containing three replicate wells (*i.e.*, n=9), should be performed. The arithmetic mean of the three wells for each test chemical and solvent control in each independent repetition is used to calculate the arithmetic mean of relative cell viability. The final arithmetic mean of the cell viability is calculated from the three independent repetitions.

28. The cell viability cut-off values for identifying test chemicals inducing serious eye damage (UN GHS/CLP Category 1) and test chemicals not requiring classification for eye irritation or serious eye damage (UN GHS/CLP No Category) are given hereafter.

Table 2: Prediction model of the STE test method

Cell viability		UN GHS/CLP Classification	Applicability
At 5%	At 0.05%		
> 70%	> 70%	No Category	Substances and mixtures, with the exception of: i) highly volatile substances with a vapor pressure over 6 kPa ¹ and ii) Solid chemicals (substances and mixtures) other than surfactants and mixtures composed only of surfactants
≤ 70%	> 70%	No prediction can be made	Not applicable
≤ 70%	≤ 70%	Category 1	Substances and mixtures ²

¹ Mixtures containing substances with vapour pressure higher than 6kPa should be evaluated with care to avoid potential under-predictions, and should be justified on a case-by-case basis.

² Based on results obtained mainly with mono-constituent substances, although a limited amount of data also exist on the testing of mixtures. The test method is nevertheless technically applicable to the testing of multi-constituent substances and mixtures. Before use of this test method on a mixture for generating data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed, when there is a regulatory requirement for testing of the mixture.

Acceptance Criteria

29. Test results are judged to be acceptable when the following criteria are all satisfied:

- Optical density of the medium control (exposed to culture medium) should be 0.3 or higher after subtraction of blank optical density.
- Viability of the solvent control should be 80% or higher relative to the medium control. If multiple solvent controls are used in each repetition, each of those controls should show cell viability greater than 80% to qualify the test chemicals tested with those solvents.
- The cell viability obtained with the positive control (0.01% SLS) should be within two standard deviations of the historical mean. The upper and lower acceptance boundaries for the positive control should be frequently updated i.e., every three months, or each time an acceptable test is conducted in laboratories where tests are conducted infrequently (i.e., less than once a month). Where a laboratory does not complete a sufficient number of experiments to establish a statistically robust positive control distribution, it is acceptable that the upper and lower acceptance boundaries established by the method developer are used, i.e., between 21.1% and 62.3%

according to its laboratory historical data, while an internal distribution is built during the first routine tests.

- d) Standard deviation of the final cell viability derived from three independent repetitions should be less than 15% for both 5% and 0.05% concentrations of the test chemical.

If one or several of these criteria is not met, the results should be discarded and another three independent repetitions should be conducted.

DATA AND REPORTING

Data

30. Data for each individual well (e.g., cell viability values) of each repetition as well as overall mean, SD, and classification are to be reported.

Test Report

31. The test report should include the following information:

Test Chemical and Control Substances

- Mono-constituent substance : chemical identification, such as IUPAC or CAS name(s), CAS registry number(s), SMILES or InChI code, structural formula, and/or other identifiers;
- Multi-constituent substance, UVCB and mixture: Characterisation as far as possible by e.g., chemical identity (see above), purity, quantitative occurrence and relevant physicochemical properties (see above) of the constituents, to the extent available;
- Physical state, volatility, pH, LogP, molecular weight, chemical class, and additional relevant physicochemical properties relevant to the conduct of the study, to the extent available;
- Purity, chemical identity of impurities as appropriate and practically feasible, etc;
- Treatment prior to testing, if applicable (e.g., warming, grinding);
- Storage conditions and stability to the extent available.

Test Method Conditions and Procedures

- Name and address of the sponsor, test facility and study director;
- Description of the test method used;
- Cell line used, its source, passage number and confluence of cells used for testing;
- Details of test procedure used;

- Number of repetitions and replicates used;
- Test chemical concentrations used (if different than the ones recommended);
- Justification for choice of solvent for each test chemical;
- Duration of exposure to the test chemical (if different than the one recommended);
- Description of any modifications of the test procedure;
- Description of evaluation and decision criteria used;
- Reference to historical positive control mean and Standard Deviation (SD):
- Demonstration of proficiency of the laboratory in performing the test method (e.g. by testing of proficiency substances) or demonstration of reproducible performance of the test method over time.

Results

- For each test chemical and control substance, and each tested concentration, tabulation should be given for the individual OD values per replicate well, the arithmetic mean OD values for each independent repetition, the % cell viability for each independent repetition, and the final arithmetic mean % cell viability and SD over the three repetitions;
- Results for the medium, solvent and positive control demonstrating suitable study acceptance criteria;
- Description of other effects observed;
- The overall derived classification with reference to the prediction model/decision criteria used.

Discussion of the Results

Conclusions

LITERATURE

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Appendix

DEFINITIONS

Accuracy: The closeness of agreement between test method results and accepted reference values. It is a measure of test method performance and one aspect of relevance. The term is often used interchangeably with concordance to mean the proportion of correct outcomes of a test method (13).

Benchmark substance: A substance used as a standard for comparison to a test chemical. A benchmark substance should have the following properties; (i) a consistent and reliable source(s); (ii) structural and functional similarity to the class of substances being tested; (iii) known physical/chemical characteristics; (iv) supporting data on known effects, and (v) known potency in the range of the desired response.

Bottom-Up Approach: A step-wise approach used for a test chemical suspected of not requiring classification for eye irritation or serious eye damage, which starts with the determination of chemicals not requiring classification (negative outcome) from other chemicals (positive outcome)

Chemical: A substance or mixture.

Eye irritation: Production of change in the eye following the application of a test chemical to the anterior surface of the eye, which are fully reversible within 21 days of application. Interchangeable with “reversible effects on the eye” and with UN GHS/CLP Category 2

False negative rate: The proportion of all positive chemicals falsely identified by a test method as negative. It is one indicator of test method performance.

False positive rate: The proportion of all negative chemicals that are falsely identified by a test method as positive. It is one indicator of test method performance.

Hazard: Inherent property of an agent or situation having the potential to cause adverse effects when an organism, system or (sub) population is exposed to that agent.

Medium control: An untreated replicate containing all components of a test system. This sample is processed with test chemical-treated samples and other control samples to determine whether the solvent interacts with the test system.

Mixture: A mixture or a solution composed of two or more substances.

Mono-constituent substance: A substance, defined by its quantitative composition, in which one main constituent is present to at least 80% (w/w).

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Thiazolyl blue tetrazolium bromide.

Multi-constituent substance: A substance, defined by its quantitative composition, in which more than one main constituent is present in a concentration $\geq 10\%$ (w/w) and $< 80\%$ (w/w). A multi-constituent substance is the result of a manufacturing process. The difference between mixture and multi-constituent substance is that a mixture is obtained by blending of two or more substances without chemical reaction. A multi-constituent substance is the result of a chemical reaction.

OD: Optical Density.

Positive control: A replicate containing all components of a test system and treated with a substance known to induce a positive response. To ensure that variability in the positive control response across time can be assessed, the magnitude of the positive response should not be excessive.

Relevance: Description of relationship of the test to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the test correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of the accuracy (concordance) of a test method (10).

Reliability: Measures of the extent that a test method can be performed reproducibly within and between laboratories over time, when performed using the same protocol. It is assessed by calculating intra- and inter-laboratory reproducibility and intra-laboratory repeatability (13).

Sensitivity: The proportion of all positive/active chemicals that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results, and is an important consideration in assessing the relevance of a test method (10).

Serious eye damage: Production of tissue damage in the eye, or serious physical decay of vision, following application of a test chemical to the anterior surface of the eye, which is not fully reversible within 21 days of application. Interchangeable with “irreversible effects on the eye” and with UN GHS/CLP Category 1.

Solvent/vehicle control: An untreated sample containing all components of a test system, including the solvent or vehicle that is processed with the test chemical-treated and other control samples to establish the baseline response for the samples treated with the test chemical dissolved in the same solvent or vehicle. When tested with a concurrent medium control, this sample also demonstrates whether the solvent or vehicle interacts with the test system.

Specificity: The proportion of all negative/inactive chemicals that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results and is an important consideration in assessing the relevance of a test method (13).

Substance: A chemical element and its compounds in the natural state or obtained by any production process, inducing any additive necessary to preserve its stability and

any impurities deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the substance or changing its composition.

Surfactant: Also called surface-active agent, this is a chemical such as a detergent, that can reduce the surface tension of a liquid and thus allow it to foam or penetrate solids; it is also known as a wetting agent.

Test chemical: Any substance or mixture tested using this Test Method.

Tiered testing strategy: A stepwise testing strategy where all existing information on a test chemical is reviewed, in a specified order, using a weight of evidence process at each tier to determine if sufficient information is available for a hazard classification decision, prior to progression to the next tier. If the irritancy potential of a test chemical can be assigned based on the existing information, no additional testing is required. If the irritancy potential of a test chemical cannot be assigned based on the existing information, a step-wise sequential animal testing procedure is performed until an unequivocal classification can be made.

Top-Down Approach: step-wise approach used for a test chemical suspected of causing serious eye damage, which starts with the determination of chemicals inducing serious eye damage (positive outcome) from other chemicals (negative outcome).

United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS): A system proposing the classification of chemicals (substances and mixtures) according to standardized types and levels of physical, health and environmental hazards, and addressing corresponding communication elements, such as pictograms, signal words, hazard statements, precautionary statements and safety data sheets, so that to convey information on their adverse effects with a view to protect people (including employers, workers, transporters, consumers and emergency responders) and the environment (1).

UN GHS/CLP Category 1: See “Serious eye damage”.

UN GHS/CLP Category 2: See “Eye irritation”.

UN GHS/CLP No Category: Chemicals that are not classified as UN GHS/CLP Category 1 or 2 (or UN GHS Category 2A or 2B).

UVCB: substances of unknown or variable composition, complex reaction products or biological materials.

B.69 RECONSTRUCTED HUMAN CORNEA-LIKE EPITHELIUM (RhCE) TEST METHOD FOR IDENTIFYING CHEMICALS NOT REQUIRING CLASSIFICATION AND LABELLING FOR EYE IRRITATION OR SERIOUS EYE DAMAGE

INTRODUCTION

1. This test method (TM) is equivalent to OECD test guideline (TG) 492 (2017). *Serious eye damage* refers to the production of tissue damage in the eye, or serious physical decay of vision, following application of a test chemical to the anterior surface of the eye, which is not fully reversible within 21 days of application, as defined by the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS) (1) and the European Union (EU) Regulation 1272/2008 on Classification, Labelling and Packaging of Substances and Mixtures (CLP)¹. Also according to UN GHS and CLP, *eye irritation* refers to the production of changes in the eye following the application of a test chemical to the anterior surface of the eye, which are fully reversible within 21 days of application. Test chemicals inducing serious eye damage are classified as UN GHS and CLP Category 1, while those inducing eye irritation are classified as UN GHS and CLP Category 2. Test chemicals not classified for eye irritation or serious eye damage are defined as those that do not meet the requirements for classification as UN GHS and CLP Category 1 or 2 (2A or 2B) i.e., they are referred to as UN GHS and CLP No Category.
2. The assessment of serious eye damage/eye irritation has typically involved the use of laboratory animals (TM B.5 (2)). The choice of the most appropriate test method and the use of this test method should be seen in the context of the OECD Guidance Document on an Integrated Approaches on Testing and Assessment for Serious Eye Damage and Eye irritation (39).
3. This test method describes an *in vitro* procedure allowing the identification of chemicals (substances and mixtures) not requiring classification and labelling for eye irritation or serious eye damage in accordance with UN GHS and CLP. It makes use of reconstructed human cornea-like epithelium (RhCE) which closely mimics the histological, morphological, biochemical and physiological properties of the human corneal epithelium. Four other *in vitro* test methods have been validated, considered

¹ Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006, OJ L 353/1, 31.12.2008

scientifically valid and adopted as TM B.47 (3), B.48 (4), B.61 (5) and B.68 (6) to address the human health endpoint serious eye damage/eye irritation.

4. Two validated tests using commercially available RhCE models are included in this test method. Validation studies for assessing eye irritation/serious eye damage have been conducted (7)(8)(9)(10)(11)(12)(13) using the EpiOcular™ Eye Irritation Test (EIT) and the SkinEthic™ Human Corneal Epithelium (HCE) Eye Irritation Test (EIT). Each of these tests makes use of commercially available RhCE tissue constructs as test system, which are referred to in the following text as the Validated Reference Methods – VRM 1 and VRM2, respectively. From these validation studies and their independent peer review (9)(12) it was concluded that the EpiOcular™ EIT and SkinEthic™ HCE EIT are able to correctly identify chemicals (both substances and mixtures) not requiring classification and labelling for eye irritation or serious eye damage according to UN GHS , and the tests were recommended as scientifically valid for that purpose (13).
5. It is currently generally accepted that, in the foreseeable future, no single *in vitro* test method will be able to fully replace the *in vivo* Draize eye test (2)(14) to predict across the full range of serious eye damage/eye irritation responses for different chemical classes. However, strategic combinations of several alternative test methods within (tiered) testing strategies such as the Bottom-Up/Top-Down approach may be able to fully replace the Draize eye test (15). The Bottom-Up approach (15) is designed to be used when, based on existing information, a chemical is expected not to cause sufficient eye irritation to require a classification, while the Top-Down approach (15) is designed to be used when, based on existing information, a chemical is expected to cause serious eye damage. The EpiOcular™ EIT and SkinEthic™ HCE EIT are recommended to identify chemicals that do not require classification for eye irritation or serious eye damage according to UN GHS/CLP (No Category) without further testing, within a testing strategy such as the Bottom-Up/Top-Down approach suggested by Scott *et al.* e.g. as an initial step in a Bottom-Up approach or as one of the last steps in a Top-Down approach (15). However, the EpiOcular™ EIT and SkinEthic™ HCE EIT are not intended to differentiate between UN GHS/CLP Category 1 (serious eye damage) and UN GHS/CLP Category 2 (eye irritation). This differentiation will need to be addressed by another tier of a test strategy (15). A test chemical that is identified as requiring classification for eye irritation/serious eye damage with EpiOcular™ EIT or SkinEthic™ HCE EIT will thus require additional testing (*in vitro* and/or *in vivo*) to reach a definitive conclusion (UN GHS/CLP No Category, Category 2 or Category 1), using e.g. TM B.47, B.48, B.61 or B.68.
6. The purpose of this test method is to describe the procedure used to evaluate the eye hazard potential of a test chemical based on its ability to induce cytotoxicity in a RhCE tissue construct, as measured by the MTT assay (16) (see paragraph 21). The

viability of the RhCE tissue following exposure to a test chemical is determined in comparison to tissues treated with the negative control substance (% viability), and is then used to predict the eye hazard potential of the test chemical.

7. Performance standards (17) are available to facilitate the validation of new or modified *in vitro* RhCE-based tests similar to EpiOcular™ EIT and SkinEthic™ HCE EIT, in accordance with the principles of the OECD Guidance Document No 34 (18), and allow for timely amendment of OECD TG 492 for their inclusion. Mutual Acceptance of Data (MAD) according to the OECD agreement will only be guaranteed for tests validated according to the performance standards, if these tests have been reviewed and included in the corresponding test guideline by the OECD.

DEFINITIONS

8. Definitions are provided in Appendix 1.

INITIAL CONSIDERATIONS AND LIMITATIONS

9. This test method is based on commercial three-dimensional RhCE tissue constructs that are produced using either primary human epidermal keratinocytes (i.e., EpiOcular™ OCL-200) or human immortalised corneal epithelial cells (i.e., SkinEthic™ HCE/S). The EpiOcular™ OCL-200 and SkinEthic™ HCE/S RhCE tissue constructs are similar to the *in vivo* corneal epithelium three-dimensional structure and are produced using cells from the species of interest (19)(20). Moreover, the tests directly measure cytotoxicity resulting from penetration of the chemical through the cornea and production of cell and tissue damage; the cytotoxic response then determines the overall *in vivo* serious eye damage/eye irritation outcome. Cell damage can occur by several modes of action (see paragraph 20), but cytotoxicity plays an important, if not the primary, mechanistic role in determining the overall serious eye damage/eye irritation response of a chemical, manifested *in vivo* mainly by corneal opacity, iritis, conjunctival redness and/or conjunctival chemosis, regardless of the physicochemical processes underlying tissue damage.
10. A wide range of chemicals, covering a large variety of chemical types, chemical classes, molecular weights, LogPs, chemical structures, etc., have been tested in the validation study underlying this test method. The EpiOcular™ EIT validation database contained 113 chemicals in total, covering 95 different organic functional groups according to an OECD QSAR toolbox analysis (8). The majority of these chemicals represented mono-constituent substances, but several multi-constituent substances (including 3 homopolymers, 5 copolymers and 10 quasi polymers) were also included in the study. In terms of physical state and UN GHS/CLP Categories, the 113 tested chemicals were distributed as follows: 13 Category 1 liquids, 15

Category 1 solids, 6 Category 2A liquids, 10 Category 2A solids, 7 Category 2B liquids, 7 Category 2B solids, 27 No Category liquids and 28 No Category solids (8). The SkinEthic™ HCE EIT validation database contained 200 chemicals in total, covering 165 different organic functional groups (8)(10)(11). The majority of these chemicals represented mono-constituent substances, but several multi-constituent substances (including 10 polymers) were also included in the study. In terms of physical state and UN GHS/CLP Categories, the 200 tested chemicals were distributed as follows: 27 Category 1 liquids, 24 Category 1 solids, 19 Category 2A liquids, 10 Category 2A solids, 9 Category 2B liquids, 8 Category 2B solids, 50 No Category liquids and 53 No Category solids (10)(11).

11. This testmethod is applicable to substances and mixtures, and to solids, liquids, semi-solids and waxes. The liquids may be aqueous or non-aqueous; solids may be soluble or insoluble in water. Whenever possible, solids should be ground to a fine powder before application; no other pre-treatment of the sample is required. Gases and aerosols have not been assessed in a validation study. While it is conceivable that these can be tested using RhCE technology, the current test method does not allow testing of gases and aerosols.
12. Test chemicals absorbing light in the same range as MTT formazan (naturally or after treatment) and test chemicals able to directly reduce the vital dye MTT (to MTT formazan) may interfere with the tissue viability measurements and need the use of adapted controls for corrections. The type of adapted controls that may be required will vary depending on the type of interference produced by the test chemical and the procedure used to quantify MTT formazan (see paragraphs 36-42).
13. Results generated in pre-validation (21)(22) and full validation (8)(10)(11) studies have demonstrated that both EpiOcular™ EIT and SkinEthic™ HCE EIT are transferable to laboratories considered to be naïve in the conduct of the assays and also to be reproducible within- and between laboratories. Based on these studies, the level of reproducibility in terms of concordance of predictions that can be expected from EpiOcular™ EIT from data on 113 chemicals is in the order of 95% within laboratories and 93% between laboratories. The level of reproducibility in terms of concordance of predictions that can be expected from SkinEthic™ HCE EIT from data on 120 chemicals is in the order of 92% within laboratories and 95% between laboratories.
14. The EpiOcular™ EIT can be used to identify chemicals that do not require classification for eye irritation or serious eye damage according to the UN GHS and CLP classification system. Considering the data obtained in the validation study (8), the EpiOcular™ EIT has an overall accuracy of 80% (based on 112 chemicals), sensitivity of 96% (based on 57 chemicals), false negative rate of 4% (based on 57

chemicals), specificity of 63% (based on 55 chemicals) and false positive rate of 37% (based on 55 chemicals), when compared to reference *in vivo* rabbit eye test data (TM B.5) (2)(14) classified according to the UN GHS and CLP classification system. A study where 97 liquid agrochemical formulations were tested with EpiOcular™ EIT demonstrated a similar performance of the test method for this type of mixtures as obtained in the validation study (23). The 97 formulations were distributed as follows: 21 Category 1, 19 Category 2A, 14 Category 2B and 43 No Category, classified according to the UN GHS classification system based on reference *in vivo* rabbit eye test data (TM B.5) (2)(14). An overall accuracy of 82% (based on 97 formulations), sensitivity of 91% (based on 54 formulations), false negative rate of 9% (based on 54 formulations), specificity of 72% (based on 43 formulations) and false positive rate of 28% (based on 43 formulations) were obtained (23).

15. The SkinEthic™ HCE EIT can be used to identify chemicals that do not require classification for eye irritation or serious eye damage according to the UN GHS and CLP classification system. Considering the data obtained in the validation study (10)(11), the SkinEthic™ HCE EIT has an overall accuracy of 84% (based on 200 chemicals), sensitivity of 95% (based on 97 chemicals), false negative rate of 5% (based on 97 chemicals), specificity of 72% (based on 103 chemicals) and false positive rate of 28% (based on 103 chemicals), when compared to reference *in vivo* rabbit eye test data (TM B.5) (2)(14) classified according to the UN GHS and CLP classification system.
16. The false negative rates obtained with both RhCE tests, with either substances or mixtures, fall within the 12% overall probability that chemicals are identified as either UN GHS and CLP Category 2 or UN GHS and CLP No Category by the *in vivo* Draize eye test, in repeated tests; this is due to the method's inherent within-test variability (24). The false positive rates obtained with both RhCE test methods with either substances or mixtures are not critical in the context of this test method since all test chemicals that produce a tissue viability equal or lower than the established cut-offs (see paragraph 44) will require further testing with other *in vitro* test methods, or as a last option in rabbits, depending on regulatory requirements, using a sequential testing strategy in a weight-of-evidence approach. These test methods can be used for all types of chemicals, whereby a negative result should be accepted for not classifying a chemical for eye irritation and serious eye damage (UN GHS and CLP No Category). The appropriate regulatory authorities should be consulted before using the EpiOcular™ EIT and SkinEthic™ HCE EIT under classification schemes other than UN GHS/CLP.
17. A limitation of this test method is that it does not allow discrimination between eye irritation/reversible effects on the eye (Category 2) and serious eye damage/irreversible effects on the eye (Category 1) as defined by UN GHS and CLP,

nor between eye irritants (optional Category 2A) and mild eye irritants (optional Category 2B), as defined by UN GHS (1). For these purposes, further testing with other *in vitro* test methods is required.

18. The term "test chemical" is used in this test method to refer to what is being tested¹ and is not related to the applicability of the RhCE test method to the testing of substances and/or mixtures.

PRINCIPLE OF THE TEST

19. The test chemical is applied topically to a minimum of two three-dimensional RhCE tissue constructs and tissue viability is measured following exposure and a post-treatment incubation period. The RhCE tissues are reconstructed from primary human epidermal keratinocytes or human immortalised corneal epithelial cells, which have been cultured for several days to form a stratified, highly differentiated squamous epithelium morphologically similar to that found in the human cornea. The EpiOcular™ RhCE tissue construct consists of at least 3 viable layers of cells and a non-keratinised surface, showing a cornea-like structure analogous to that found *in vivo*. The SkinEthic™ HCE RhCE tissue construct consists of at least 4 viable layers of cells including columnar basal cells, transitional wing cells and superficial squamous cells similar to that of the normal human corneal epithelium (20)(26).
20. Chemical-induced serious eye damage/eye irritation, manifested *in vivo* mainly by corneal opacity, iritis, conjunctival redness and/or conjunctival chemosis, is the result of a cascade of events beginning with penetration of the chemical through the cornea and/or conjunctiva and production of damage to the cells. Cell damage can occur by several modes of action, including: cell membrane lysis (e.g. by surfactants, organic solvents); coagulation of macromolecules (particularly proteins) (e.g. by surfactants, organic solvents, alkalis and acids); saponification of lipids (e.g. by alkalis); and alkylation or other covalent interactions with macromolecules (e.g. by bleaches, peroxides and alkylators) (15)(27)(28). However, it has been shown that cytotoxicity plays an important, if not the primary, mechanistic role in determining the overall serious eye damage/eye irritation response of a chemical regardless of the physicochemical processes underlying tissue damage (29)(30). Moreover, the serious eye damage/eye irritation potential of a chemical is principally determined by the extent of initial injury (31), which correlates with the extent of cell death (29) and

¹ In June 2013, the OECD Joint Meeting agreed that where possible, a more consistent use of the term "test chemical" describing what is being tested should now be applied in new and updated OECD test guidelines.

with the extent of the subsequent responses and eventual outcomes (32). Thus, slight irritants generally only affect the superficial corneal epithelium, the mild and moderate irritants damage principally the epithelium and superficial stroma and the severe irritants damage the epithelium, deep stroma and at times the corneal endothelium (30)(33). The measurement of viability of the RhCE tissue construct after topical exposure to a test chemical to identify chemicals not requiring classification for serious eye damage/eye irritancy (UN GHS and CLP No Category) is based on the assumption that all chemicals inducing serious eye damage or eye irritation will induce cytotoxicity in the corneal epithelium and/or conjunctiva.

21. RhCE tissue viability is classically measured by enzymatic conversion of the vital dye MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Thiazolyl blue tetrazolium bromide; CAS number 298-93-1] by the viable cells of the tissue into a blue MTT formazan salt that is quantitatively measured after extraction from tissues (16). Chemicals not requiring classification and labelling according to UN GHS/CLP (No Category) are identified as those that do not decrease tissue viability below a defined threshold (i.e., tissue viability > 60%, in EpiOcular™ EIT and SkinEthic™ HCE EITL¹, or > 50%, in SkinEthic™ HCE EITS²) (see paragraph 44).

DEMONSTRATION OF PROFICIENCY

22. Prior to routine use of RhCE tests for regulatory purposes, laboratories should demonstrate technical proficiency by correctly predicting the fifteen proficiency chemicals listed in Table 1. These chemicals were selected from the chemicals used in the validation studies of the VRMs (8)(10)(11). The selection includes, to the extent possible, chemicals that: (i) cover different physical states; (ii) cover the full range of *in vivo* serious eye damage/eye irritation responses based on high quality results obtained in the reference *in vivo* rabbit eye test (TM B.5) (2)(14) and the UN GHS classification system (i.e., Categories 1, 2A, 2B, or No Category) (1) and CLP classification system (i.e., Categories 1, 2 or No Category) ; (iii) cover the various *in vivo* drivers of classification (24)(25); (iv) are representative of the chemical classes used in the validation study (8)(10)(11); (v) cover a good and wide representation of organic functional groups (8)(10)(11); (vi) have chemical structures that are well-defined (8)(10)(11); (vii) are coloured and/or direct MTT reducers; (viii) produced

¹ EITL: EIT for liquids in the case of SkinEthic™ HCE

² EITS: EIT for solids in the case of SkinEthic™ HCE

reproducible results in RhCE test methods during their validations; (ix) were correctly predicted by RhCE test methods during their validation studies; (x) cover the full range of *in vitro* responses based on high quality RhCE test methods data (0 to 100% viability); (xi) are commercially available; and (xii) are not associated with prohibitive acquisition and/or disposal costs. In situations where a listed chemical is unavailable or cannot be used for other justified reasons, another chemical fulfilling the criteria described above, e.g. from the chemicals used in the validation of the VRM, could be used. Such deviations should however be justified.

Table 1: List of proficiency chemicals

Chemical Name	CASRN	Organic Functional Group ¹	Physical State	VRM1 viability (%) ²	VRM2 viability (%) ³	VRM Prediction	MTT Reducer	Colour interf.
<i>In Vivo Category 1⁴</i>								
Methylthioglycolate	2365-48-2	Carboxylic acid ester; Thioalcohol	L	10.9±6.4	5.5±7.4	No prediction can be made	Y (strong)	N
Hydroxyethyl acrylate	818-61-1	Acrylate; Alcohol	L	7.5±4.7 ⁵	1.6±1.0	No prediction can be made	N	N
2,5-Dimethyl-2,5-hexanediol	110-03-2	Alcohol	S	2.3±0.2	0.2±0.1	No prediction can be made	N	N
Sodium oxalate	62-76-0	Oxocarboxylic acid	S	29.0±1.2	5.3±4.1	No prediction can be made	N	N
<i>In Vivo Category 2A⁴</i>								
2,4,11,13-Tetraazatetradecane-diimidamide, N,N"-bis(4-chlorophenyl)-3,12-diimino-, di-D-gluconate (20%, aqueous) ⁶	18472-51-0	Aromatic heterocyclic halide; Aryl halide; Dihydroxyl group; Guanidine	L	4.0±1.1	1.3±0.6	No prediction can be made	N	Y (weak)
Sodium benzoate	532-32-1	Aryl; Carboxylic acid	S	3.5±2.6	0.6±0.1	No prediction can be made	N	N
<i>In Vivo Category 2B⁴</i>								
Diethyl toluamide	134-62-3	Benzamide	L	15.6±6.3	2.8±0.9	No prediction can be made	N	N
2,2-Dimethyl-3-methylenebicyclo [2.2.1] heptane	79-92-5	Alkane, branched with tertiary carbon; Alkene; Bicycloheptane; Bridged-ring carbocycles; Cycloalkane	S	4.7±1.5	15.8±1.1	No prediction can be made	N	N
<i>In Vivo No Category⁴</i>								
1-Ethyl-3-methylimidazolium ethylsulphate	342573-75-5	Alkoxy; Ammonium salt; Aryl; Imidazole; Sulphate	L	79.9±6.4	79.4±6.2	No Cat	N	N
Dicaprylyl ether	629-82-3	Alkoxy; Ether	L	97.8±4.3	95.2±3.0	No Cat	N	N

Chemical Name	CASRN	Organic Functional Group ¹	Physical State	VRM1 viability (%) ²	VRM2 viability (%) ³	VRM Prediction	MTT Reducer	Colour interf.
Piperonyl butoxide	51-03-6	Alkoxy; Benzodioxole; Benzyl; Ether	L	104.2±4.2	96.5±3.5	No Cat	N	N
Polyethylene glycol (PEG-40) hydrogenated castor oil	61788-85-0	Acylal; Alcohol; Allyl; Ether	Viscous	77.6±5.4	89.1±2.9	No Cat	N	N
1-(4-Chlorophenyl)-3-(3,4-dichlorophenyl) urea	101-20-2	Aromatic heterocyclic halide; Aryl halide; Urea derivatives	S	106.7±5.3	101.9±6.6	No Cat	N	N
2,2'-Methylene-bis-(6-(2H-benzotriazol-2-yl)-4-(1,1,3,3-tetramethylbutyl)-phenol)	103597-45-1	Alkane branched with quaternary carbon; Fused carbocyclic aromatic; Fused saturated heterocycles; Precursors quinoid compounds; tert-Butyl	S	102.7±13.4	97.7±5.6	No Cat	N	N
Potassium tetrafluoroborate	14075-53-7	Inorganic Salt	S	88.6±3.3	92.9±5.1	No Cat	N	N

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; UN GHS = United Nations Globally Harmonized System of Classification and Labelling of Chemicals (1); VRM1 = Validated Reference Method, EpiOcular™ EIT; VRM2 = Validated Reference Method, SkinEthic™ HCE EIT; Colour interf. = colour interference with the standard absorbance (Optical Density (OD)) measurement of MTT formazan.

¹Organic functional group assigned according to an OECD Toolbox 3.1 nested analysis (8).

²Based on results obtained with EpiOcular™ EIT in the EURL ECVAM/Cosmetics Europe Eye Irritation Validation Study (EIVS) (8).

³Based on results obtained with SkinEthic™ HCE EIT in the validation study (10)(11).

⁴Based on results from the *in vivo* rabbit eye test (TM B.5/OECD TG 405) (2)(14) and using the UN GHS .

⁵Based on results obtained in the CEFIC CONsortium for *in vitro* Eye Irritation testing strategy (CON4EI) Study.

⁶Classification as 2A or 2B depends on the interpretation of the UN GHS criterion for distinguishing between these two categories, i.e., 1 out of 3 vs 2 out of 3 animals with effects at day 7 necessary to generate a Category 2A classification. The *in vivo* study included 3 animals. All endpoints apart from corneal opacity in one animal recovered to a score of zero by day 7 or earlier. The one animal that did not fully recover by day 7 had a corneal opacity score of 1 (at day 7) that fully recovered at day 9.

23. As part of the proficiency testing, it is recommended that users verify the barrier properties of the tissues after receipt as specified by the RhCE tissue construct producer (see paragraphs 25, 27 and 30). This is particularly important if tissues are shipped over long distance / time periods. Once a test has been successfully established and proficiency in its use has been acquired and demonstrated, such verification will not be necessary on a routine basis. However, when using a test routinely, it is recommended to continue to assess the barrier properties at regular intervals.

PROCEDURE

24. The tests currently covered by this test method are the scientifically valid EpiOcular™ EIT and SkinEthic™ HCE EIT (9)(12)(13), referred to as the Validated Reference Method (VRM1 and VRM2, respectively). The Standard Operating Procedures (SOP) for the RhCE test methods are available and should be employed when implementing and using the test methods in a laboratory (34)(35). The following paragraphs and Appendix 2 describe the main components and procedures of the RhCE tests.

RHCE TEST METHOD COMPONENTS

General conditions

25. Relevant human-derived cells should be used to reconstruct the cornea-like epithelium three-dimensional tissue, which should be composed of progressively stratified but not cornified cells. The RhCE tissue construct is prepared in inserts with a porous synthetic membrane through which nutrients can pass to the cells. Multiple layers of viable, non-keratinised epithelial cells should be present in the reconstructed cornea-like epithelium. The RhCE tissue construct should have the epithelial surface in direct contact with air so as to allow for direct topical exposure of test chemicals in a fashion similar to how the corneal epithelium would be exposed *in vivo*. The RhCE tissue construct should form a functional barrier with sufficient robustness to resist rapid penetration of cytotoxic benchmark substances, e.g. Triton X-100 or sodium dodecyl sulphate (SDS). The barrier function should be demonstrated and may be assessed by determination of either the exposure time required to reduce tissue viability by 50% (ET₅₀) upon application of a benchmark substance at a specified, fixed concentration (e.g. 100 µl of 0.3% (v/v) Triton X-100), or the concentration at which a benchmark substance reduces the viability of the tissues by 50% (IC₅₀) following a fixed exposure time (e.g. 30 minutes treatment with 50 µl SDS) (see paragraph 30). The containment properties of the RhCE tissue construct should prevent the passage of test chemical around the edge of the viable tissue, which could

lead to poor modelling of corneal exposure. The human-derived cells used to establish the RhCE tissue construct should be free of contamination by bacteria, viruses, mycoplasma, and fungi. The sterility of the tissue construct should be checked by the supplier for absence of contamination by fungi and bacteria.

Functional conditions

Viability

26. The assay used for quantifying tissue viability is the MTT assay (16). Viable cells of the RhCE tissue construct reduce the vital dye MTT into a blue MTT formazan precipitate, which is then extracted from the tissue using isopropanol (or a similar solvent). The extracted MTT formazan may be quantified using either a standard absorbance (Optical Density (OD)) measurement or an HPLC/UPLC-spectrophotometry procedure (36). The OD of the extraction solvent alone should be sufficiently small, i.e. $OD < 0.1$. Users of the RhCE tissue construct should ensure that each batch of the RhCE tissue construct used meets defined criteria for the negative control. Acceptability ranges for the negative control OD values for the VRMs are given in Table 2. An HPLC/UPLC-spectrophotometry user should use the negative control OD ranges provided in Table 2 as the acceptance criterion for the negative control. It should be documented in the test report that the tissues treated with the negative control substance are stable in culture (provide similar tissue viability measurements) for the duration of the test exposure period. A similar procedure should be followed by the tissue producer as part of the quality control tissue batch release, but in this case different acceptance criteria than those specified in Table 2 may apply. An acceptability range (upper and lower limit) for the negative control OD values (in the QC test method conditions) should be established by the RhCE tissue construct developer/supplier.

Table 2: Acceptability ranges for negative control OD values (for the test users)

Test	Lower acceptance limit	Upper acceptance limit
EpiOcular™ EIT (OCL-200) – VRM1 (for both the liquids and the solids protocols)	$> 0.8^1$	< 2.5
SkinEthic™ HCE EIT (HCE/S) – VRM2 (for both the liquids and the solids protocols)	> 1.0	≤ 2.5

¹This acceptance limit considers the possibility of extended shipping/storage time (e.g. > 4 days), which has been shown not to impact on the performance of the test method (37).

Barrier function

27. The RhCE tissue construct should be sufficiently thick and robust to resist the rapid penetration of cytotoxic benchmark substances, as estimated e.g. by ET_{50} (Triton X-100) or by IC_{50} (SDS) (Table 3). The barrier function of each batch of the RhCE

tissue construct used should be demonstrated by the RhCE tissue construct developer/vendor upon supply of the tissues to the end user (see paragraph 30).

Morphology

28. Histological examination of the RhCE tissue construct should demonstrate human cornea-like epithelium structure (including at least 3 layers of viable epithelial cells and a non-keratinised surface). For the VRMs, appropriate morphology has been established by the developer/supplier and therefore does not need to be demonstrated again by a test method user for each tissue batch used.

Reproducibility

29. The results of the positive and negative controls of the test method should demonstrate reproducibility over time.

Quality control (QC)

30. The RhCE tissue construct should only be used if the developer/supplier demonstrates that each batch of the RhCE tissue construct used meets defined production release criteria, among which those for viability (paragraph 26) and barrier function (see paragraph 27) are the most relevant. An acceptability range (upper and lower limits) for the barrier functions as measured by the ET₅₀ or IC₅₀ (see paragraphs 25 and 26) should be established by the RhCE tissue construct developer/supplier. The ET₅₀ and IC₅₀ acceptability range used as QC batch release criterion by the developer/supplier of the RhCE tissue constructs (used in the VRMs) is given in Table 3. Data demonstrating compliance with all production release criteria should be provided by the RhCE tissue construct developer/supplier to the test method users so that they are able to include this information in the test report. Only results produced with tissues fulfilling all of these production release criteria can be accepted for reliable prediction of chemicals not requiring classification and labelling for eye irritation or serious eye damage in accordance with UN GHS/CLP.

Table 3: QC batch release criterion

Test	Lower acceptance limit	Upper acceptance limit
EpiOcular™ EIT (OCL-200) – VRM1 (100 µl of 0.3% (v/v) Triton X-100)	ET ₅₀ = 12.2 min	ET ₅₀ = 37.5 min
SkinEthic™ HCE EIT (HCE/S) – VRM2 (30 minutes treatment with 50 µl SDS)	IC ₅₀ = 1 mg/ml	IC ₅₀ = 3.2 mg/ml

Application of the Test Chemical and Control Substances

31. At least two tissue replicates should be used for each test chemical and each control substance in each run. Two different treatment protocols are used, one for liquid test chemicals and one for solid test chemicals (34)(35). For both methods and protocols,

the tissue construct surface should be moistened with calcium and magnesium-free Dulbecco's Phosphate Buffered Saline ($\text{Ca}^{2+}/\text{Mg}^{2+}$ -free DPBS) before application of test chemicals, to mimic the wet conditions of human eye. The treatment of the tissues is initiated with exposure to the test chemical(s) and control substances. For both treatment protocols in both VRMs, a sufficient amount of test chemical or control substance should be applied to uniformly cover the epithelial surface while avoiding an infinite dose (see paragraphs 32 and 33) (Appendix 2).

32. Test chemicals that can be pipetted at 37°C or lower temperatures (using a positive displacement pipette, if needed) are treated as liquids in the VRMs, otherwise they should be treated as solids (see paragraph 33). In the VRMs, liquid test chemical are evenly spread over the tissue surface (i.e. a minimum of 60 $\mu\text{l}/\text{cm}^2$ application) (see Appendix 2, (33)(34)). Capillary effects (surface tension effects) that may occur due to the low volumes applied to the insert (on the tissue surface) should be avoided to the extent possible to guarantee the correct dosing of the tissue. Tissues treated with liquid test chemicals are incubated for 30 min at standard culture conditions (37±2°C, 5±1% CO_2 , ≥95% RH). At the end of the exposure period, the liquid test chemical and the control substances should be carefully removed from the tissue surface by extensive rinsing with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free DPBS at room temperature. This rinsing step is followed by a post-exposure immersion in fresh medium at room temperature (to remove any test chemical absorbed into the tissue) for a pre-defined period of time that varies depending on the VRM used. For VMR1 only, a post-exposure incubation in fresh medium at standard culture conditions is applied prior to performing the MTT assay (see Appendix 2, (34)(35)).
33. Test chemicals that cannot be pipetted at temperatures up to 37°C are treated as solids in the VRMs. The amount of test chemical applied should be sufficient to cover the entire surface of the tissue, i.e. a minimum of 60 mg/cm^2 application should be used (Appendix 2). Whenever possible, solids should be tested as a fine powder. Tissues treated with solid test chemicals are incubated for a pre-defined period of time (depending on the VRM used) at standard culture conditions (see Appendix 2, (34)(35)). At the end of the exposure period, the solid test chemical and the control substances should be carefully removed from the tissue surface by extensive rinsing with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free DPBS at room temperature. This rinsing step is followed by a post-exposure immersion in fresh medium at room temperature (to remove any test chemical absorbed into the tissue) for a pre-defined period of time that varies depending on the VRM used, and a post-exposure incubation in fresh medium at standard culture conditions, prior to performing the MTT assay (see Appendix 2, (34)(35)).
34. Concurrent negative and positive controls should be included in each run to demonstrate that the viability (determined with the negative control) and the

sensitivity (determined with the positive control) of the tissues are within acceptance ranges defined based on historical data. The concurrent negative control also provides the baseline (100% tissue viability) to calculate the relative percent viability of the tissues treated with the test chemical (%Viability_{test}). The recommended positive control substance to be used with the VRMs is neat methyl acetate (CAS No 79-20-9, commercially available from e.g. Sigma-Aldrich, Cat# 45997; liquid). The recommended negative control substances to be used with the VRM1 and VRM2 are ultrapure H₂O and Ca²⁺/Mg²⁺-free DPBS, respectively. These were the control substances used in the validation studies of the VRMs and are those for which most historical data exist. The use of suitable alternative positive or negative control substances should be scientifically and adequately justified. Negative and positive controls should be tested with the same protocol(s) as the one(s) used for the test chemicals included in the run (i.e. for liquids and/or solids). This application should be followed by the treatment exposure, rinsing, a post-exposure immersion, and post-exposure incubation where applicable, as described for controls run concurrently to liquid test chemicals (see paragraph 32) or for controls run concurrently to solid test chemicals (see paragraph 33), prior to performing the MTT assay (see paragraph 35) (34)(35). One single set of negative and positive controls is sufficient for all test chemicals of the same physical state (liquids or solids) included in the same run.

Tissue Viability Measurements

35. The MTT assay is a standardised quantitative method (16) that should be used to measure tissue viability under this test method. It is compatible with use in a three-dimensional tissue construct. The MTT assay is performed immediately following the post-exposure incubation period. In the VRMs, the RhCE tissue construct sample is placed in 0.3 ml of MTT solution at 1 mg/ml for 180±15 min at standard culture conditions. The vital dye MTT is reduced into a blue MTT formazan precipitate by the viable cells of the RhCE tissue construct. The precipitated blue MTT formazan product is then extracted from the tissue using an appropriate volume of isopropanol (or a similar solvent) (34)(35). Tissues tested with liquid test chemicals should be extracted from both the top and the bottom of the tissues, while tissues tested with solid test chemicals and coloured liquids should be extracted from the bottom of the tissue only (to minimise any potential contamination of the isopropanol extraction solution with any test chemical that may have remained on the tissue). Tissues tested with liquid test chemicals that are not readily washed off may also be extracted from the bottom of the tissue only. The concurrently tested negative and positive control substances should be treated similarly to the tested chemical. The extracted MTT formazan may be quantified either by a standard absorbance (OD) measurement at 570 nm using a filter band pass of maximum ±30 nm or by using an HPLC/UPLC-spectrophotometry procedure (see paragraph 42) (11)(36).

36. Optical properties of the test chemical or its chemical action on MTT may interfere with the measurement of MTT formazan leading to a false estimate of tissue viability. Test chemicals may interfere with the measurement of MTT formazan by direct reduction of the MTT into blue MTT formazan and/or by colour interference if the test chemical absorbs, naturally or due to treatment procedures, in the same OD range as MTT formazan (i.e., around 570 nm). Pre-checks should be performed before testing to allow identification of potential direct MTT reducers and/or colour interfering chemicals and additional controls should be used to detect and correct for potential interference from such test chemicals (see paragraphs 37-41). This is especially important when a specific test chemical is not completely removed from the RhCE tissue construct by rinsing or when it penetrates the cornea-like epithelium and is therefore present in the RhCE tissue constructs when the MTT assay is performed. For test chemicals absorbing light in the same range as MTT formazan (naturally or after treatment), which are not compatible with the standard absorbance (OD) measurement of MTT formazan due to too strong interference, i.e., strong absorption at 570 ± 30 nm, an HPLC/UPLC-spectrophotometry procedure to measure MTT formazan may be employed (see paragraphs 41 and 42) (11)(36). A detailed description of how to detect and correct for direct MTT reduction and interferences by colouring agents is available in the VRMs SOPs (34)(35). Illustrative flowcharts providing guidance on how to identify and handle direct MTT-reducers and/or colour interfering chemicals for VRM1 and VRM2 are also provided in Appendices III and IV, respectively.
37. To identify potential interference by test chemicals absorbing light in the same range as MTT formazan (naturally or after treatment) and decide on the need for additional controls, the test chemical is added to water and/or isopropanol and incubated for an appropriate time at room temperature (see Appendix 2, (34)(35)). If the test chemical in water and/or isopropanol absorbs sufficient light in the range of 570 ± 20 nm for VRM1 (see Appendix 3), or if a coloured solution is obtained when mixing the test chemical with water for VRM2 (see Appendix 4), the test chemical is presumed to interfere with the standard absorbance (OD) measurement of MTT formazan and further colourant controls should be performed or, alternatively, an HPLC/UPLC-spectrophotometry procedure should be used in which case these controls are not required (see paragraphs 41 and 42 and Appendices III and IV)(34)(35). When performing the standard absorbance (OD) measurement, each interfering test chemical should be applied on at least two viable tissue replicates, which undergo the entire testing procedure but are incubated with medium instead of MTT solution during the MTT incubation step, to generate a non-specific colour in living tissues (NSC_{living}) control (34)(35). The NSC_{living} control needs to be performed concurrently to the testing of the coloured test chemical and, in case of multiple testing, an independent NSC_{living} control needs to be conducted with each test performed (in each run) due to

the inherent biological variability of living tissues. True tissue viability is calculated as: the percent tissue viability obtained with living tissues exposed to the interfering test chemical and incubated with MTT solution (%Viability_{test}) minus the percent non-specific colour obtained with living tissues exposed to the interfering test chemical and incubated with medium without MTT, run concurrently to the test being corrected (%NSC_{living}), i.e., True tissue viability = [%Viability_{test}] - [%NSC_{living}].

38. To identify direct MTT reducers, each test chemical should be added to freshly prepared MTT solution. An appropriate amount of test chemical is added to a MTT solution and the mixture is incubated for approximately 3 hours at standard culture conditions (see Appendices III and IV)(34)(35). If the MTT mixture containing the test chemical (or suspension for insoluble test chemicals) turns blue/purple, the test chemical is presumed to directly reduce MTT and a further functional check on non-viable RhCE tissue constructs should be performed, independently of using the standard absorbance (OD) measurement or an HPLC/UPLC-spectrophotometry procedure. This additional functional check employs killed tissues that possess only residual metabolic activity but absorb and retain the test chemical in a similar way as viable tissues. Killed tissues of VRM1 are prepared by exposure to low temperature ("freeze-killed"). Killed tissues of VRM2 are prepared by prolonged incubation (e.g. at least 24±1 hours) in water followed by storage to low temperature ("water-killed"). Each MTT reducing test chemical is applied on at least two killed tissue replicates, which undergo the entire testing procedure, to generate a non-specific MTT reduction (NSMTT) control (34)(35). A single NSMTT control is sufficient per test chemical regardless of the number of independent tests/runs performed. True tissue viability is calculated as: the percent tissue viability obtained with living tissues exposed to the MTT reducer (%Viability_{test}) minus the percent non-specific MTT reduction obtained with the killed tissues exposed to the same MTT reducer, calculated relative to the negative control run concurrently to the test being corrected (%NSMTT), i.e., True tissue viability = [%Viability_{test}] - [%NSMTT].
39. Test chemicals that are identified as producing both colour interference (see paragraph 37) and direct MTT reduction (see paragraph 38) will also require a third set of controls when performing the standard absorbance (OD) measurement, apart from the NSMTT and NSC_{living} controls described in the previous paragraphs. This is usually the case with darkly coloured test chemicals absorbing light in the range of 570±30 nm (e.g. blue, purple, black) because their intrinsic colour impedes the assessment of their capacity to directly reduce MTT as described in paragraph 38. This forces the use of NSMTT controls, by default, together with the NSC_{living} controls. Test chemicals for which both NSMTT and NSC_{living} controls are performed may be absorbed and retained by both living and killed tissues. Therefore, in this case, the NSMTT control may not only correct for potential direct MTT reduction by the

test chemical, but also for colour interference arising from the absorption and retention of the test chemical by killed tissues. This could lead to double correction for colour interference since the NSC_{living} control already corrects for colour interference arising from the absorption and retention of the test chemical by living tissues. To avoid a possible double correction for colour interference, a third control for non-specific colour in killed tissues (NSC_{killed}) needs to be performed (see Appendices III and IV)(34)(35). In this additional control, the test chemical is applied on at least two killed tissue replicates, which undergo the entire testing procedure but are incubated with medium instead of MTT solution during the MTT incubation step. A single NSC_{killed} control is sufficient per test chemical regardless of the number of independent tests/runs performed, but should be performed concurrently to the NSMTT control and with the same tissue batch. True tissue viability is calculated as: the percent tissue viability obtained with living tissues exposed to the test chemical (%Viability_{test}) minus %NSMTT minus %NSC_{living} plus the percent non-specific colour obtained with killed tissues exposed to the interfering test chemical and incubated with medium without MTT, calculated relative to the negative control ran concurrently to the test being corrected (%NSC_{killed}), i.e., True tissue viability = [%Viability_{test}] - [%NSMTT] - [%NSC_{living}] + [%NSC_{killed}].

40. It is important to note that non-specific MTT reduction and non-specific colour interferences may increase the OD (when performing standard absorbance measurements) of the tissue extract above the linearity range of the spectrophotometer and that non-specific MTT reduction can also increase the MTT formazan peak area (when performing HPLC/UPLC-spectrophotometry measurements) of the tissue extract above the linearity range of the spectrophotometer. On this basis, it is important for each laboratory to determine the OD/peak area linearity range of their spectrophotometer with e.g. MTT formazan (CAS # 57360-69-7), commercially available from e.g. Sigma-Aldrich (Cat# M2003), before initiating the testing of test chemicals for regulatory purposes.
41. The standard absorbance (OD) measurement using a spectrophotometer is appropriate to assess direct MTT-reducers and colour interfering test chemicals, when the observed interference with the measurement of MTT formazan is not too strong (i.e., the ODs of the tissue extracts obtained with the test chemical without any correction for direct MTT reduction and/or colour interference are within the linear range of the spectrophotometer). Nevertheless, results for test chemicals producing %NSMTT and/or %NSC_{living} ≥ 60% (VRM1, and VRM2 for liquids' protocol) or 50% (VRM2 for solids' protocol) of the negative control should be taken with caution as this is the established cut-off used in the VRMs to distinguish classified from not classified chemicals (see paragraph 44). Standard absorbance (OD) can however not be measured when the interference with the measurement of MTT formazan is too strong

(*i.e.*, leading to uncorrected ODs of the test tissue extracts falling outside of the linear range of the spectrophotometer). Coloured test chemicals or test chemicals that become coloured in contact with water or isopropanol that interfere too strongly with the standard absorbance (OD) measurement of MTT formazan may still be assessed using HPLC/UPLC-spectrophotometry (see Appendices III and IV). This is because the HPLC/UPLC system allows for the separation of the MTT formazan from the chemical before its quantification (36). For this reason, NSC_{living} or NSC_{killed} controls are never required when using HPLC/UPLC-spectrophotometry, independently of the chemical being tested. NSMTT controls should nevertheless be used if the test chemical is suspected to directly reduce MTT (following the procedure described in paragraph 38). NSMTT controls should also be used with test chemicals having a colour (intrinsic or appearing when in water) that impedes the assessment of their capacity to directly reduce MTT as described in paragraph 38. When using HPLC/UPLC-spectrophotometry to measure MTT formazan, the percent tissue viability is calculated as percent MTT formazan peak area obtained with living tissues exposed to the test chemical relative to the MTT formazan peak obtained with the concurrent negative control. For test chemicals able to directly reduce MTT, true tissue viability is calculated as: %Viability_{test} minus %NSMTT, as described in the last sentence of paragraph 38. Finally, it should be noted that direct MTT-reducers or direct MTT-reducers that are also colour interfering, which are retained in the tissues after treatment and reduce MTT so strongly that they lead to ODs (using standard OD measurement) or peak areas (using UPLC/HPLC-spectrophotometry) of the tested tissue extracts that fall outside of the linearity range of the spectrophotometer cannot be assessed with RhCE test methods, although these are expected to occur in only very rare situations.

42. HPLC/UPLC-spectrophotometry may be used with all types of test chemicals (coloured, non-coloured, MTT-reducers and non-MTT reducers) for measurement of MTT formazan (11)(36). Due to the diversity of HPLC/UPLC-spectrophotometry systems, it is not feasible for each user to establish the exact same system conditions. As such, qualification of the HPLC/UPLC-spectrophotometry system should be demonstrated before its use to quantify MTT formazan from tissue extracts by meeting the acceptance criteria for a set of standard qualification parameters based on those described in the U.S. Food and Drug Administration guidance for industry on bioanalytical method validation (36)(38). These key parameters and their acceptance criteria are shown in Appendix 5. Once the acceptance criteria defined in Appendix 5 have been met, the HPLC/UPLC-spectrophotometry system is considered qualified and ready to measure MTT formazan under the experimental conditions described in this test method.

Acceptance Criteria

43. For each run using RhCE tissue batches that met the quality control (see paragraph 30), tissues treated with the negative control substance should exhibit OD reflecting the quality of the tissues that followed shipment, receipt steps and all protocol processes and should not be outside the historically established boundaries described in Table 2 (see paragraph 26). Similarly, tissues treated with the positive control substance, i.e., methyl acetate, should show a mean tissue viability $< 50\%$ relative to the negative control in the VRM1 with either the liquids' or the solids' protocols, and $\leq 30\%$ (liquids' protocol) or $\leq 20\%$ (solids' protocol) relative to the negative control in the VRM2, thus reflecting the ability of the tissues to respond to an irritant test chemical under the conditions of the test method (34)(35). The variability between tissue replicates of test chemicals and control substances should fall within the accepted limits (i.e., the difference of viability between two tissue replicates should be less than 20% or the standard deviation (SD) between three tissue replicates should not exceed 18%). If either the negative control or positive control included in a run is outside of the accepted ranges, the run is considered "non-qualified" and should be repeated. If the variability between tissue replicates of a test chemical is outside of the accepted range, the test must be considered "non-qualified" and the test chemical should be re-tested.

Interpretation of Results and Prediction Model

44. The OD values/peak areas obtained with the replicate tissue extracts for each test chemical should be used to calculate the mean percent tissue viability (mean between tissue replicates) normalised to the negative control, which is set at 100%. The percentage tissue viability cut-off value for identifying test chemicals not requiring classification for eye irritation or serious eye damage (UN GHS and CLP No Category) is given in Table 4. Results should thus be interpreted as follows:

- The test chemical is identified as not requiring classification and labelling according to UN GHS and CLP (No Category) if the mean percent tissue viability after exposure and post-exposure incubation is more than ($>$) the established percentage tissue viability cut-off value, as shown in Table 4. In this case no further testing in other test methods is required.
- If the mean percent tissue viability after exposure and post-exposure incubation is less than or equal ($\leq$) to the established percentage tissue viability cut-off value, no prediction can be made, as shown in Table 4. In this case, further testing with other test methods will be required because RhCE test methods show a certain number of false positive results (see paragraphs 14-15) and cannot resolve between UN GHS and CLP Categories 1 and 2 (see paragraph 17).

Table 4: Prediction Models according to UN GHS and CLP classification

VRM	No Category	No prediction can be made
VRM 1 - EpiOcular™ EIT (for both protocols)	Mean tissue viability > 60%	Mean tissue viability ≤ 60%
VRM 2 - SkinEthic™ HCE EIT (for the liquids' protocol)	Mean tissue viability > 60%	Mean tissue viability ≤ 60%
VRM2 - SkinEthic™ HCE EIT (for the solids' protocol)	Mean tissue viability > 50%	Mean tissue viability ≤ 50%

45. A single test composed of at least two tissue replicates should be sufficient for a test chemical when the result is unequivocal. However, in cases of borderline results, such as non-concordant replicate measurements and/or mean percent tissue viability equal to 60±5% (VRM1, and VRM2 for liquids' protocol) or 50±5% (VRM2 for solids' protocol), a second test should be considered, as well as a third one in case of discordant results between the first two tests.
46. Different percentage tissue viability cut-off values distinguishing classified from non-classified test chemicals may be considered for specific types of mixtures, where appropriate and justifiable, in order to increase the overall performance of the test method for those types of mixtures (see paragraph 14). Benchmark chemicals may be useful for evaluating the serious eye damage/eye irritation potential of unknown test chemicals or product class, or for evaluating the relative ocular toxicity potential of a classified chemical within a specific range of positive responses.

DATA AND REPORTING

Data

47. Data from individual replicate tissues in a run (e.g. OD values/MTT formazan peak areas and calculated percent tissue viability data for the test chemical and controls, and the final RhCE test method prediction) should be reported in tabular form for each test chemical, including data from repeat tests, as appropriate. In addition, mean percent tissue viability and difference of viability between two tissue replicates (if n=2 replicate tissues) or SD (if n≥3 replicate tissues) for each individual test chemical and control should be reported. Any observed interferences of a test chemical with the measurement of MTT formazan through direct MTT reduction and/or coloured interference should be reported for each tested chemical.

Test Report

48. The test report should include the following information:

Test Chemical

Mono-constituent substance

- Chemical identification, such as IUPAC or CAS name(s), CAS registry number(s), SMILES or InChI code, structural formula, and/or other identifiers;
- Physical state, volatility, pH, LogP, molecular weight, chemical class, and additional relevant physicochemical properties relevant to the conduct of the study, to the extent available;
- Purity, chemical identity of impurities as appropriate and practically feasible, *etc.*;
- Treatment prior to testing, if applicable (e.g. warming, grinding);
- Storage conditions and stability to the extent available.

Multi-constituent substance, UVCB and mixture

- Characterisation as far as possible by e.g. chemical identity (see above), purity, quantitative occurrence and relevant physicochemical properties (see above) of the constituents, to the extent available;
- Physical state and additional relevant physicochemical properties relevant to the conduct of the study, to the extent available;
- Purity, chemical identity of impurities as appropriate and practically feasible, *etc.*;
- Treatment prior to testing, if applicable (e.g. warming, grinding);
- Storage conditions and stability to the extent available.

Positive and Negative Control Substances

- Chemical identification, such as IUPAC or CAS name(s), CAS registry number(s), SMILES or InChI code, structural formula, and/or other identifiers;
- Physical state, volatility, molecular weight, chemical class, and additional relevant physicochemical properties relevant to the conduct of the study, to the extent available;
- Purity, chemical identity of impurities as appropriate and practically feasible, *etc.*;
- Treatment prior to testing, if applicable (e.g. warming, grinding);
- Storage conditions and stability to the extent available;
- Justification for the use of a different negative control than ultrapure H₂O or Ca²⁺/Mg²⁺-free DPBS, if applicable;
- Justification for the use of a different positive control than neat methyl acetate, if applicable;

- Reference to historical positive and negative control results demonstrating suitable run acceptance criteria.

Information Concerning the Sponsor and the Test Facility

- Name and address of the sponsor, test facility and study director.

RhCE Tissue Construct and Protocol Used (providing rationale for the choices, if applicable)

Test Method Conditions

- RhCE tissue construct used, including batch number;
- Wavelength and band pass (if applicable) used for quantifying MTT formazan, and linearity range of measuring device (e.g. spectrophotometer);
- Description of the method used to quantify MTT formazan;
- Description of the HPLC/UPLC-spectrophotometry system used, if applicable;
- Complete supporting information for the specific RhCE tissue construct used including its performance. This should include, but is not limited to:
 - i) Viability quality control (supplier)
 - ii) Viability under test method conditions (user);
 - iii) Barrier function quality control;
 - iv) Morphology, if available;
 - v) Reproducibility and predictive capacity;
 - vi) Other quality controls (QC) of the RhCE tissue construct, if available;
- Reference to historical data of the RhCE tissue construct. This should include, but is not limited to: Acceptability of the QC data with reference to historical batch data;
- Statement that the testing facility has demonstrated proficiency in the use of the test method before routine use by testing of the proficiency chemicals;

Run and Test Acceptance Criteria

- Positive and negative control means and acceptance ranges based on historical data;
- Acceptable variability between tissue replicates for positive and negative controls;
- Acceptable variability between tissue replicates for the test chemical;

Test Procedure

- Details of the test procedure used;
- Doses of test chemical and control substances used;
- Duration and temperature of exposure, post-exposure immersion and post-exposure incubation periods (where applicable);

- Description of any modifications to the test procedure;
- Indication of controls used for direct MTT-reducers and/or colouring test chemicals, if applicable;
- Number of tissue replicates used per test chemical and controls (positive control, negative control, NSMTT, NSCliving and NSCKilled, if applicable);

Results

- Tabulation of data from individual test chemicals and control substances for each run (including repeat experiments where applicable) and each replicate measurement, including OD value or MTT formazan peak area, percent tissue viability, mean percent tissue viability, Difference between tissue replicates or SD, and final prediction;
- If applicable, results of controls used for direct MTT-reducers and/or coloured test chemicals, including OD value or MTT formazan peak area, %NSMTT, %NSCliving, %NSCKilled, Difference between tissue replicates or SD, final correct percent tissue viability, and final prediction;
- Results obtained with the test chemical(s) and control substances in relation to the define run and test acceptance criteria;
- Description of other effects observed, e.g. coloration of the tissues by a coloured test chemical;

Discussion of the Results

Conclusion

LITERATURE

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- (2) Chapter B.5 of this Annex, Acute Eye Irritation/Corrosion.
- (3) Chapter B.47 of this Annex, Bovine Corneal Opacity and Permeability Test Method for Identifying i) Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring Classification for Eye Irritation or Serious Eye Damage.
- (4) Chapter B.48 of this Annex, Isolated Chicken Eye Test Method for Identifying i) Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring Classification.
- (5) Chapter B.61 of this Annex, Fluorescein Leakage Test Method for Identifying Ocular Corrosives and Severe Irritants.
- (6) Chapter B.68 of this Annex, Short Time Exposure *In Vitro* Test Method for Identifying i) Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring Classification for Eye Irritation or Serious Eye Damage.
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Appendix 1

DEFINITIONS

Accuracy: The closeness of agreement between test method results and accepted reference values. It is a measure of test method performance and one aspect of “relevance.” The term is often used interchangeably with “concordance”, to mean the proportion of correct outcomes of a test method (18).

Benchmark chemical: A chemical used as a standard for comparison to a test chemical. A benchmark chemical should have the following properties: (i) consistent and reliable source(s) for its identification and characterisation; (ii) structural, functional and/or chemical or product class similarity to the chemical(s) being tested; (iii) known physicochemical characteristics; (iv) supporting data on known effects; and (v) known potency in the range of the desired response.

Bottom-Up approach: Step-wise approach used for a test chemical suspected of not requiring classification and labelling for eye irritation or serious eye damage, which starts with the determination of chemicals not requiring classification and labelling (negative outcome) from other chemicals (positive outcome).

Chemical: A substance or mixture.

Concordance: See "Accuracy".

Cornea: The transparent part of the front of the eyeball that covers the iris and pupil and admits light to the interior.

CV: Coefficient of Variation.

Dev: Deviation.

EIT: Eye Irritation Test.

EURL ECVAM: European Union Reference Laboratory for Alternatives to Animal Testing.

Eye irritation: Production of changes in the eye following the application of a test chemical to the anterior surface of the eye, which are fully reversible within 21 days of application. Interchangeable with “Reversible effects on the eye” and with “UN GHS/CLP Category 2”.

ET₅₀: Exposure time required to reduce tissue viability by 50% upon application of a benchmark chemical at a specified, fixed concentration.

False negative rate: The proportion of all positive substances falsely identified by a test method as negative. It is one indicator of test method performance.

False positive rate: The proportion of all negative substances that are falsely identified by a

test method as positive. It is one indicator of test method performance.

Hazard: Inherent property of an agent or situation having the potential to cause adverse effects when an organism, system or (sub) population is exposed to that agent.

HCE: SkinEthic™ Human Corneal Epithelium.

HPLC: High Performance Liquid Chromatography.

IC₅₀: Concentration at which a benchmark chemical reduces the viability of the tissues by 50% following a fixed exposure time (e.g. 30 minutes treatment with SDS).

Infinite dose: Amount of test chemical applied to the RhCE tissue construct exceeding the amount required to completely and uniformly cover the epithelial surface.

Irreversible effects on the eye: See “Serious eye damage”.

LLOQ: Lower Limit of Quantification.

LogP: Logarithm of the octanol-water partitioning coefficient

Mixture: A mixture or a solution composed of two or more substances.

Mono-constituent substance: A substance, defined by its quantitative composition, in which one main constituent is present to at least 80% (w/w).

Multi-constituent substance: A substance, defined by its quantitative composition, in which more than one main constituent is present in a concentration $\geq 10\%$ (w/w) and $< 80\%$ (w/w). A multi-constituent substance is the result of a manufacturing process. The difference between mixture and multi-constituent substance is that a mixture is obtained by blending of two or more substances without chemical reaction. A multi-constituent substance is the result of a chemical reaction.

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Thiazolyl blue tetrazolium bromide.

Negative control: A sample containing all components of a test system and treated with a substance known not to induce a positive response in the test system. This sample is processed with test chemical-treated samples and other control samples and is used to determine 100% tissue viability.

Not Classified: Chemicals that are not classified for Eye irritation (UN GHS/CLP Category 2, UN GHS Category 2A or 2B) or Serious eye damage (UN GHS/CLP Category 1). Interchangeable with “UN GHS/CLP No Category”.

NSC_{killed}: Non-Specific Colour in killed tissues.

NSC_{living}: Non-Specific Colour in living tissues.

NSMTT: Non-Specific MTT reduction.

OD: Optical Density.

Performance standards: Standards, based on a validated test method which was considered scientifically valid, that provide a basis for evaluating the comparability of a proposed test method that is mechanistically and functionally similar. Included are: (i) essential test method components; (ii) a minimum list of Reference Chemicals selected from among the chemicals used to demonstrate the acceptable performance of the validated test method; and (iii) the comparable levels of accuracy and reliability, based on what was obtained for the validated test method, that the proposed test method should demonstrate when evaluated using the minimum list of Reference Chemicals (18).

Positive control: A sample containing all components of a test system and treated with a substance known to induce a positive response in the test system. This sample is processed with test chemical-treated samples and other control samples. To ensure that variability in the positive control response across time can be assessed, the magnitude of the positive response should not be excessive.

Relevance: Description of relationship of the test to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the test correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of the accuracy (concordance) of a test method (18).

Reliability: Measures of the extent that a test method can be performed reproducibly within and between laboratories over time, when performed using the same protocol. It is assessed by calculating intra- and inter-laboratory reproducibility and intra-laboratory repeatability (18).

Replacement test: A test which is designed to substitute for a test that is in routine use and accepted for hazard identification and/or risk assessment, and which has been determined to provide equivalent or improved protection of human or animal health or the environment, as applicable, compared to the accepted test, for all possible testing situations and chemicals (18).

Reproducibility: The agreement among results obtained from repeated testing of the same test chemical using the same test protocol (See "Reliability") (18).

Reversible effects on the eye: See "Eye irritation".

RhCE: Reconstructed human Cornea-like Epithelium.

Run: A run consists of one or more test chemicals tested concurrently with a negative control and with a positive control.

SD: Standard Deviation.

Sensitivity: The proportion of all positive/active test chemicals that are correctly classified

by the test. It is a measure of accuracy for a test method that produces categorical results, and is an important consideration in assessing the relevance of a test method (18).

Serious eye damage: Production of tissue damage in the eye, or serious physical decay of vision, following application of a test substance to the anterior surface of the eye, which is not fully reversible within 21 days of application. Interchangeable with “Irreversible effects on the eye” and with “UN GHS and CLP Category 1”.

Standard Operating Procedures (SOP): Formal, written procedures that describe in detail how specific routine, and test-specific, laboratory operations should be performed. They are required by GLP.

Specificity: The proportion of all negative/inactive test chemicals that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results and is an important consideration in assessing the relevance of a test method (18).

Substance: A chemical element and its compounds in the natural state or obtained by any production process, inducing any additive necessary to preserve its stability and any impurities deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the substance or changing its composition.

Test: A single test chemical concurrently tested in a minimum of two tissue replicates as defined in the corresponding SOP.

Tissue viability: Parameter measuring total activity of a cell population in a reconstructed tissue as their ability to reduce the vital dye MTT, which, depending on the endpoint measured and the test design used, correlates with the total number and/or vitality of living cells.

Top-Down approach: Step-wise approach used for a chemical suspected of causing serious eye damage, which starts with the determination of chemicals inducing serious eye damage (positive outcome) from other chemicals (negative outcome).

Test chemical: Any substance or mixture tested using this test method.

Tiered testing strategy: A stepwise testing strategy, which uses test methods in a sequential manner. All existing information on a test chemical is reviewed at each tier, using a weight-of-evidence process, to determine if sufficient information is available for a hazard classification decision, prior to progression to the next tier in the strategy. If the hazard potential/potency of a test chemical can be assigned based on the existing information at a given tier, no additional testing is required (18).

ULOQ: Upper Limit of Quantification.

United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS): A system proposing the classification of chemicals (substances and mixtures) according to standardised types and levels of physical, health and environmental hazards, and addressing corresponding communication elements, such as

pictograms, signal words, hazard statements, precautionary statements and safety data sheets, so that to convey information on their adverse effects with a view to protect people (including employers, workers, transporters, consumers and emergency responders) and the environment (1).

UN GHS and CLP Category 1: See “Serious eye damage”.

UN GHS and CLP Category 2: See “Eye irritation”.

UN GHS and CLP No Category: Chemicals that do not meet the requirements for classification as UN GHS/CLP Category 1 or 2 (or UN GHS Category 2A or 2B). Interchangeable with “Not Classified”.

UPLC: Ultra-High Performance Liquid Chromatography.

UVCB: substances of unknown or variable composition, complex reaction products or biological materials.

Valid test method: A test method considered to have sufficient relevance and reliability for a specific purpose and which is based on scientifically sound principles. A test method is never valid in an absolute sense, but only in relation to a defined purpose (18).

Validated test method: A test method for which validation studies have been completed to determine the relevance (including accuracy) and reliability for a specific purpose. It is important to note that a validated test method may not have sufficient performance in terms of accuracy and reliability to be found acceptable for the proposed purpose (18).

VRM: Validated Reference Method.

VRM1: EpiOcular™ EIT is referred as the Validated Reference Method 1.

VRM2: SkinEthic™ HCE EIT is referred to as the Validated Reference Method 2.

Weight-of-evidence: The process of considering the strengths and weaknesses of various pieces of information in reaching and supporting a conclusion concerning the hazard potential of a test substance.

Appendix 2

MAIN TEST COMPONENTS OF THE RHCE TESTS VALIDATED FOR IDENTIFYING CHEMICALS NOT REQUIRING CLASSIFICATION AND LABELLING FOR EYE IRRITATION OR SEERIOUS EYE DAMAGE

Test Components	EpiOcular™ EIT (VRM 1)		SkinEthic™ HCE EIT (VRM 2)	
Protocols	Liquids (pipetteable at 37±1°C or lower temperatures for 15 min)	Solids (not pipetteable)	Liquids and viscous (pipetteable)	Solids (not pipetteable)
Model surface	0.6 cm ²	0.6 cm ²	0.5 cm ²	0.5 cm ²
Number of tissue replicates	At least 2	At least 2	At least 2	At least 2
Pre-check for colour interference	50 µl + 1 ml H₂O for 60 min at 37±2°C, 5±1% CO₂, ≥95% RH (non-coloured test chemicals), or 50 µl + 2 ml isopropanol mixed for 2-3h at RT (coloured test chemicals) → if the OD of the test chemical at 570±20 nm, after subtraction of the OD for isopropanol or water is > 0.08 (which corresponds to approximately 5% of the mean OD of the negative control), living adapted controls should be performed.	50 mg + 1 ml H₂O for 60 min at 37±2°C, 5±1% CO₂, ≥95% RH (non-coloured test chemicals) and/or 50 mg + 2 ml isopropanol mixed for 2-3h at RT (colored and non-colored test chemicals) → if the OD of the test chemical at 570±20 nm after subtraction of the OD for isopropanol or water is > 0.08 (which corresponds to approximately 5% of the mean OD of the negative control), living adapted controls should be performed.	10 µl + 90 µl H₂O mixed for 30±2 min at Room Temperature (RT, 18-28°C) → if test chemical is coloured, living adapted controls should be performed	10 mg + 90 µl H₂O mixed for 30±2 min at RT → if test chemical is coloured, living adapted controls should be performed

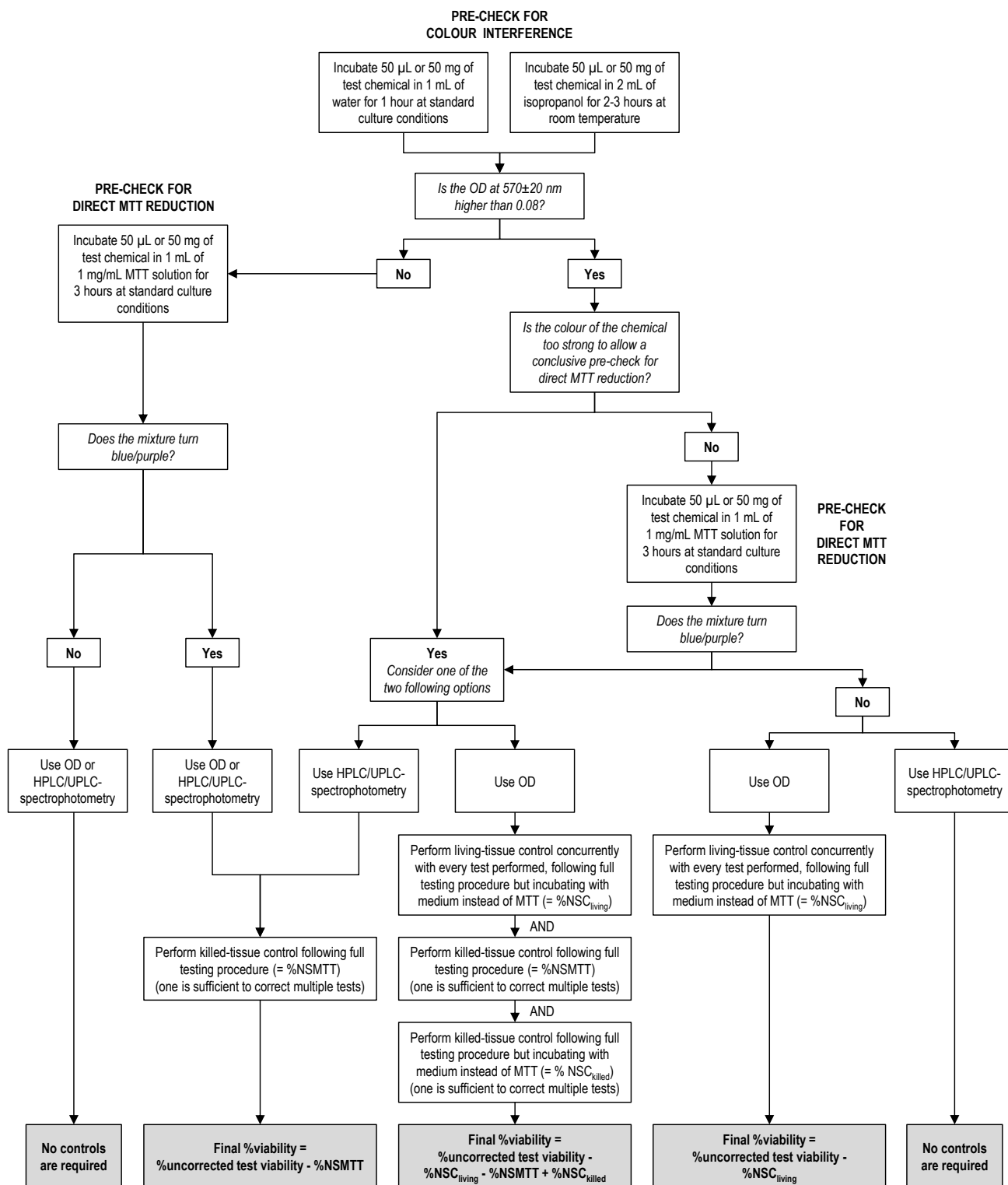
Test Components	EpiOcular™ EIT (VRM 1)		SkinEthic™ HCE EIT (VRM 2)	
Pre-check for direct MTT reduction	50 µl + 1 ml MTT 1 mg/ml solution for 180±15 min at 37±2°C, 5±1% CO ₂ , ≥95% RH → if solution turns blue/purple, freeze-killed adapted controls should be performed (50 µl of sterile deionised water in MTT solution is used as negative control)	50 mg + 1 ml MTT 1 mg/ml solution for 180±15 min at 37±2°C, 5±1% CO ₂ , ≥95% RH → if solution turns blue/purple, freeze-killed adapted controls should be performed (50 µl of sterile deionised water in MTT solution is used as negative control)	30 µl + 300 µl MTT 1 mg/ml solution for 180± 15 min at 37±2°C, 5±1% CO ₂ , ≥95% RH → if solution turns blue/purple, water-killed adapted controls should be performed (30 µl of sterile deionised water in MTT solution is used as negative control)	30 mg + 300 µl MTT 1 mg/ml solution for 180± 15 min at 37±2°C, 5±1% CO ₂ , ≥95% RH → if solution turns blue/purple, water-killed adapted controls should be performed (30 µl of sterile deionised water in MTT solution is used as negative control)
Pre-treatment	20 µl Ca ²⁺ /Mg ²⁺ -free DPBS for 30 ± 2 min at 37±2°C, 5±1% CO ₂ , ≥95% RH, protected from light.	20 µl Ca ²⁺ /Mg ²⁺ -free DPBS for 30±2 min at 37±2°C, 5±1% CO ₂ , ≥95% RH, protected from light.	-	-
Treatment doses and application	50 µl (83.3 µl/cm ²)	50 mg (83.3 mg/cm ²) using a calibrated tool (e.g. a levelled spoonful calibrated to hold 50 mg of sodium chloride).	10 µl Ca ²⁺ /Mg ²⁺ -free DPBS + 30 ± 2 µl (60 µl/cm ²) For viscous, use a nylon mesh	30 µl Ca ²⁺ /Mg ²⁺ -free DPBS + 30 ± 2 mg (60 mg/cm ²)
Exposure time and temperature	30 min (± 2 min) in culture medium at 37±2°C, 5±1% CO ₂ , ≥95% RH	6 hours (± 0.25 h) in culture medium at 37±2°C, 5±1% CO ₂ , ≥95% RH	30 min (± 2 min) in culture medium at 37±2°C, 5±1% CO ₂ , ≥95% RH	4 hours (± 0.1 h) in culture medium at 37±2°C, 5±1% CO ₂ , ≥95% RH
Rinsing at room temperature	3 times in 100 ml of Ca ²⁺ /Mg ²⁺ -free DPBS	3 times in 100 ml of Ca ²⁺ /Mg ²⁺ -free DPBS	20 ml Ca ²⁺ /Mg ²⁺ -free DPBS	25 ml Ca ²⁺ /Mg ²⁺ -free DPBS
Post-exposure immersion	12 min (± 2 min) at RT in culture medium	25 min (± 2 min) at RT in culture medium	30 min (± 2 min) at 37°C, 5% CO ₂ , 95% RH in culture medium	30 min (± 2 min) at RT in culture medium
Post-exposure incubation	120 min (± 15 min) in culture medium at 37±2°C, 5±1% CO ₂ , ≥95% RH	18 h (± 0.25 h) in culture medium at 37±2°C, 5±1% CO ₂ , ≥95% RH	none	18 h (± 0.5 h) in culture medium at 37±2°C, 5±1% CO ₂ , ≥95% RH

Test Components	EpiOcular™ EIT (VRM 1)		SkinEthic™ HCE EIT (VRM 2)	
Negative control	50 µl H ₂ O Tested concurrently	50 µl H ₂ O Tested concurrently	30 ± 2µl Ca ²⁺ /Mg ²⁺ -free DPBS Tested concurrently	30 ± 2µl Ca ²⁺ /Mg ²⁺ -free DPBS Tested concurrently
Positive control	50 µl Methyl acetate Tested concurrently	50 µl Methyl acetate Tested concurrently	30 ± 2µl Methyl acetate Tested concurrently	30 ± 2µl Methyl acetate Tested concurrently
MTT solution	300 µl 1 mg/ml	300 µl 1 mg/ml	300 µl 1 mg/ml	300 µl 1 mg/ml
MTT incubation time and temperature	180 min (± 15 min) at 37±2°C, 5±1% CO ₂ , ≥95% RH	180 min (± 15 min) at 37±2°C, 5±1% CO ₂ , ≥95% RH	180 min (± 15 min) at 37±2°C, 5±1% CO ₂ , ≥95% RH	180 min (± 15 min) at 37±2°C, 5±1% CO ₂ , ≥95% RH
Extraction solvent	2 ml isopropanol (extraction from top and bottom of insert by piercing the tissue)	2 ml isopropanol (extraction from bottom of insert by piercing the tissue)	1.5 ml isopropanol (extraction from top and bottom of insert)	1.5 ml isopropanol (extraction from bottom of insert)
Extraction time and temperature	2-3 h with shaking (~120 rpm) at RT or overnight at 4-10°C	2-3 h with shaking (~120 rpm) at RT or overnight at 4-10°C	4 h with shaking (~120 rpm) at RT or at least overnight without shaking at 4-10°C	At least 2 h with shaking (~120 rpm) at RT
OD reading	570 nm (550 - 590 nm) without reference filter	570 nm (550-590 nm) without reference filter	570 nm (540 - 600 nm) without reference filter	570 nm (540 - 600 nm) without reference filter
Tissue Control Quality	Treatment with 100 µl of 0.3% (v/v) Triton X-100 12.2 min ≤ ET ₅₀ ≤ 37.5 min	Treatment with 100 µl of 0.3% (v/v) Triton X-100 12.2 min ≤ ET ₅₀ ≤ 37.5 min	30 min treatment with SDS (50 µl) 1.0 mg/ml ≤ IC ₅₀ ≤ 3.5 mg/ml	30 min treatment with SDS (50 µl) 1.0 mg/ml ≤ IC ₅₀ ≤ 3.2 mg/ml

Test Components	EpiOcular™ EIT (VRM 1)		SkinEthic™ HCE EIT (VRM 2)	
Acceptance Criteria	1. Mean OD of the tissue replicates treated with the negative control should be > 0.8 and < 2.5 2. Mean viability of the tissue replicates exposed for 30 min with the positive control, expressed as % of the negative control, should be < 50% 3. The difference of viability between two tissue replicates should be less than 20%.	1. Mean OD of the tissue replicates treated with the negative control should be > 0.8 and < 2.5 2. Mean viability of the tissue replicates exposed for 6 hours with the positive control, expressed as % of the negative control, should be < 50% 3. The difference of viability between two tissue replicates should be less than 20%.	1. Mean OD of the tissue replicates treated with the negative control should be > 1.0 and ≤ 2.5 2. Mean viability of the tissue replicates exposed for 30 min with the positive control, expressed as % of the negative control, should be ≤ 30% 3. The difference of viability between two tissue replicates should be less than 20%.	1. Mean OD of the tissue replicates treated with the negative control should be > 1.0 and ≤ 2.5 2. Mean viability of the tissue replicates exposed for 4 hours with the positive control, expressed as % of the negative control, should be ≤ 20% 3. The difference of viability between two tissue replicates should be less than 20%.

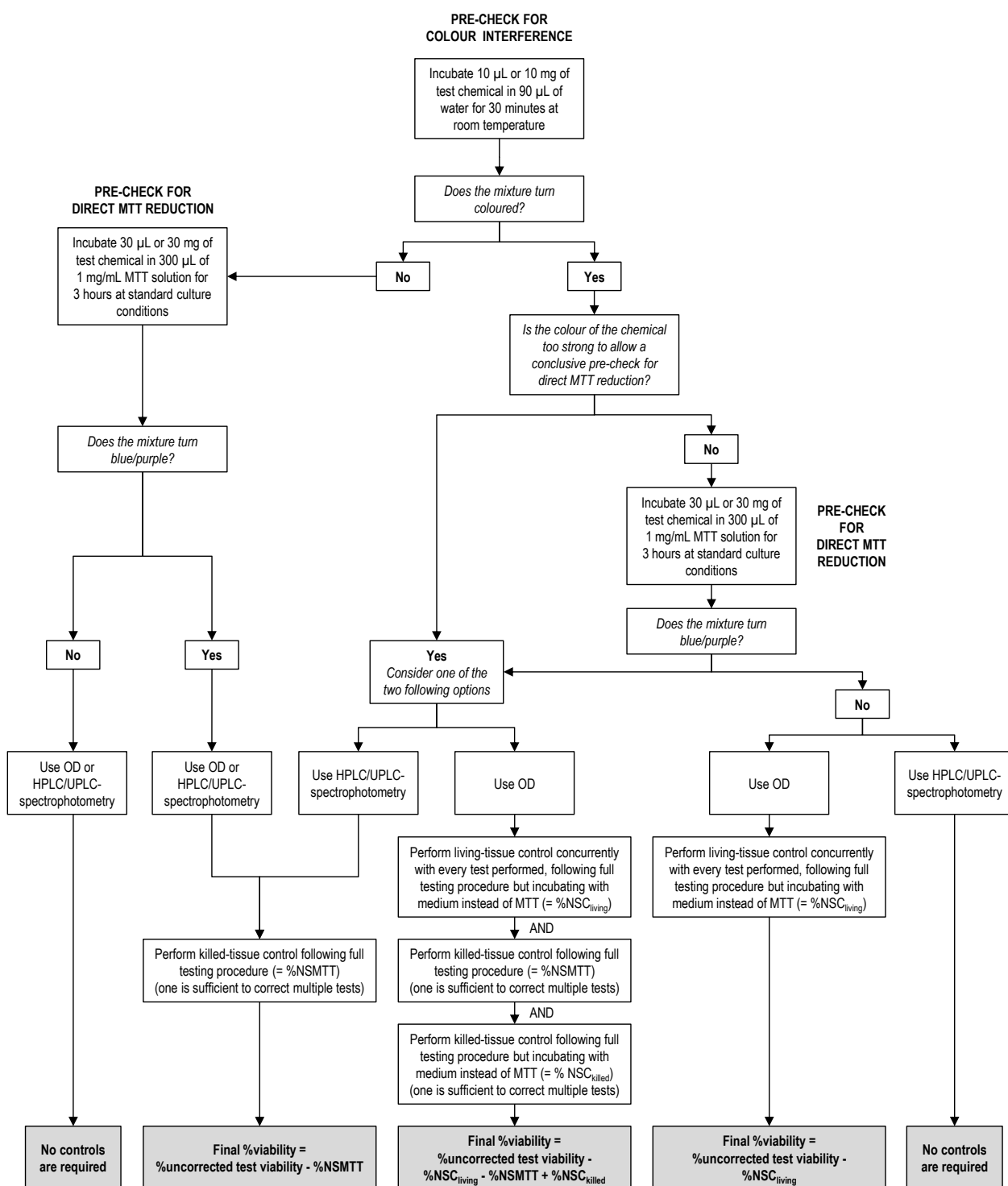
Appendix 3

ILLUSTRATIVE FLOWCHART PROVIDING GUIDANCE ON HOW TO IDENTIFY AND HANDLE DIRECT MTT-REDUCERS AND/OR COLOUR INTERFERING CHEMICALS, BASED ON THE VRM1 SOP



Appendix 4

ILLUSTRATIVE FLOWCHART PROVIDING GUIDANCE ON HOW TO IDENTIFY AND HANDLE DIRECT MTT-REDUCERS AND/OR COLOUR INTERFERING CHEMICALS, BASED ON THE VRM2 SOP



Appendix 5

KEY PARAMETERS AND ACCEPTANCE CRITERIA FOR QUALIFICATION OF AN HPLC/UPLC-SPECTROPHOTOMETRY SYSTEM FOR MEASUREMENT OF MTT FORMAZAN EXTRACTED FROM RHCE TISSUE CONSTRUCTS

Parameter	Protocol Derived from FDA Guidance (36)(38)	Acceptance Criteria
Selectivity	Analysis of isopropanol, living blank (isopropanol extract from living RhCE tissue constructs without any treatment), dead blank (isopropanol extract from killed RhCE tissue constructs without any treatment), and of a dye (e.g. methylene blue)	$\text{Area}_{\text{interference}} \leq 20\% \text{ of } \text{Area}_{\text{LLOQ}}^1$
Precision	Quality Controls (i.e., MTT formazan at 1.6 µg/ml, 16 µg/ml and 160 µg/ml) in isopropanol (n=5)	$\text{CV} \leq 15\% \text{ or } \leq 20\% \text{ for the LLOQ}$
Accuracy	Quality Controls in isopropanol (n=5)	$\% \text{Dev} \leq 15\% \text{ or } \leq 20\% \text{ for LLOQ}$
Matrix Effect	Quality Controls in living blank (n=5)	$85\% \leq \% \text{Matrix Effect} \leq 115\%$
Carryover	Analysis of isopropanol after an ULOQ ² standard	$\text{Area}_{\text{interference}} \leq 20\% \text{ of } \text{Area}_{\text{LLOQ}}$
Reproducibility (intra-day)	3 independent calibration curves (based on 6 consecutive 1/3 dilutions of MTT formazan in isopropanol starting at ULOQ, i.e., 200 µg/ml); Quality Controls in isopropanol (n=5)	Calibration Curves: $\% \text{Dev} \leq 15\% \text{ or } \leq 20\% \text{ for LLOQ}$ Quality Controls: $\% \text{Dev} \leq 15\% \text{ and } \text{CV} \leq 15\%$
Reproducibility (inter-day)	Day 1: 1 calibration curve and Quality Controls in isopropanol (n=3) Day 2: 1 calibration curve and Quality Controls in isopropanol (n=3) Day 3: 1 calibration curve and Quality Controls in isopropanol (n=3)	
Short Term Stability of MTT Formazan in RhCE Tissue Extract	Quality Controls in living blank (n=3) analysed the day of the preparation and after 24 hours of storage at room temperature	$\% \text{Dev} \leq 15\%$
Long Term Stability of MTT Formazan in RhCE Tissue Extract, if required	Quality Controls in living blank (n=3) analysed the day of the preparation and after several days of storage at -20°C	$\% \text{Dev} \leq 15\%$

¹ LLOQ: Lower Limit of Quantification, defined to cover 1-2% tissue viability, i.e., 0.8 µg/ml.

² ULOQ: Upper Limit of Quantification, defined to be at least two times higher than the highest expected MTT formazan concentration in isopropanol extracts from negative controls (~70 µg/ml in the VRM), i.e., 200 µg/ml.

B.70 HUMAN RECOMBINANT ESTROGEN RECEPTOR (hrER) *IN VITRO* ASSAYS TO DETECT CHEMICALS WITH ER BINDING AFFINITY

GENERAL INTRODUCTION

OECD Performance-Based Test Guideline

1. This test method is equivalent to OECD test guideline (TG) 493 (2015). TG 493 is a performance-based test guideline (PBTG), describing the methodology for human recombinant *in vitro* assays to detect substances with estrogen receptor binding affinity (hrER binding assays). It comprises two mechanistically and functionally similar assays for the identification of estrogen receptor (i.e. ER α) binders and should facilitate the development of new similar or modified assays in accordance with the principles for validation set forth in the OECD Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment (1). The fully validated reference test methods (Appendix 2 and Appendix 3) that provide the basis for this PBTG are:

- The Freyberger-Wilson (FW) *In Vitro* Estrogen Receptor (ER) Binding Assay Using a Full Length Human Recombinant ER α (2), and
- The Chemical Evaluation and Research Institute (CERI) *In Vitro* Estrogen Receptor Binding Assay Using a Human Recombinant Ligand Binding Domain Protein (2).

Performance standards (PS) (3) are available to facilitate the development and validation of similar test methods for the same hazard endpoint and allow for timely amendment of PBTG 493 so that new similar assays can be added to an updated PBTG. However, similar test assays will only be added after review and agreement by OECD that performance standards are met. The assays included in TG 493 can be used indiscriminately to address OECD member countries' requirements for test results on estrogen receptor binding while benefiting from the OECD Mutual Acceptance of Data.

Background and principles of the assays included in this test method

2. The OECD initiated a high-priority activity in 1998 to revise existing, and to develop new test guidelines for the screening and testing of potential endocrine disrupting chemicals. The OECD conceptual framework (CF) for testing and assessment of potential endocrine disrupting chemicals was revised in 2012. The original and revised CFs are included as Annexes in the Guidance Document on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption (4). The CF comprises five levels, each level corresponding to a different level of biological complexity. The ER binding assays described in this test method are level 2, which includes "*in vitro* assays providing data about selected endocrine mechanism(s)/pathway(s)". This test method is for *in vitro* receptor binding assays designed to identify ligands for the human estrogen receptor alpha (ER α).

3. The relevance of the *in vitro* ER binding assay to biological functions has been clearly demonstrated. ER binding assays are designed to identify chemicals that have the potential to disrupt the estrogen hormone pathway, and have been used extensively during the past two decades to characterise ER tissue distribution as well as to identify ER agonists/antagonists. These assays reflect the ligand-receptor interaction which is the initial step of the estrogen signalling pathway and essential for reproduction function in all vertebrates.
4. The interaction of estrogens with ERs can affect transcription of estrogen-controlled genes and induce non-genomic effects, which can lead to the induction or inhibition of cellular processes, including those necessary for cell proliferation, normal foetal development, and reproductive function (5) (6) (7). Perturbation of normal estrogenic systems may have the potential to trigger adverse effects on normal development (ontogenesis), reproductive health and the integrity of the reproductive system. Inappropriate ER signalling can lead to effects such as increased risk of hormone dependent cancer, impaired fertility, and alterations in foetal growth and development (8).
5. *In vitro* binding assays are based on a direct interaction of a substance with a specific receptor ligand binding site that regulates the gene transcription. The key component of the human recombinant estrogen receptor alpha (hrER α) binding assay measures the ability of a radiolabelled ligand ([³H]17 β -estradiol) to bind with the ER in the presence of increasing concentrations of a test chemical (i.e. competitor). Test chemicals that possess a high affinity for the ER compete with the radiolabelled ligand at a lower concentration as compared with those chemicals with lower affinity for the receptor. This assay consists of two major components: a saturation binding experiment to characterise receptor-ligand interaction parameters and document ER specificity, followed by a competitive binding experiment that characterises the competition between a test chemical and a radiolabelled ligand for binding to the ER.
6. Validation studies of the CERI and the FW binding assays have demonstrated their relevance and reliability for their intended purpose (2).
7. Definitions and abbreviations used in this test method are described in Appendix 1.

Scope and limitations related to the receptor binding assays

8. These assays are being proposed for screening and prioritisation purposes, but can also provide information for a molecular initiation event (MIE) that can be used in a weight of evidence approach. They address chemical binding to the ER α ligand binding domain in an *in vitro* system. Thus, results should not be directly extrapolated to the complex signalling and regulation of the intact endocrine system *in vivo*.
9. Binding of the natural ligand, 17 β -estradiol, is the initial step of a series of molecular events that activates the transcription of target genes and ultimately, culminates with a

physiological change (9). Thus binding to the ER α ligand binding domain is considered one of the key mechanisms of ER mediated endocrine disruption (ED), although there are other mechanisms through which ED can occur, including (i) interactions with sites of ER α other than the ligand binding pocket, (ii) interactions with other receptors relevant for estrogen signalling, ER β and G-protein coupled estrogen receptor, other receptors and enzymatic systems within the endocrine system, (iii) hormone synthesis, (iv) metabolic activation and/or inactivation of hormones, (v) distribution of hormones to target tissues, and (vi) clearance of hormones from the body. None of the assays under this test method address these modes of action.

10. This test method addresses the ability of substances to bind to human ER α and does not distinguish between ER α agonists or antagonists. These assays does not address either further downstream events such as gene transcription or physiological changes. Considering that only single mono-constituent substances were used during the validation, the applicability to test mixtures has not been addressed. The assays are nevertheless theoretically applicable to the testing of multi-constituent substances and mixtures. Before use of the test method on a mixture for generating data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed, when there is a regulatory requirement for testing of the mixture.
11. The cell free receptor systems have no intrinsic metabolic capability and they were not validated in combination with metabolic enzyme systems. However, it might be possible to incorporate metabolic activity in a study design but this would require further validation efforts.
12. Chemicals that may denature the protein (i.e. receptor protein), such as surfactant or chemicals that can change the pH of the assay buffer, may not be tested or may only be tested at concentrations devoid of such interactions. Otherwise, the concentration range that can be tested in the assays for a test chemical is limited by its solubility in the assay buffer.
13. For informational purposes, Table 1 provides the test results for the 24 substances that were tested in both of the fully validated assays described in this test method. Of these substances, 17 are classified as ER binders and 6 as non-binders based upon published reports, including *in vitro* assays for ER transcriptional activation and/or the uterotrophic assay (9) (10) (11) (12) (13) (14) (15). In reference to the data summarised in Table 1, there was almost 100% agreement between the two assays on the classifications of all the substances up to 10⁻⁴M, and each substance was correctly classified as an ER binder or non-binder. Supplementary information on this group of substances as well as additional substances tested in the ER binding assays during the validation studies is provided in the Performance Standards for the hrER binding assay (3), Appendix 2 (Tables 1, 2 and 3).

Table 1: Classification of substances as ER binders or non-binders when tested in the FW and CERI hrER Binding Assays with comparison with expected response

	Substance Name	CAS RN	Expected Response	FW Assay		CERI Assay		MESH Chemical Class	Product Class
				Concentration Range (M)	Classification	Concentration Range (M)	Classification		
1	17 β -Estradiol	50-28-2	<i>Binder</i>	1x10 ⁻¹¹ – 1x10 ⁻⁶	Binder	1x10 ⁻¹¹ – 1x10 ⁻⁶	Binder	Steroid	Pharmaceutical, Veterinary Agent
2	Norethynodrel	68-23-5	<i>Binder</i>	3x10 ⁻⁹ – 30x10 ⁻⁴	Binder	3x10 ⁻⁹ – 30x10 ⁻⁴	Binder	Steroid	Pharmaceutical, Veterinary Agent
3	Norethindrone	68-22-4	<i>Binder</i>	3x10 ⁻⁹ – 30x10 ⁻⁴	Binder	3x10 ⁻⁹ – 30x10 ⁻⁴	Binder	Steroid	Pharmaceutical, Veterinary Agent
4	Di- <i>n</i> -butyl phthalate	84-74-2	<i>Non-binder*</i>	1x10 ⁻¹⁰ – 1x10 ⁻⁴	Non-Binder ^{*†}	1x10 ⁻¹⁰ – 1x10 ⁻⁴	Non-Binder ^{*†}	Hydrocarbon (cyclic), Ester	Plasticiser, Chemical Intermediate
5	DES	56-53-1	<i>Binder</i>	1x10 ⁻¹⁰ – 1x10 ⁻³	Binder	1x10 ⁻¹⁰ – 1x10 ⁻³	Binder	Hydrocarbon (Cyclic), Phenol	Pharmaceutical, Veterinary Agent
6	17 α -ethynylestradiol	57-63-6	<i>Binder</i>	1x10 ⁻¹⁰ – 1x10 ⁻³	Binder	1x10 ⁻¹⁰ – 1x10 ⁻³	Binder	Steroid	Pharmaceutical, Veterinary Agent
7	Meso-Hexestrol	84-16-2	<i>Binder</i>	1x10 ⁻¹⁰ – 1x10 ⁻³	Binder	1x10 ⁻¹⁰ – 1x10 ⁻³	Binder	Hydrocarbon (Cyclic), Phenol	Pharmaceutical, Veterinary Agent
8	Genistein	446-72-0	<i>Binder</i>	1x10 ⁻¹⁰ – 1x10 ⁻³	Binder	1x10 ⁻¹⁰ – 1x10 ⁻³	Binder	Hydrocarbon (heterocyclic), Flavonoid	Natural Product
9	Equol	531-95-3	<i>Binder</i>	1x10 ⁻¹⁰ – 1x10 ⁻³	Binder	1x10 ⁻¹⁰ – 1x10 ⁻³	Binder	Phytoestrogen Metabolite	Natural Product
10	Butyl paraben (<i>n</i> -butyl-4-hydroxybenzoate)	94-26-8	<i>Binder</i>	1x10 ⁻¹⁰ – 1x10 ⁻³	Binder	1x10 ⁻¹⁰ – 1x10 ⁻³	Binder	Paraben	Preservative
11	Nonylphenol (mixture)	84852-15-3	<i>Binder</i>	1x10 ⁻¹⁰ – 1x10 ⁻³	Binder	1x10 ⁻¹⁰ – 1x10 ⁻³	Binder	Alkylphenol	Intermediate Compound
12	<i>o,p'</i> -DDT	789-02-6	<i>Binder</i>	1x10 ⁻¹⁰ – 1x10 ⁻³	Binder	1x10 ⁻¹⁰ – 1x10 ⁻³	Binder	Organochlorine	Insecticide
13	Corticosterone	50-22-6	<i>Non-binder*</i>	1x10 ⁻¹⁰ – 1x10 ⁻⁴	Non-binder	1x10 ⁻¹⁰ – 1x10 ⁻⁴	Non-Binder	Steroid	Natural Product
14	Zearalenone	17924-92-4	<i>Binder</i>	1x10 ⁻¹⁰ – 1x10 ⁻³	Binder	1x10 ⁻¹⁰ – 1x10 ⁻³	Binder	Hydrocarbon (heterocyclic), Lactone	Natural Product
15	Tamoxifen	10540-29-1	<i>Binder</i>	1x10 ⁻¹⁰ – 1x10 ⁻³	Binder	1x10 ⁻¹⁰ – 1x10 ⁻³	Binder	Hydrocarbon, (Cyclic)	Pharmaceutical, Veterinary Agent
16	5 α -dihydrotestosterone	521-18-6	<i>Binder</i>	1x10 ⁻¹⁰ – 1x10 ⁻³	Binder	1x10 ⁻¹⁰ – 1x10 ⁻³	Binder	Steroid, Nonphenolic	Natural Product
17	Bisphenol A	80-05-7	<i>Binder</i>	1x10 ⁻¹⁰ – 1x10 ⁻³	Binder	1x10 ⁻¹⁰ – 1x10 ⁻³	Binder	Phenol	Chemical Intermediate
18	4- <i>n</i> -heptylphenol	1987-50-4	<i>Binder</i>	1x10 ⁻¹⁰ – 1x10 ⁻³	Equivocal ^a	1x10 ⁻¹⁰ – 1x10 ⁻³	Binder	Alkylphenol	Intermediate
19	Kepone (Chlordecone)	143-50-0	<i>Binder</i>	1x10 ⁻¹⁰ – 1x10 ⁻³	Binder	1x10 ⁻¹⁰ – 1x10 ⁻³	Binder	Hydrocarbon, (Halogenated)	Pesticide
20	Benz(a)anthracene	56-55-3	<i>Non-Binder</i>	1x10 ⁻¹⁰ – 1x10 ⁻³	Non-Binder ^b	1x10 ⁻¹⁰ – 1x10 ⁻³	Non-Binder ^b	Aromatic Hydrocarbon	Intermediate
21	Enterolactone	78473-71-9	<i>Binder</i>	1x10 ⁻¹⁰ – 1x10 ⁻³	Binder	1x10 ⁻¹⁰ – 1x10 ⁻³	Binder	Phytoestrogen	Natural Product

22	Progesterone	57-83-0	<i>Non-binder*</i>	$1 \times 10^{-10} - 1 \times 10^{-4}$	Non-Binder	$1 \times 10^{-10} - 1 \times 10^{-4}$	Non-Binder	Steroid	Natural Product
23	Octyltriethoxysilane	2943-75-1	<i>Non-binder</i>	$1 \times 10^{-10} - 1 \times 10^{-3}$	Non-Binder	$1 \times 10^{-10} - 1 \times 10^{-3}$	Non-Binder	Silane	Surface Modifier
24	Atrazine	1912-24-9	<i>Non-binder*</i>	$1 \times 10^{-10} - 1 \times 10^{-4}$	Non-Binder	$1 \times 10^{-10} - 1 \times 10^{-4}$	Non-Binder	Heterocyclic compound	Herbicide

*Limit of solubility $< 1 \times 10^{-4}$ M.

*The use and classification of di-n-butyl phthalate (DBP) as a non-binder was based on testing up to 10^{-4} M because the substance had been observed to be insoluble at 10^{-3} M (e.g. turbidity) in some laboratories during the pre-validation studies.

† During the validation study, di-n-butyl phthalate (DBP) was tested as a coded test substance at concentrations up to 10^{-3} M. Under these conditions, some laboratories observed either a decrease in radioligand binding at the highest concentration (10^{-3} M) and/or an ambiguous curve fit. For these runs, DBP was classified as ‘equivocal’ or ‘binder’ in 3/5 laboratories using the CERI assay and 5/6 laboratories using the FW assay (see Reference (2), Sections IV.B.3a,b and VI.A).

^a Classification was not consistent with expected classification. Classification of 4-*n*-heptylphenol as ‘equivocal’ or ‘non-binder’ by 3/5 labs resulted in an average classification of equivocal. Closer inspection revealed that this was due to chemical solubility limitations that prevented the production of a full binding curve.

^b During the validation study, benz(a)anthracene was reclassified as a non-binder (i.e. negative) based on published literature demonstrating that the *in vitro* estrogenic activity reported for this substance (16) is primarily dependent upon its metabolic activation (17)(18). Enzymatic metabolic activation of the substance would not be anticipated in the cell free hrER binding assays as used in this inter-laboratory validation study. Thus, the correct classification for this substance is a ‘non-binder’ when used under the experimental conditions for the FW and CERI assays.

hrER BINDING ASSAY COMPONENTS

Essential Assay Components

14. This test method applies to assays using an ER receptor and a suitably strong ligand to the receptor that can be used as a marker/tracer for the assay and can be displaced with increasing concentrations of a test chemical. Binding assays contain the following two major components: 1) saturation binding and 2) competitive binding. The saturation binding assay is used to confirm the specificity and activity of the receptor preparations, while the competitive binding experiment is used to evaluate the ability of a test chemical to bind to hrER.

Controls

15. The basis for the proposed concurrent reference estrogen and controls should be described. Concurrent controls (solvent (vehicle), positive (ER binder; strong and weak affinity), negative (non-binder)), as appropriate, serve as an indication that the assay is operative under the test conditions and provide a basis for experiment-to-experiment comparisons; they are usually part of the acceptability criteria for a given experiment (1). Full concentration curves for the reference estrogen and controls (i.e. weak binder and non-binder) should be used in one plate during each run. All other plates should contain: 1) a high- (approximately full displacement of radiolabelled ligand) and medium- (approximately the IC_{50}) concentration each of E2 and weak binder in triplicate; 2) solvent control and non-specific binding, each in triplicate.

Standard Quality Control Procedures

16. Standard quality control procedures should be performed as described for each assay to ensure active receptors, the correct chemical concentrations, tolerance bounds remain stable through multiple replications, and retain the ability to provide the expected ER-binding responses over time.

Demonstration of Laboratory Proficiency

17. Prior to testing unknown chemicals with any of the assays under this test method , each laboratory should demonstrate proficiency in using the assay by performing saturation assays to confirm specificity and activity of the ER preparation, and competitive binding assays with the reference estrogen and controls (weak binder and non-binder). A historical database with results for the reference estrogen and controls generated from 3-5 independent experiments conducted on different days should be established by the laboratory. These experiments will be the foundation for the reference estrogen and historical controls for the laboratory and will be used as a partial assessment of assay acceptability for future runs.
18. The responsiveness of the test system will also be confirmed by testing the proficiency substances listed in Table 2. The list of proficiency substances is a subset of the reference substances provided in the Performance Standards for the ER binding assays (3). These substances are commercially available, represent the classes of chemicals commonly associated with ER binding activity, exhibit a suitable range of potency expected for ER binders (i.e. strong to weak) and non-binders (i.e. negatives). For each proficiency substance, concentrations tested should cover the range provided in Table 2. At least three experiments should be performed for each substance and results should be in concordance with expected chemical activity. Each experiment should be conducted independently (i.e. with fresh dilutions of receptor, chemicals, and reagent), with three replicates for each concentration. Proficiency is demonstrated by correct classification (positive/negative) of each proficiency substance. Proficiency testing should be performed by each technician when learning the assays.

Table 2: List of controls and proficiency substances for the hrER competitive binding assays¹

No	Substance Name	CAS RN ²	Expected Response ^{3,4}	Test concentration range (M)	MeSH chemical class ⁵	Product class ⁶
Controls (Reference estrogen, weak binder, non-binder)						
1	17 β -estradiol	50-28-2	Binder	1x10 ⁻¹¹ – 1x10 ⁻⁶	Steroid	Pharmaceutical, Veterinary agent
2	Norethynodrel (or) Norethindrone	68-23-5 (or) 68-22-4	Binder	3x10 ⁻⁹ – 30x10 ⁻⁶	Steroid	Pharmaceutical, Veterinary agent
3	Octyltriethoxysilane	2943-75-1	Non-binder	1x10 ⁻¹⁰ – 1x10 ⁻³	Silane	Surface modifier
Proficiency substances⁶						
4	Diethylstilbestrol	56-53-1	Binder	1x10 ⁻¹¹ – 1x10 ⁻⁶	Hydrocarbon (cyclic), Phenol	Pharmaceutical, Veterinary agent
5	17 α -ethynylestradiol	57-63-6	Binder	1x10 ⁻¹¹ – 1x10 ⁻⁶	Steroid	Pharmaceutical, Veterinary agent
6	meso-Hexestrol	84-16-2	Binder	1x10 ⁻¹¹ – 1x10 ⁻⁶	Hydrocarbon (cyclic), Phenol	Pharmaceutical, Veterinary agent
7	Tamoxifen	10540-29-1	Binder	1x10 ⁻¹¹ – 1x10 ⁻⁶	Hydrocarbon (cyclic)	Pharmaceutical, Veterinary agent
8	Genistein	446-72-0	Binder	1x10 ⁻¹⁰ – 1x10 ⁻³	Heterocyclic compound, Flavonoid,	Natural product
9	Bisphenol A	80-05-7	Binder	1x10 ⁻¹⁰ – 1x10 ⁻³	Phenol	Chemical intermediate
10	Zearalonone	17924-92-4	Binder	1x10 ⁻¹¹ – 1x10 ⁻³	Heterocyclic compound, Lactone	Natural Product

11	Butyl paraben	94-26-8	Binder	$1 \times 10^{-11} - 1 \times 10^{-3}$	Carboxylic acid, Phenol	Preservative
12	Atrazine	1912-24-9	Non-binder	$1 \times 10^{-11} - 1 \times 10^{-6}$	Heterocyclic compound	Herbicide
13	Di-n-butylphthalate (DBP) ⁷	84-74-2	Non-binder ⁸	$1 \times 10^{-10} - 1 \times 10^{-4}$	Hydrocarbon (cyclic), Ester	Plasticiser, Chemical intermediate
14	Corticosterone	50-22-6	Non-binder	$1 \times 10^{-11} - 1 \times 10^{-4}$	Steroid	Natural product

¹If a proficiency substance is no longer commercially available, a substance with the same ER binding classification, comparable potency, and chemical class can be used.

² Abbreviations: CAS RN = Chemical Abstracts Service Registry Number.

³Classification as an ER α Binder or Non-binder during the validation study for the CER1 and FW hrER Binding Assays (2).

⁴ER binding activity was based upon the ICCVAM Background Review Documents (BRD) for ER Binding and TA assays (9) as well as empirical data and other information obtained from referenced studies published and reviewed (10) (11) (12) (13) (14) (15).

⁵ Substances were assigned into one or more chemical classes using the U.S. National Library of Medicine's Medical Subject Headings (MeSH), an internationally recognised standardised classification scheme (available at: <http://www.nlm.nih.gov/mesh>).

⁶ Substances were assigned into one or more product classes using the U.S. National Library of Medicine's Hazardous Substances Database (available at: <http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB>).

⁷ DBP can be used as an alternate control non-binder tested with maximum concentration of 10^{-4} M.

⁸ Limit of solubility for this substance is 10^{-4} M. The use and classification of di-n-butyl phthalate (DBP) as a non-binder has been based on testing up to 10^{-4} M because the substance had been observed to be insoluble at 10^{-3} M (e.g. turbidity) in some laboratories during the pre-validation studies.

Solubility Testing and Concentration Range Finding for Test Chemicals

19. A preliminary test should be conducted to determine the limit of solubility for each test chemical and to identify the appropriate concentration range to use when conducting the test. The limit of solubility of each test chemical is to be initially determined in the solvent and further confirmed under assay conditions. The final concentration tested in the assay should not exceed 1 mM. Range finder testing consists of a solvent control along with eight, log serial dilutions, starting at the maximum acceptable concentration (e.g. 1 mM or lower, based upon the limit of solubility), and the presence of cloudiness or precipitate noted. Concentrations in the second and third experiments should be adjusted as appropriate to better characterise the concentration-response curve.

Test Run Acceptability Criteria

20. Acceptance or rejection of a test run is based on the evaluation of results obtained for the reference estrogen and control used for each experiment. First, for plate 1, the full concentration curves for the reference controls from each experiment should meet the measures of performance with curve-fit parameters (e.g. IC_{50} and Hillslope) based upon the results reported for the respective protocols for the CER1 and FW assays (Appendix 2 and 3), and the historical control data from the laboratory conducting the test. All controls (reference estrogen, weak binder, and non-binder) should be correctly classified for each experiment. Secondly, the controls on all subsequent plates need to be assessed for consistency with plate 1. A sufficient range of concentrations of the test chemical should be used to clearly define the top of the competitive binding curve. Variability among replicates at each concentration of the test chemical as well as among the three independent runs should be reasonable and scientifically defensible. The ability to consistently conduct the assay should be demonstrated by the development and maintenance of a historical database for the reference estrogen and controls. Standard deviations (SD) or coefficients of variation (CV) for the means of reference estrogen and control weak binder curves fitting parameters from multiple experiments may be used as a measure of within-laboratory reproducibility. Professional judgment should be applied when reviewing the plate control results from each run as well as for each test chemical.

In addition, the following principles regarding acceptability criteria should be met:

- Data should be sufficient for a quantitative assessment of ER binding
- The concentrations tested should remain within the solubility range of the test chemical.

Analysis of data

21. The defined data analysis procedure for saturation and competitive binding data should adhere to the key principles for characterising receptor-ligand interactions. Typically, saturation binding data are analysed using a non-linear regression model that accounts for

total and non-specific binding. A correction for ligand depletion (e.g. Swillens, 1995 (19)) may be needed when determining B_{max} and K_d. Data from competitive binding assays are typically transformed (e.g. percent specific binding and concentration of test chemical (log M)). Estimates of log (IC₅₀) for each test chemical should be determined using an appropriate nonlinear curve fitting software to fit a four parameter Hill equation. Following an initial analysis, the curve fit parameters and a visual review of how well the binding data fit the generated competitive binding curve should be conducted. In some cases, additional analysis may be needed to obtain the best curve fit (e.g. constraining top and/or bottom of curve, use of 10% rule, see Appendix 4 and Reference 2 (Section III.A.2)).

22. Meeting the acceptability criteria (paragraph 20) indicates the assay system is operating properly, but it does not ensure that any particular test will produce accurate data. Replicating the correct results of the first test is the best indication that accurate data were produced.

General Data Interpretation Criteria

23. There is currently no universally agreed method for interpreting ER binding data. However, both qualitative (e.g. binder/non-binder) and/or quantitative (e.g. log IC₅₀, Relative Binding Affinity (RBA), etc.) assessments of hrER-mediated activity should be based on empirical data and sound scientific judgment.

Test Report

24. The test report should include the following information:

Assay:

- assay used;

Control/Reference/Test chemical

- source, lot number, limit date for use, if available
- stability of the test chemical itself, if known;
- solubility and stability of the test chemical in solvent, if known.
- measurement of pH, osmolality and precipitate in the culture medium to which the test chemical was added, as appropriate.

Mono-constituent substance:

- physical appearance, water solubility, and additional relevant physicochemical properties;
- chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc.

Multi-constituent substance, UVCBs and mixtures:

- characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents.

Solvent/Vehicle:

- characterisation (nature, supplier and lot);
- justification for choice of solvent/vehicle;
- solubility and stability of the test chemical in solvent/vehicle, if known;

Receptors:

- source of receptors (supplier, catalog No, lot, species of receptor, active receptor concentration provided from supplier, certification from supplier)
- characterisation of receptors (including saturation binding results): K_d , B_{max} ,
- storage of receptors
- radiolabelled ligand:
- supplier, catalog No., lot, specific activity

Test conditions:

- solubility limitations under assay conditions;
- composition of binding buffer;
- concentration of receptor;
- concentration of tracer (i.e. radiolabelled ligand);
- concentrations of test chemical;
- percent vehicle in final assay;
- incubation temperature and time;
- method of bound/free separation;
- positive and negative controls/reference substances;
- criteria for considering tests as positive, negative or equivocal;

Acceptability check:

- actual IC_{50} and Hill slope values for concurrent positive controls/reference substances;

Results:

- raw and bound/free data;
- denaturing confirmation check, if appropriate;
- if it exists, the lowest effective concentration (LEC);

- RBA and/or IC_{50} values, as appropriate;
- concentration-response relationship, where possible;
- statistical analyses, if any, together with a measure of error and confidence (e.g. SEM, SD, CV or 95% CI) and a description of how these values were obtained;

Discussion of the results:

- application of 10% rule

Conclusion

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Appendix 1

DEFINITIONS AND ABBREVIATIONS

10% Rule: Option to exclude from the analyses data points where the mean of the replicates for the percent [^3H]17 β -estradiol specific bound is 10% or more above that observed for the mean value at a lower concentration (see Appendix 4).

Acceptability criteria: Minimum standards for the performance of experimental controls and reference standards. All acceptability criteria should be met for an experiment to be considered valid.

Accuracy (concordance): The closeness of agreement between assay results and an accepted reference values. It is a measure of assay performance and one aspect of relevance. The term is often used interchangeably with “concordance” to mean the proportion of correct outcomes of an assay (1).

CF: The OECD Conceptual Framework for the Testing and Evaluation of Endocrine Disrupters.

Chemical: A substance or a mixture.

CV: Coefficient of variation

E2: 17 β -estradiol

ED: Endocrine disruption

hER α : Human estrogen receptor alpha

ER: Estrogen receptor

Estrogenic activity: The capability of a chemical to mimic 17 β -estradiol in its ability to bind estrogen receptors. Binding to the hER α can be detected with this test method.

IC₅₀: The half maximal effective concentration of an inhibitory test chemical.

ICCVAM: The Interagency Coordinating Committee on the Validation of Alternative Methods.

Inter-laboratory reproducibility: A measure of the extent to which different qualified laboratories, using the same protocol and testing the same substances, can produce qualitatively and quantitatively similar results. Interlaboratory reproducibility is determined during the prevalidation and validation processes, and indicates the extent to which an assay can be successfully transferred between laboratories, also referred to as between-laboratory reproducibility (1).

Intra-laboratory reproducibility: A determination of the extent that qualified people within the same laboratory can successfully replicate results using a specific protocol at different times. Also referred to as “within-laboratory reproducibility” (1).

LEC: Lowest effective concentration is the lowest concentration of test chemical that produces a

response (*i.e.* the lowest test chemical concentration at which the fold induction is statistically different from the concurrent vehicle control).

Me-too test: A colloquial expression for an assay that is structurally and functionally similar to a validated and accepted reference test method. Interchangeably used with similar test method

PBTG: Performance-Based Test Guideline

Performance standards: Standards, based on a validated test method, that provide a basis for evaluating the comparability of a proposed assay that is mechanistically and functionally similar. Included are (1) essential assay components; (2) a minimum list of reference chemicals selected from among the chemicals used to demonstrate the acceptable performance of the validated test method; and (3) the comparable levels of accuracy and reliability, based on what was obtained for the validated test method, that the proposed assay should demonstrate when evaluated using the minimum list of reference chemicals (1).

Proficiency substances: A subset of the Reference substances included in the Performance Standards that can be used by laboratories to demonstrate technical competence with a standardised assay. Selection criteria for these substances typically include that they represent the range of responses, are commercially available, and have high quality reference data available.

Proficiency: The demonstrated ability to properly conduct an assay prior to testing unknown substances.

Reference estrogen: 17 β -estradiol (E2, CAS 50-28-2).

Reference test methods: The assays upon which PBTG 493 is based.

RBA: Relative Binding Affinity. The RBA of a substance is calculated as a percent of the log (IC₅₀) for the substance relative to the log (IC₅₀) for 17 β -estradiol

Relevance: Description of relationship of an assay to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the assay correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of the accuracy (concordance) of an assay (1).

Reliability: Measure of the extent that an assay can be performed reproducibly within and between laboratories over time, when performed using the same protocol. It is assessed by calculating intra- and inter-laboratory reproducibility.

SD: Standard deviation.

Test chemical: Any substance or mixture tested using this test method.

Validated test method: An assay for which validation studies have been completed to determine the relevance (including accuracy) and reliability for a specific purpose. It is important to note that a validated test method may not have sufficient performance in terms of accuracy and reliability to be found acceptable for the proposed purpose (1).

Validation: The process by which the reliability and relevance of a particular approach, method,

assay, process or assessment is established for a defined purpose (1).

Appendix 2

THE FREYBERGER-WILSON *IN VITRO* ESTROGEN RECEPTOR (ER α) SATURATION AND COMPETITIVE BINDING ASSAYS USING FULL LENGTH RECOMBINANT ER α

INITIAL CONSIDERATIONS AND LIMITATIONS (SEE ALSO GENERAL INTRODUCTION)

1. This *in vitro* Estrogen Receptor (ER α) saturation and competitive binding assay uses full length human receptor ER α (hrER α) that is produced in and isolated from baculovirus-infected insect cells. The protocol, developed by Freyberger and Wilson, underwent an international multi-laboratory validation study (2) which has demonstrated its relevance and reliability for the intended purpose of the assay.
2. This assay is a screening procedure for identifying substances that can bind to the full length hrER α . It is used to determine the ability of a test chemical to compete with 17 β -estradiol for binding to hrER α . Quantitative assay results may include the IC₅₀ (a measure of the concentration of test chemical needed to displace half of the [³H]-17 β -estradiol from the hrER α) and the relative binding affinities of test chemicals for the hrER α compared to 17 β -estradiol. For chemical screening purposes, acceptable qualitative assay results may include classifications of test chemicals as either hrER α binders, non-binders, or equivocal based upon criteria described for the binding curves.
3. The assay uses a radioactive ligand that requires a radioactive materials license for the laboratory. All procedures with radioisotopes and hazardous chemicals should follow the regulations and procedures as described by national legislation.
4. The “GENERAL INTRODUCTION” and “hrER BINDING ASSAY COMPONENTS” should be read before using this assay for regulatory purposes. Definitions and abbreviations used in this TG are described in Appendix 1.

PRINCIPLES OF THE ASSAY (SEE ALSO GENERAL INTRODUCTION)

5. The hrER α binding assay measures the ability of a radiolabelled ligand ([³H]17 β -estradiol) to bind with the ER in the presence of increasing concentrations of a test chemical (i.e. competitor). Test chemicals that possess a high affinity for the ER compete with the radiolabelled ligand at a lower concentration as compared with those chemicals with lower affinity for the receptor.
6. This assay consists of two major components: a saturation binding experiment to characterise receptor-ligand interaction parameters, followed by a competitive binding experiment that characterises the competition between a test chemical and a radiolabelled ligand for binding to the ER.

7. The purpose of the saturation binding experiment is to characterise a particular batch of receptors for binding affinity and number in preparation for the competitive binding experiment. The saturation binding experiment measures, under equilibrium conditions, the affinity of a fixed concentration of the estrogen receptor for its natural ligand (represented by the dissociation constant, K_d), and the concentration of active receptor sites (B_{max}).
8. The competitive binding experiment measures the affinity of a substance to compete with $[3H]17\beta$ -estradiol for binding to the ER. The affinity is quantified by the concentration of test chemical that, at equilibrium, inhibits 50% of the specific binding of the $[3H]17\beta$ -estradiol (termed the “inhibitory concentration 50%” or IC_{50}). This can also be evaluated using the relative binding affinity (RBA, relative to the IC_{50} of estradiol measured separately in the same run). The competitive binding experiment measures the binding of $[3H]17\beta$ -estradiol at a fixed concentration in the presence of a wide range (eight orders of magnitude) of test chemical concentrations. The data are then fit, where possible, to a form of the Hill equation (Hill, 1910) that describes the displacement of the radioligand by a one-site competitive binder. The extent of displacement of the radiolabelled estradiol at equilibrium is used to characterise the test chemical as a binder, non-binder, or generating an equivocal response.

PROCEDURE

Demonstration of Acceptable hrER α Protein Performance

9. Prior to routinely conducting the saturation and competitive binding assays, each new batch of hrER α should be shown to be performing correctly in the laboratory in which it will be used. A two-step process should be used to demonstrate performance. These steps are the following:
 - Conduct a saturation $[^3H]$ - 17β -estradiol binding assay to demonstrate hrER α specificity and saturation. Nonlinear regression analysis of these data (e.g. BioSoft; McPherson, 1985; Motulsky, 1995) and the subsequent Scatchard plot should document hrER α binding affinity of the $[^3H]$ - 17β -estradiol (K_d) and the number of receptors (B_{max}) for each batch of hrER α .
 - Conduct a competitive binding assay using the control substances (reference estrogen (17β -estradiol)), a weak binder (e.g. norethynodrel or norethindrone), and a non-binder (octyltriethoxysilane, OTES). Each laboratory should establish an historical database to document the consistency of IC_{50} and other relevant values for the reference estrogen and weak binder among experiments and different batches of hrER α . The parameters of the competitive binding curves for the control substances should be within the limits of the 95% confidence interval (see Table 1) that were developed using data from laboratories that participated in the validation study for this assay (2).

Table 1: Performance criteria developed for the reference estrogen and weak binder, FW hrER Binding Assay.

Substance	Parameter	Mean ^a	Standard Deviation (n)	95% Confidence Intervals ^b	
				Lower Limit	Upper Limit
17 β -estradiol	Top (%)	100.44	10.84 (67)	97.8	103.1
	Bottom (%)	0.29	1.25 (67)	-0.01	0.60
	Hill Slope	-1.06	0.20 (67)	-1.11	-1.02
	LogIC ₅₀ (M)	-8.92 ^c	0.18 (67)	-8.97	-8.88
Norethynodrel	Top (%)	99.42	8.90 (68)	97.27	101.60
	Bottom (%)	2.02	3.42 (68)	1.19	2.84
	Hill Slope	-1.01	0.38 (68)	-1.10	-0.92
	Log IC ₅₀ (M)	-6.39	0.27 (68)	-6.46	-6.33
Norethindrone ^c	Top (%)	96.14	8.44 (27)	92.80	99.48
	Bottom (%)	2.38	5.02 (27)	0.40	4.37
	Hill Slope	-1.41	0.32 (27)	-1.53	-1.28
	LogIC ₅₀ (M)	-5.73	0.27 (27)	-5.84	-5.62

^aMean (n) $\pm$ Standard Deviation (SD) were calculated using curve fit parameter estimates (4-parameter Hill Equation) for control runs conducted in four laboratories during the validation study (see Annex N of Reference 2).

^b The 95% confidence intervals are provided as a guide for acceptability criteria.

^c Testing of norethindrone was optional for Subtask 4 during validation study (see Reference 2, see Subtask 4). Thus, the mean $\pm$ SD (n) were calculated using curve fit estimates (4-parameter Hill equation) for control runs conducted in two laboratories.

The range for the IC₅₀ will be dependent upon the K_d of the receptor preparation and concentration of radiolabelled ligand used within each laboratory. Appropriate adjustment for the range of the IC₅₀ based upon the conditions used to conduct the assay will be acceptable.

Demonstration of laboratory proficiency

- See paragraphs 17 and 18 and Table 2 in “hrER BINDING ASSAY COMPONENTS” of this test method. Each assay (saturation and competitive binding) should consist of three independent runs (i.e. with fresh dilutions of receptor, chemicals, and reagents) on different days, and each run should contain three replicates.

Determination of Receptor (hrER α) Concentration

- The concentration of active receptor varies slightly by batch and storage conditions. For this reason, the concentration of active receptor as received from the supplier should be determined. This will yield the appropriate concentration of active receptor at the time of the run.
- Under conditions corresponding to competitive binding (i.e. 1 nM [³H]-estradiol), nominal concentrations of 0.25, 0.5, 0.75, and 1 nM receptor should be incubated in the absence (total binding) and presence (non-specific binding) of 1 μ M unlabelled estradiol. Specific binding, calculated as the difference of total and non-specific binding, is plotted against the

nominal receptor concentration. The concentration of receptor that gives specific binding values corresponding to 20% of added radiolabel is related to the corresponding nominal receptor concentration, and this receptor concentration should be used for saturation and competitive binding experiments. Frequently, a final hrER concentration of 0.5 nM will comply with this condition.

13. If the 20% criterion repeatedly cannot be met, the experimental set up should be checked for potential errors. Failure to achieve the 20% criterion may indicate that there is very little active receptor in the recombinant batch, and the use of another receptor batch should then be considered.

Saturation assay

14. Eight increasing concentrations of [³H]17β-estradiol should be evaluated in triplicate, under the following three conditions (see Table 2):
 - In the absence of unlabelled 17β-estradiol and presence of ER. This is the determination of total binding by measure of the radioactivity in the wells that have only [3H]17β-estradiol.
 - In the presence of a 1000- fold excess concentration of unlabelled 17β-estradiol over labelled 17β-estradiol and presence of ER. The intent of this condition is to saturate the active binding sites with unlabelled 17β-estradiol, and by measuring the radioactivity in the wells, determine the non-specific binding. Any remaining hot estradiol that can bind to the receptor is considered to be binding at a non-specific site as the cold estradiol should be at such a high concentration that it is bound to all of the available specific sites on the receptor.
 - In the absence of unlabelled 17β-estradiol and absence of ER (determination of total radioactivity)

Preparation of [³H]-17β-estradiol and unlabelled 17β-estradiol solutions

15. Dilutions of [³H]-17β-estradiol should be prepared by adding assay buffer to a 12 nM stock solution of [³H]-17β-estradiol to obtain concentrations initially ranging from 0.12nM to 12 nM. By adding 40 µl of these solutions to the respective assay wells of a 96-well microtiter plate (in a final volume of 160 µl), the final assay concentrations, ranging from 0.03 to 3.0 nM, will be obtained. Preparation of assay buffer, [³H]-17β-estradiol stock solution and dilutions and determination of the concentrations are described in depth in the FW protocol (2).
16. Dilutions of ethanolic 17β-estradiol solutions should be prepared by adding assay buffer to achieve eight increasing concentrations initially ranging from 0.06 µM to 6 µM. By adding 80 µl of these solutions to the respective assay wells of a 96-well microtiter plate (in a final volume of 160 µl), the final assay concentrations, ranging from 0.03µM to 3µM, will be obtained. The final concentration of unlabelled 17β-estradiol in the individual non-specific binding assay wells should be 1000-fold of the labelled [³H]-17β- estradiol concentration.

Preparation of unlabelled 17 β -estradiol dilutions is described in depth in the FW protocol (2).

17. The nominal concentration of receptor that gives specific binding of 20 $\pm$ 5% should be used (see paragraphs 12-13). The hrER α solution should be prepared immediately prior to use.

18. The 96-well microtiter plates are prepared as illustrated in Table 2, with 3 replicates per concentration. Example of plate concentration and volume assignment of [³H]-17 β -estradiol, unlabelled 17 β -estradiol, buffer and receptor are provided in Appendix 2.2.

Table 2: Saturation Binding Assay Microtiter Plate Layout

	<i>1</i>	<i>2</i>	<i>3</i>	<i>4</i>	<i>5</i>	<i>6</i>	<i>7</i>	<i>8</i>	<i>9</i>	<i>10</i>	<i>11</i>	<i>12</i>	
<i>A</i>	0.03 nM [³ H] E2 + ER			0.06 nM [³ H] E2 + ER			0.08 nM [³ H] E2 + ER			0.10 nM [³ H] E2 + ER			Total Binding (Solvent)
<i>B</i>	0.30 nM [³ H] E2 + ER			0.60 nM [³ H] E2 + ER			1.0 nM [³ H] E2 + ER			3.0 nM [³ H] E2 + ER			
<i>C</i>													
<i>D</i>	0.03 nM [³ H] E2 + ER + 0.03 μM E2			0.06 nM [³ H] E2 + ER + 0.06 μM E2			0.08 nM [³ H] E2 + ER + 0.08 μM E2			0.10 nM [³ H] E2 + ER + 0.10 μM E2			Non-Specific Binding
<i>E</i>	0.30 nM [³ H] E2 + ER + 0.30 μM E2			0.60 nM [³ H] E2 + ER + 0.60 μM E2			1.0 nM [³ H] E2 + ER + 1.0 μM E2			3.0 nM [³ H] E2 + ER + 3.0 μM E2			
<i>F</i>													
<i>G</i>													
<i>H</i>													

[³H] E2: [³H]-17 β -estradiol

ER: estrogen receptor

E2: unlabelled 17 β -estradiol

19. Assay microtiter plates should be incubated at 2° to 8°C for 16 to 20 hours and placed on a rotator during the incubation period.

Measurement of [³H]-17 β -Estradiol bound to hrER α

20. [³H]-17 β -Estradiol bound to hrER α should be separated from free [³H]-17 β -Estradiol by adding 80 μ l of cold DCC suspension to each well, shaking the microtiter plates for 10 minutes and centrifugating for 10 minutes at about 2500 RPM. To minimise dissociation of bound [³H]-17 β -estradiol from the hrER α during this process, it is extremely important that the buffers and assay wells be kept between 2 and 8°C and that each step be conducted quickly. A shaker for microtiter plates is necessary to process plates efficiently and quickly.

21. 50 μ l of supernatant containing the hrER α -bound [³H]-17 β -estradiol should then be taken with extreme care, to avoid any contamination of the wells by touching DCC, and should be placed on a second microtiter plate.

22. 200 µl of scintillation fluid, capable of converting the kinetic energy of nuclear emissions into light energy, should then be added to each well (A1-B12 and D1 to E12). Wells G1-H12 (identified as total dpms) represent serial dilutions of the [³H]-17β-estradiol (40 µl) that should be delivered directly into the scintillation fluid in the wells of the measurement plate as indicated in Table 3, i.e. these wells contain only 200 µl of scintillation fluid and the appropriate dilution of [³H]-17β-estradiol. These measures demonstrate how much [³H]-17β-estradiol in dpms was added to each set of wells for the total binding and non-specific binding.

Table 3: Saturation Binding Assay Microtiter Plate Layout, Radioactivity Measurement

	1	2	3	4	5	6	7	8	9	10	11	12	
<i>A</i>	0.03 nM [³ H] E2 + ER			0.06 nM [³ H] E2 + ER			0.08 nM [³ H] E2 + ER			0.10 nM [³ H] E2 + ER			Total Binding (Solvent)
<i>B</i>	0.30 nM [³ H] E2 + ER			0.60 nM [³ H] E2 + ER			1.0 nM [³ H] E2 + ER			3.0 nM [³ H] E2 + ER			
<i>C</i>													
<i>D</i>	0.03 nM [³ H] E2 + ER + 0.03 μM E2			0.06 nM [³ H] E2 + ER + 0.06 μM E2			0.08 nM [³ H] E2 + ER + 0.08 μM E2			0.10 nM [³ H] E2 + ER + 0.10 μM E2			Non-Specific Binding
<i>E</i>	0.30 nM [³ H] E2 + ER + 0.30 μM E2			0.60 nM [³ H] E2 + ER + 0.60 μM E2			1.0 nM [³ H] E2 + ER + 1.0 μM E2			3.0 nM [³ H] E2 + ER + 3.0 μM E2			
<i>F</i>													
<i>G</i>	0.03 nM [³ H] E2 (total dpms)			0.06 nM [³ H] E2			0.08 nM [³ H] E2			0.10 nM [³ H] E2			Total dpms*
<i>H</i>	0.30 nM [³ H] E2			0.60 nM [³ H] E2			1.0 nM [³ H] E2			3.0 nM [³ H] E2			

[³H] E2: [³H]-17β-estradiol

ER: estrogen receptor

E2: unlabelled 17β-estradiol

dpms: disintegrations per minute

*The hot serial dilutions of [³H]-labelled estradiol here should be directly added into 200 µl of scintillation fluid in wells G1 – H12.

23. Measurement should start with a delay of at least 2 hours and counting time should be 40 minutes per well. A microtiter plate scintillation counter should be used for determination of dpm/well with quench correction. Alternatively, if a scintillation counter for a microtiter plate is not available, samples may be measured in a conventional counter. Under these conditions, a reduction of counting time may be considered.

Competitive binding assay

24. The competitive binding assay measures the binding of a single concentration of [³H]-17β-estradiol in the presence of increasing concentrations of a test chemical. Three concurrent replicates should be used at each concentration within one run. In addition, three non-

concurrent runs should be performed for each chemical tested. The assay should be set up in one or more 96-well microtiter plates

Controls

25. When performing the assay, concurrent solvent and controls (i.e. reference estrogen, weak binder, and non-binder) should be included in each experiment. Full concentration curves for the reference estrogen and controls (i.e. weak binder and non-binder) should be used in one plate during each run. All other plates should contain (i) a high- (maximum displacement) and medium- (approximately the IC₅₀) concentration each of E2 and weak binder in triplicate; (ii) solvent control and non-specific binding, each at least in triplicate. Procedures for the preparation of assay buffer, controls, [³H]-17β-estradiol, hrERα and test chemical solutions are described in Reference 2 (Annex K, see FW Assay Protocol).

Solvent control:

26. The solvent control indicates that the solvent does not interact with the test system and also measures total binding (TB). Ethanol is the preferred solvent. Alternatively, if the highest concentration of the test chemical is not soluble in ethanol, DMSO may be used. The concentration of ethanol or DMSO, if used, in the final assay wells is 1.5% and may not exceed 2%.

Buffer control:

27. The buffer control (BC) should contain neither solvent nor test chemical, but all of the other components of the assay. The results of the buffer control are compared to the solvent control to verify that the solvent used does not affect the assay system.

Strong binder (reference estrogen)

28. 17β-estradiol (CAS 50-28-2) is the endogenous ligand and binds with high affinity to the ER, alpha subtype. A standard curve using unlabelled 17β-estradiol should be prepared for each hrERα competitive binding assay, to allow for an assessment of variability when conducting the assay over time within the same laboratory. Eight solutions of unlabelled 17β-estradiol should be prepared in ethanol, with concentrations in the assay wells ranging from 100 nM – 10 pM (-7[logM] to -11[logM]), spaced as follows: (-7[logM], -8[logM], -8.5[logM], -9[logM], -9.5[logM], -10[logM], -11[logM]). The highest concentration of unlabelled 17β-estradiol (1 μM) also serves as the non-specific binding indicator. This concentration is distinguished by the label “NSB” in Table 4 even though it is also part of the standard curve.

Weak binder

29. A weak binder (norethynodrel (CAS68-23-5) or norethindrone (CAS 68-22-4)) should be included to demonstrate the sensitivity of each experiment and to allow an assessment of variability when conducting the assay over time. Eight solutions of the weak binder should be prepared in ethanol, with concentrations in the assay wells ranging from 3 nM to 30 μM

(-8.5[logM] to -4.5[logM]), spaced as follows: -4.5[logM], -5[logM], -5.5[logM], -6[logM], -6.5[logM], -7[logM], -7.5[logM], -8.5[logM].

Non binder

30. Octyltriethoxysilane (OTES, CAS 2943-75-1) should be used as the negative control (non-binder). It provides assurance that the assay as run, will detect when test chemicals do not bind to the hrER α . Eight solutions of the non-binder should be prepared in ethanol, with concentrations in the assay wells ranging from 0.1nM to 1000 μ M (-10[logM] to -3[logM]), in log increments. Di-*n*-butyl phthalate (DBP) can be used as an alternate control non-binder. Its maximum solubility has been shown to be -4[logM].

hrER α concentration

31. The amount of receptor that gives specific binding of 20 $\pm$ 5% of 1 nM radioligand should be used (see paragraphs 12-13 of Appendix 2). The hrER α solution should be prepared immediately prior to use.

[3H]-17 β -estradiol

32. The concentration of [³H]-17 β -estradiol in the assay wells should be of 1.0 nM.

Test Chemicals

33. In the first instance, it is necessary to conduct a solubility test to determine the limit of solubility for each test chemical and to identify the appropriate concentration range to use when conducting the test protocol. The limit of solubility of each test chemical is to be initially determined in the solvent and further confirmed under assay conditions. The final concentration tested in the assay should not exceed 1 mM. Range finder testing consists of a solvent control along with 8 log serial dilutions, starting at the maximum acceptable concentration (e.g. 1 mM or lower, based upon the limit of solubility), and the presence of cloudiness or precipitate noted (see also paragraph 35). The test chemical should be tested using 8 log concentration spaced curves as defined by the preceding range finding test. Concentrations in the second and third experiments should be adjusted as appropriate to better characterise the concentration-response curve.
34. Dilutions of the test chemical should be prepared in the appropriate solvent (see paragraph 26 of Appendix 2). If the highest concentration of the test chemical is not soluble in either ethanol or DMSO, and adding more solvent would cause the solvent concentration in the final tube to be greater than the acceptable limit, the highest concentration may be reduced to the next lower concentration. In this case, an additional concentration may be added at the low end of the concentration series. Other concentrations in the series should remain unchanged.
35. The test chemical solutions should be closely monitored when added to the assay well, as the test chemical may precipitate upon addition to the assay well. The data for all wells that

contain precipitate should be excluded from curve-fitting, and the reason for exclusion of the data noted.

36. If there is prior existing information from other sources that provide a $\log(\text{IC}_{50})$ of a test chemical, it may be appropriate to geometrically space the dilutions (i.e. 0.5 log units around the expected $\log(\text{IC}_{50})$). The final result should reflect sufficient spread of concentrations on either side of the $\log(\text{IC}_{50})$, including the “top” and “bottom”, such that the binding curve can be adequately characterised.

Assay plate organisation

37. Labelled microtiter plates should be prepared considering sextuple incubations with codes for the solvent control, the highest concentration of the reference estrogen which also serves as the non-specific binding (NSB) indicator, and the buffer control and considering triplicate incubations with codes for each of the eight concentrations of the non-binding control (octyltriethoxysilane), the 7 lower concentrations for the reference estrogen, the eight concentrations dose levels of the weak binder, and the 8 concentrations of each test chemical (TC). An example layout of the plate diagram for the full concentration curves for the reference estrogen and control is given below in Table 4. Additional microtiter plates are used for the test chemicals and should include plate controls, i.e. 1) a high- (maximum displacement) and medium- (approximately the IC_{50}) concentration each of E2 and weak binder in triplicate; 2) solvent control and non-specific binding, each in sextuple (Table 5). An example of a competitive assay microtiter plate layout worksheet using three unknown test chemicals is provided in Appendix 2.3. The concentrations indicated in Tables 4 and 5 are the final concentrations of the assay. The maximum concentration for E2 should be 1×10^{-7} M and for the weak binder, the highest concentration used for the weak binder on plate 1 should be used. The IC_{50} concentration has to be determined by the laboratory based on their historical control database. It is expected that this value would be similar to that observed in the validation studies (see Table 1).

Table 4: Competitive Binding Assay Microtiter Plate Layout, Full Concentration Curves for Reference Estrogen and Controls (Plate 1).

	1	2	3	4	5	6	7	8	9	10	11	12
A	TB (Solvent only)			TB (Solvent only)			NSB			NSB		
B	E2 (1×10^{-7})			E2 (1×10^{-8})			E2 ($1 \times 10^{-8.5}$)			E2 (1×10^{-9})		
C	E2 ($1 \times 10^{-9.5}$)			E2 (1×10^{-10})			E2 (1×10^{-11})			Blank*		
D	NE ($1 \times 10^{-4.5}$)			NE (1×10^{-5})			NE ($1 \times 10^{-5.5}$)			NE (1×10^{-6})		
E	NE ($1 \times 10^{-6.5}$)			NE (1×10^{-7})			NE ($1 \times 10^{-7.5}$)			NE ($1 \times 10^{-8.5}$)		
F	OTES (1×10^{-3})			OTES (1×10^{-4})			OTES (1×10^{-5})			OTES (1×10^{-6})		
G	OTES (1×10^{-7})			OTES (1×10^{-8})			OTES (1×10^{-9})			OTES (1×10^{-10})		
H	Blank (for hot)**			Blank (for hot)**			Buffer control			Buffer control		

In this example, the weak binder is norethinodrel (NE)

* real blank, well not used

** blank not used during the incubation, but used to confirm the total radioactivity added.

Table 5: Competitive Binding Assay Microtiter Plate Layout, Full Concentration Curves for Test Chemicals and Plate Controls.

	1	2	3	4	5	6	7	8	9	10	11	12
A	TB (Solvent only)			TB (Solvent only)			NSB			NSB		
B	TC1 (1×10^{-3})			TC1 (1×10^{-4})			TC1 (1×10^{-5})			TC1 (1×10^{-6})		
C	TC1 (1×10^{-7})			TC1 (1×10^{-8})			TC1 (1×10^{-9})			TC1 (1×10^{-10})		
D	TC2 (1×10^{-3})			TC2 (1×10^{-4})			TC2 (1×10^{-5})			TC2 (1×10^{-6})		
E	TC2 (1×10^{-7})			TC2 (1×10^{-8})			TC2 (1×10^{-9})			TC2 (1×10^{-10})		
F	TC3 (1×10^{-3})			TC3 (1×10^{-4})			TC3 (1×10^{-5})			TC3 (1×10^{-6})		
G	TC3 (1×10^{-7})			TC3 (1×10^{-8})			TC3 (1×10^{-9})			TC3 (1×10^{-10})		
H	NE (IC_{50})			NE ($1 \times 10^{-4.5}$)			E2 (IC_{50})			E2 (1×10^{-7})		

In this example, the weak binder is norethinodrel (NE)

Completion of competitive binding assay

38. As shown in Table 6, 80 μ l of the solvent control, buffer control, reference estrogen, weak binder, non-binder, and test chemicals prepared in assay buffer should be added to the wells. Then, 40 μ l of a 4 nM [^{3} H]-17 β -estradiol solution should be added to each well. After gentle rotation for 10 to 15 minutes between 2° to 8°C, 40 μ l of hrER α solution should be added. Assay microtiter plates should be incubated at 2° to 8°C for 16 to 20 hours, and placed on a rotator during the incubation period.

Table 6: Volume of Assay Components for hrER Competitive Binding Assay, Microtiter Plates

Volume (μ l)	Constituent
80	Unlabelled 17 β -estradiol, norethynodrel, OTES, test chemicals, solvent or buffer
40	4 nM [^{3} H]-17 β -estradiol solution
40	hrER α solution, concentration as determined
160	Total volume in each assay well

39. The quantification of [^{3} H]-17 β -Estradiol bound to hrER α , following separation of [^{3} H]-17 β -Estradiol bound to hrER α from free [^{3} H]-17 β -Estradiol by adding 80 μ l of cold DCC

suspension to each well, should then be performed as described in paragraphs 20-23 for the saturation binding assay.

40. Wells H1-6 (identified as blank (for hot) in table 4) represent the dpms of the [³H]-labelled-estradiol in 40 µl. The 40 µl aliquot should be delivered directly into the scintillation fluid in wells H1 – H6.

Acceptability criteria

Saturation binding assay

41. The specific binding curve should reach a plateau as increasing concentrations of [³H]-17β-estradiol were used, indicating saturation of hrERα with ligand.
42. The specific binding at 1 nM of [3H]-17β-estradiol should be inside the acceptable range 15% to 25% of the average measured total radioactivity added across runs. Occasional slight excursions outside of this range are acceptable, but if runs are consistently outside this range or a particular run is significantly outside this range, the protein concentration should be adjusted and the saturation assay repeated.
43. The data should produce a linear Scatchard plot.
44. The non-specific binding should not be excessive. The value for non-specific binding should typically be <35% of the total binding. However, the ratio might occasionally exceed this limit when measuring very low dpm for the lowest concentration of radiolabelled 17β-Estradiol tested.

Competitive binding assay

45. Increasing concentrations of unlabelled 17β-estradiol should displace [³H]-17β- estradiol from the receptor in a manner consistent with a one-site competitive binding.
46. The IC₅₀ value for the reference estrogen (i.e. 17β-estradiol) should be approximately equal to the molar concentration of [³H]-17β-estradiol plus the K_d determined from the saturation binding assay.
47. The total specific binding should be consistently within the acceptable range of 20 ± 5 % when the average measured concentration of total radioactivity added to each well was 1 nM across runs. Occasional slight excursions outside of this range are acceptable, but if runs are consistently outside this range or a particular run is significantly outside this range, the protein concentration should be adjusted.
48. The solvent should not alter the sensitivity or reproducibility of the assay. The results of the solvent control (TB wells) are compared to the buffer control to verify that the solvent used does not affect the assay system. The results of the TB and Buffer control should be comparable if there is no effect of the solvent on the assay.

49. The non-binder should not displace more than 25% of the [^3H]-17 β -estradiol from the hrER α when tested up to 10^{-3} M (OTES) or 10^{-4} M (DBP).
50. Performance criteria were developed for the reference estrogen and two weak binders (e.g. norethynodrel, norethindrone) using data from the validation study of the FW hrER Binding Assay (Annex N of Reference 2). 95% confidence intervals are provided for the mean (n) $\pm$ SD for all control runs across the laboratories participating in the validation study. 95% confidence intervals were calculated for the curve fit parameters (i.e. top, bottom, Hillslope, logIC $_{50}$) for the reference estrogen and weak binders and for the log $_{10}$ RBA of the weak binders relative to the reference estrogen and are provided as performance criteria for the positive controls. Table 1 provides expected ranges for the curve fit parameters that can be used as performance criteria. In practice, the range of the IC $_{50}$ may vary slightly based upon the K $_d$ of receptor preparation and ligand concentration.
51. No performance criteria was developed for curve fit parameters for the test chemicals because of the wide array of existing potential test chemicals and variation in potential affinities and outcomes (e.g. Full curve, partial curve, no curve fit). However, professional judgment should be applied when reviewing results from each run for a test chemical. A sufficient range of concentrations of the test chemical should be used to clearly define the top (e.g. 90 - 100% of binding) of the competitive curve. Variability among replicates at each concentration of test chemical as well as among the 3 non-concurrent runs should be reasonable and scientifically defensible. Controls from each run for a test chemical should approach the measures of performance reported for this FW assay and be consistent historical control data from each respective laboratory.

ANALYSIS OF DATA

Saturation binding assay

52. Both total and non-specific binding are measured. From these values, specific binding of increasing concentrations of [^3H]-17 β -estradiol under equilibrium conditions is calculated by subtracting non-specific from total. A graph of specific binding versus [^3H]-17 β -estradiol concentration should reach a plateau for maximum specific binding indicative of saturation of the hrER α with the [^3H]-17 β -estradiol. In addition, analysis of the data should document the binding of the [^3H]-17 β - estradiol to a single, high-affinity binding site. Non-specific, total, and specific binding should be displayed on a saturation binding curve. Further analysis of these data should use a non-linear regression analysis (e.g. BioSoft; McPherson, 1985; Motulsky, 1995) with a final display of the data as a Scatchard plot.
53. The data analysis should determine B $_{\text{max}}$ and K $_d$ from the total binding data alone, using the assumption that non- specific binding is linear, unless justification is given for using a different method. In addition, robust regression should be used when determining the best fit unless justification is given. The method chosen for robust regression should be stated.

Correction for ligand depletion (e.g. using the method of Swillens 1995) should always be used when determining B_{max} and K_d from saturation binding data.

Competitive binding assay

54. The competitive binding curve is plotted as specific [^3H]-17 β -estradiol binding versus the concentration (log10 units) of the competitor. The concentration of the test chemical that inhibits 50% of the maximum specific [^3H]-17 β -estradiol binding is the IC_{50} value.
55. Estimates of log(IC_{50}) values for the positive controls (e.g. reference estrogen and weak binder) should be determined using an appropriate nonlinear curve fitting software to fit a four parameter Hill equation (e.g. BioSoft; McPherson, 1985; Motulsky, 1995). The top, bottom, slope, and log(IC_{50}) should generally be left unconstrained when fitting these curves. Robust regression should be used when determining the best fit unless justification is given. Correction for ligand depletion should not be used. Following the initial analysis, each binding curve should be reviewed to ensure appropriate fit to the model. The relative binding affinity (RBA) for the weak binder should be calculated as a percent of the log (IC_{50}) for the weak binder relative to the log (IC_{50}) for 17 β -estradiol. Results from the positive controls and the non-binder control should be evaluated using the measures of the assay performance in paragraphs 45-50 in this Appendix 2.
56. Data for all test chemicals should be analysed using a step-wise approach to ensure that data are appropriately analysed and that each competitive binding curve is properly classified. It is recommended that each run for a test chemical initially undergo a standardised data analysis that is identical to that used for the reference estrogen and weak binder controls (see paragraph 55 above). Once completed, a technical review of the curve fit parameters as well as a visual review of how well the data fit the generated competitive binding curve for each run should be conducted. During this technical review, the observations of a concentration dependent decrease in the percent [^3H]-17 β -estradiol specifically bound, low variability among the technical replicates at each chemical concentration, and consistency in fit parameters among the three runs are a good indication that the assay and data analyses were conducted appropriately.

Data interpretation

57. Providing that all acceptability criteria are fulfilled, a test chemical is considered to be a binder for the hrER α if a binding curve can be fit and the lowest point on the response curve within the range of the data is less than 50% (Figure 1).
58. Providing that all acceptability criteria are fulfilled, a test chemical is considered to be a non-binder for the hrER α if:
 - A binding curve can be fit and the lowest point on the fitted response curve within the range of the data is above 75%, or

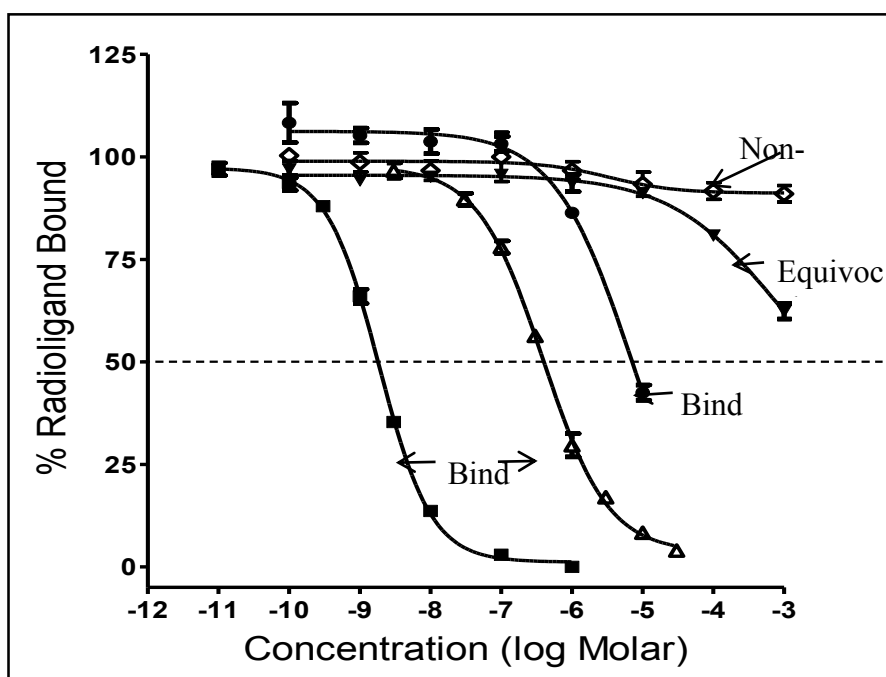
- A binding curve cannot be fit and the lowest unsmoothed average percent binding among the concentration groups in the data is above 75%.

59. Test chemicals are considered equivocal if none of the above conditions are met (e.g. the lowest point on the fitted response curve is between 76 – 51%).

Table 7: Criteria for assigning classification based upon competitive binding curve for a test chemical.

Classification	Criteria
Binder ^a	A binding curve can be fit. The lowest point on the response curve within the range of the data is less than 50%.
Non-binder ^b	If a binding curve can be fit, the lowest point on the fitted response curve within the range of the data is above 75%. If a binding curve cannot be fit, the lowest unsmoothed average percent binding among the concentration groups in the data is above 75%.
Equivocal ^c	Any testable run that is neither a binder nor a non-binder (e.g. The lowest point on the fitted response curve is between 76 – 51%).

Figure 1: Examples of test chemical classification using competitive binding curve.



60. Multiple runs conducted within a laboratory for a test chemical are combined by assigning numeric values to each run and averaging across the runs as shown in Table 8. Results for the combined runs within each laboratory are compared with the expected classification for each test chemical.

Table 8: Method for classification of test chemical using multiple runs within a laboratory

To assign value to each run:	
Classification	Numeric Value
Binder	2
Equivocal	1
Non-binder	0
To classify average of numeric value across runs:	
Classification	Numeric Value
Binder	Average ≥ 1.5
Equivocal	$0.5 \leq \text{Average} < 1.5$
Non-binder	Average < 0.5

TEST REPORT

61. See paragraph 24 of “**hrER BINDING ASSAY COMPONENTS**” of this test method.

Appendix 2.1

LIST OF TERMS

[³H]E2: 17 β -Estradiol radiolabelled with tritium

DCC: Dextran-coated charcoal

E2: Unlabelled 17 β -estradiol (inert)

Assay buffer: 10 mM Tris, 10 mg Bovine Serum Albumin /ml, 2 mM DTT, 10% glycerol, 0.2 mM leupeptin, pH 7.5

hrER α : Human recombinant estrogen receptor alpha

Replicate: One of multiple wells that contain the same contents at the same concentrations and are assayed concurrently within a single run. In this protocol, each concentration of test chemical is tested in triplicate; that is, there are three replicates that are assayed simultaneously at each concentration of test chemical.

Run: A complete set of concurrently-run microtiter plate assay wells that provides all the information necessary to characterise binding of a test chemical to the hrER α (viz., total [³H]-17 β -estradiol added to the assay well, maximum binding of [³H]-17 β -estradiol to the hrER α , nonspecific binding, and total binding at various concentrations of test chemical). A run could consist of as few as one assay well (i.e. replicate) per concentration, but since this protocol requires assaying in triplicate, one run consists of three assay wells per concentration. In addition, this protocol requires three independent (i.e. non-concurrent) runs per chemical.

Appendix 2.2

TYPICAL [³H]-17B-ESTRADIOL SATURATION ASSAY WITH THREE REPLICATE WELLS

Typical [³ H]-17β-Estradiol Saturation Assay with Three Replicate Wells											
Position	Replicate	Well Type Code	Hot E2 Initial Concentration (nM)	Hot E2 Volume (μl)	Hot E2 Final Concentration (nM)	Cold E2 Initial Concentration (μM)	Cold E2 Volume (μl)	Cold E2 Final Concentration (μM)	Buffer Volume (μl)	Receptor Volume (μl)	Total volume in wells
A1	1	H	0.12	40	0.03	—	—	—	80	40	160
A2	2	H	0.12	40	0.03	—	—	—	80	40	160
A3	3	H	0.12	40	0.03	—	—	—	80	40	160
A4	1	H	0.24	40	0.06	—	—	—	80	40	160
A5	2	H	0.24	40	0.06	—	—	—	80	40	160
A6	3	H	0.24	40	0.06	—	—	—	80	40	160
A7	1	H	0.32	40	0.08	—	—	—	80	40	160
A8	2	H	0.32	40	0.08	—	—	—	80	40	160
A9	3	H	0.32	40	0.08	—	—	—	80	40	160
A10	1	H	0.40	40	0.10	—	—	—	80	40	160
A11	2	H	0.40	40	0.10	—	—	—	80	40	160
A12	3	H	0.40	40	0.10	—	—	—	80	40	160
B1	1	H	1.20	40	0.30	—	—	—	80	40	160
B2	2	H	1.20	40	0.30	—	—	—	80	40	160
B3	3	H	1.20	40	0.30	—	—	—	80	40	160
B4	1	H	2.40	40	0.60	—	—	—	80	40	160
B5	2	H	2.40	40	0.60	—	—	—	80	40	160
B6	3	H	2.40	40	0.60	—	—	—	80	40	160
B7	1	H	4.00	40	1.00	—	—	—	80	40	160
B8	2	H	4.00	40	1.00	—	—	—	80	40	160
B9	3	H	4.00	40	1.00	—	—	—	80	40	160
B10	1	H	12.00	40	3.00	—	—	—	80	40	160
B11	2	H	12.00	40	3.00	—	—	—	80	40	160
B12	3	H	12.00	40	3.00	—	—	—	80	40	160
D1	1	HC	0.12	40	0.03	0.06	80	0.03	—	40	160
D2	2	HC	0.12	40	0.03	0.06	80	0.03	—	40	160
D3	3	HC	0.12	40	0.03	0.06	80	0.03	—	40	160
D4	1	HC	0.24	40	0.06	0.12	80	0.06	—	40	160
D5	2	HC	0.24	40	0.06	0.12	80	0.06	—	40	160
D6	3	HC	0.24	40	0.06	0.12	80	0.06	—	40	160
D7	1	HC	0.32	40	0.08	0.16	80	0.08	—	40	160
D8	2	HC	0.32	40	0.08	0.16	80	0.08	—	40	160
D9	3	HC	0.32	40	0.08	0.16	80	0.08	—	40	160
D10	1	HC	0.40	40	0.10	0.2	80	0.1	—	40	160
D11	2	HC	0.40	40	0.10	0.2	80	0.1	—	40	160
D12	3	HC	0.40	40	0.10	0.2	80	0.1	—	40	160

Typical [3H]-17β-Estradiol Saturation Assay with Three Replicate Wells											
Position	Replicate	Well Type Code	Hot E2 Initial Concentration (nM)	Hot E2 Volume (μl)	Hot E2 Final Concentration (nM)	Cold E2 Initial Concentration (μM)	Cold E2 Volume (μl)	Cold E2 Final Concentration (μM)	Buffer Volume (μl)	Receptor Volume (μl)	Total volume in wells
E1	1	HC	1.20	40	0.30	0.6	80	0.3	—	40	160
E2	2	HC	1.20	40	0.30	0.6	80	0.3	—	40	160
E3	3	HC	1.20	40	0.30	0.6	80	0.3	—	40	160
E4	1	HC	2.40	40	0.60	1.2	80	0.6	—	40	160
E5	2	HC	2.40	40	0.60	1.2	80	0.6	—	40	160
E6	3	HC	2.40	40	0.60	1.2	80	0.6	—	40	160
E7	1	HC	4.00	40	1.00	2	80	1	—	40	160
E8	2	HC	4.00	40	1.00	2	80	1	—	40	160
E9	3	HC	4.00	40	1.00	2	80	1	—	40	160
E10	1	HC	12.00	40	3.00	6	80	3	—	40	160
E11	2	HC	12.00	40	3.00	6	80	3	—	40	160
E12	3	HC	12.00	40	3.00	6	80	3	—	40	160
G1	1	Hot	0.12	40	0.03	—	—	—	—	—	40
G2	2	Hot	0.12	40	0.03	—	—	—	—	—	40
G3	3	Hot	0.12	40	0.03	—	—	—	—	—	40
G4	1	Hot	0.24	40	0.06	—	—	—	—	—	40
G5	2	Hot	0.24	40	0.06	—	—	—	—	—	40
G6	3	Hot	0.24	40	0.06	—	—	—	—	—	40
G7	1	Hot	0.32	40	0.08	—	—	—	—	—	40
G8	2	Hot	0.32	40	0.08	—	—	—	—	—	40
G9	3	Hot	0.32	40	0.08	—	—	—	—	—	40
G10	1	Hot	0.40	40	0.10	—	—	—	—	—	40
G11	2	Hot	0.40	40	0.10	—	—	—	—	—	40
G12	3	Hot	0.40	40	0.10	—	—	—	—	—	40
H1	1	Hot	1.20	40	0.30	—	—	—	—	—	40
H2	2	Hot	1.20	40	0.30	—	—	—	—	—	40
H3	3	Hot	1.20	40	0.30	—	—	—	—	—	40
H4	1	Hot	2.40	40	0.60	—	—	—	—	—	40
H5	2	Hot	2.40	40	0.60	—	—	—	—	—	40
H6	3	Hot	2.40	40	0.60	—	—	—	—	—	40
H7	1	Hot	4.00	40	1.00	—	—	—	—	—	40
H8	2	Hot	4.00	40	1.00	—	—	—	—	—	40
H9	3	Hot	4.00	40	1.00	—	—	—	—	—	40
H10	1	Hot	12.00	40	3.00	—	—	—	—	—	40
H11	2	Hot	12.00	40	3.00	—	—	—	—	—	40
H12	3	Hot	12.00	40	3.00	—	—	—	—	—	40

Note that the "hot" wells are empty during incubation. The 40 μl are added only for scintillation counting.

Appendix 2.3: Competitive Binding Assay Well Layout

Plate	Position	Replicate	Well type	Well code	Concentration code	Competitor Initial Concentration (M)	hrER stock (μl)	Buffer Volume (μl)	Tracer (Hot E2) Volume (μL)	Volume from dilution plate (μL)	Final Volume (μl)	Competitor Final Concentration (M)
S	A1	1	total binding	TB	TB1	-	40		40	80	160	-
S	A2	2	total binding	TB	TB2	-	40		40	80	160	-
S	A3	3	total binding	TB	TB3	-	40		40	80	160	-
S	A4	1	total binding	TB	TB4	-	40		40	80	160	-
S	A5	2	total binding	TB	TB5	-	40		40	80	160	-
S	A6	3	total binding	TB	TB6	-	40		40	80	160	-
S	A7	1	cold E2 (high)	NSB	S0	2.00E-06	40	-	40	80	160	1.0E-06
S	A8	2	cold E2 (high)	NSB	S0	2.00E-06	40	-	40	80	160	1.0E-06
S	A9	3	cold E2 (high)	NSB	S0	2.00E-06	40	-	40	80	160	1.0E-06
S	A10	1	cold E2 (high)	NSB	S0	2.00E-06	40	-	40	80	160	1.0E-06
S	A11	2	cold E2 (high)	NSB	S0	2.00E-06	40	-	40	80	160	1.0E-06
S	A12	3	cold E2 (high)	NSB	S0	2.00E-06	40	-	40	80	160	1.0E-06
S	B1	1	cold E2	S	S1	2.00E-07	40	-	40	80	160	1.0E-07
S	B2	2	cold E2	S	S1	2.00E-07	40	-	40	80	160	1.0E-07
S	B3	3	cold E2	S	S1	2.00E-07	40	-	40	80	160	1.0E-07
S	B4	1	cold E2	S	S2	2.00E-08	40	-	40	80	160	1.0E-08
S	B5	2	cold E2	S	S2	2.00E-08	40	-	40	80	160	1.0E-08
S	B6	3	cold E2	S	S2	2.00E-08	40	-	40	80	160	1.0E-08
S	B7	1	cold E2	S	S3	6.00E-09	40	-	40	80	160	3.0E-09
S	B8	2	cold E2	S	S3	6.00E-09	40	-	40	80	160	3.0E-09
S	B9	3	cold E2	S	S3	6.00E-09	40	-	40	80	160	3.0E-09
S	B10	1	cold E2	S	S4	2.00E-09	40	-	40	80	160	1.0E-09
S	B11	2	cold E2	S	S4	2.00E-09	40	-	40	80	160	1.0E-09
S	B12	3	cold E2	S	S4	2.00E-09	40	-	40	80	160	1.0E-09
S	C1	1	cold E2	S	S5	6.00E-10	40	-	40	80	160	3.0E-10
S	C2	2	cold E2	S	S5	6.00E-10	40	-	40	80	160	3.0E-10
S	C3	3	cold E2	S	S5	6.00E-10	40	-	40	80	160	3.0E-10
S	C4	1	cold E2	S	S6	2.00E-10	40	-	40	80	160	1.0E-10
S	C5	2	cold E2	S	S6	2.00E-10	40	-	40	80	160	1.0E-10
S	C6	3	cold E2	S	S6	2.00E-10	40	-	40	80	160	1.0E-10
S	C7	1	cold E2	S	S7	2.00E-11	40	-	40	80	160	1.0E-11
S	C8	2	cold E2	S	S7	2.00E-11	40	-	40	80	160	1.0E-11
S	C9	3	cold E2	S	S7	2.00E-11	40	-	40	80	160	1.0E-11
S	C10	1	blank	blank	B1	-	-	160	-	-	160	-
S	C11	2	blank	blank	B2	-	-	160	-	-	160	-
S	C12	3	blank	blank	B3	-	-	160	-	-	160	-
S	D1	1	norethynodrel	NE	WP1	6.00E-05	40	-	40	80	160	3.0E-05
S	D2	1	norethynodrel	NE	WP1	6.00E-05	40	-	40	80	160	3.0E-05
S	D3	1	norethynodrel	NE	WP1	6.00E-05	40	-	40	80	160	3.0E-05
S	D4	1	norethynodrel	NE	WP2	2.00E-05	40	-	40	80	160	1.0E-05
S	D5	1	norethynodrel	NE	WP2	2.00E-05	40	-	40	80	160	1.0E-05
S	D6	1	norethynodrel	NE	WP2	2.00E-05	40	-	40	80	160	1.0E-05
S	D7	1	norethynodrel	NE	WP3	6.00E-06	40	-	40	80	160	3.0E-06
S	D8	1	norethynodrel	NE	WP3	6.00E-06	40	-	40	80	160	3.0E-06
S	D9	1	norethynodrel	NE	WP3	6.00E-06	40	-	40	80	160	3.0E-06
S	D10	1	norethynodrel	NE	WP4	2.00E-06	40	-	40	80	160	1.0E-06
S	D11	1	norethynodrel	NE	WP4	2.00E-06	40	-	40	80	160	1.0E-06
S	D12	1	norethynodrel	NE	WP4	2.00E-06	40	-	40	80	160	1.0E-06

Competitive Binding Assay Well Layout												
Plate	Position	Replicate	Well type	Well code	Concentration code	Competitor Initial Concentration (M)	hrER stock (μl)	Buffer Volume (μl)	Tracer (Hot E2) Volume (μL)	Volume from dilution plate (μL)	Final Volume (μl)	Competitor Final Concentration (M)
S	E1	1	norethynodrel	NE	WP	6.00E-07	40		40	80	160	3.0E-07
S	E2	2	norethynodrel	NE	WP	6.00E-07	40		40	80	160	3.0E-07
S	E3	3	norethynodrel	NE	WP	6.00E-07	40		40	80	160	3.0E-07
S	E4	1	norethynodrel	NE	WP	2.00E-07	40		40	80	160	1.0E-07
S	E5	2	norethynodrel	NE	WP	2.00E-07	40		40	80	160	1.0E-07
S	E6	3	norethynodrel	NE	WP	2.00E-07	40		40	80	160	1.0E-07
S	E7	1	norethynodrel	NE	WP	6.00E-08	40	-	40	80	160	3.0E-08
S	E8	2	norethynodrel	NE	WP	6.00E-08	40	-	40	80	160	3.0E-08
S	E9	3	norethynodrel	NE	WP	6.00E-08	40	-	40	80	160	3.0E-08
S	E10	1	norethynodrel	NE	WP	6.00E-09	40	-	40	80	160	3.0E-09
S	E11	2	norethynodrel	NE	WP	6.00E-09	40	-	40	80	160	3.0E-09
S	E12	3	norethynodrel	NE	WP	6.00E-09	40	-	40	80	160	3.0E-09
S	F1	1	OTES	N	OTES	2.00E-03	40	-	40	80	160	1.0E-03
S	F2	2	OTES	N	OTES	2.00E-03	40	-	40	80	160	1.0E-03
S	F3	3	OTES	N	OTES	2.00E-03	40	-	40	80	160	1.0E-03
S	F4	1	OTES	N	OTES	2.00E-04	40	-	40	80	160	1.0E-04
S	F5	2	OTES	N	OTES	2.00E-04	40	-	40	80	160	1.0E-04
S	F6	3	OTES	N	OTES	2.00E-04	40	-	40	80	160	1.0E-04
S	F7	1	OTES	N	OTES	2.00E-05	40	-	40	80	160	3.0E-05
S	F8	2	OTES	N	OTES	2.00E-05	40	-	40	80	160	3.0E-05
S	F9	3	OTES	N	OTES	2.00E-05	40	-	40	80	160	3.0E-05
S	F10	1	OTES	N	OTES	2.00E-06	40	-	40	80	160	1.0E-06
S	F11	2	OTES	N	OTES	2.00E-06	40	-	40	80	160	1.0E-06
S	F12	3	OTES	N	OTES	2.00E-06	40	-	40	80	160	1.0E-06
S	G1	1	OTES	N	OTES	2.00E-07	40	-	40	80	160	3.0E-07
S	G2	2	OTES	N	OTES	2.00E-07	40	-	40	80	160	3.0E-07
S	G3	3	OTES	N	OTES	2.00E-07	40	-	40	80	160	3.0E-07
S	G4	1	OTES	N	OTES	2.00E-08	40	-	40	80	160	1.0E-08
S	G5	2	OTES	N	OTES	2.00E-08	40	-	40	80	160	1.0E-08
S	G6	3	OTES	N	OTES	2.00E-08	40	-	40	80	160	1.0E-08
S	G7	1	OTES	N	OTES	2.00E-09	40	-	40	80	160	1.0E-09
S	G8	2	OTES	N	OTES	2.00E-09	40	-	40	80	160	1.0E-09
S	G9	3	OTES	N	OTES	2.00E-09	40	-	40	80	160	1.0E-09
S	G10	1	OTES	N	OTES	2.00E-10	40	-	40	-	160	1.0E-10
S	G11	2	OTES	N	OTES	2.00E-10	40	-	40	-	160	1.0E-10
S	G12	3	OTES	N	OTES	2.00E-10	40	-	40	-	160	1.0E-10
S	H1	1	hot	H	H	-	-	-	40	-	40	-
S	H2	1	hot	H	H	-	-	-	40	-	40	-
S	H3	1	hot	H	H	-	-	-	40	-	40	-
S	H4	1	hot	H	H	-	-	-	40	-	40	-
S	H5	1	hot	H	H	-	-	-	40	-	40	-
S	H6	1	hot	H	H	-	-	-	40	-	40	-
S	H7	1	buffer control	BC	BC	-	40	80	40	-	160	-
S	H8	1	buffer control	BC	BC	-	40	80	40	-	160	-
S	H9	1	buffer control	BC	BC	-	40	80	40	-	160	-
S	H10	1	buffer control	BC	BC	-	40	80	40	-	160	-
S	H11	1	buffer control	BC	BC	-	40	80	40	-	160	-
S	H12	1	buffer control	BC	BC	-	40	80	40	-	160	-

Note that the "hot" wells are empty during incubation. The 40 μl are added only for scintillation counting.

Competitive Binding Assay Well Layout													
Plate	Position	Replicate	Well type	Well Code	Concentration Code	Competitor Initial Concentration (M)	hrER stock (μL)	Buffer Volume (μL)	Tracer (Hot E2) Volume (μL)	Volume from dilution plate (μL)	Final Volume (μL)	Competitor Final Concentration (M)	
P1	A1	1	total binding	TB	TBB1B1	-	40	-	40	80	160	-	
P1	A2	2	total binding	TB	TB2	-	40	-	40	80	160	-	
P1	A3	3	total binding	TB	TB3	-	40	-	40	80	160	-	
P1	A4	1	total binding	TB	TB4	-	40	-	40	80	160	-	
P1	A5	2	total binding	TB	TB5	-	40	-	40	80	160	-	
P1	A6	3	total binding	TB	TB6	-	40	-	40	80	160	-	
P1	A7	1	cold E2 (high)	NSB	S0	2.00E-06	40	-	40	80	160	1.0E-06	
P1	A8	2	cold E2 (high)	NSB	S0	2.00E-06	40	-	40	80	160	1.0E-06	
P1	A9	3	cold E2 (high)	NSB	S0	2.00E-06	40	-	40	80	160	1.0E-06	
P1	A10	1	cold E2 (high)	NSB	S0	2.00E-06	40	-	40	80	160	1.0E-06	
P1	A11	2	cold E2 (high)	NSB	S0	2.00E-06	40	-	40	80	160	1.0E-06	
P1	A12	3	cold E2 (high)	NSB	S0	2.00E-06	40	-	40	80	160	1.0E-06	
P1	B1	1	Test Chemical 1	TC1	1	2.00E-03	40	0	40	80	160	1.0E-03	
P1	B2	2	Test Chemical 1	TC1	1	2.00E-03	40	0	40	80	160	1.0E-03	
P1	B3	3	Test Chemical 1	TC1	1	2.00E-03	40	0	40	80	160	1.0E-03	
P1	B4	1	Test Chemical 1	TC1	2	2.00E-04	40	0	40	80	160	1.0E-04	
P1	B5	2	Test Chemical 1	TC1	2	2.00E-04	40	0	40	80	160	1.0E-04	
P1	B6	3	Test Chemical 1	TC1	2	2.00E-04	40	0	40	80	160	1.0E-04	
P1	B7	1	Test Chemical 1	TC1	3	2.00E-05	40	0	40	80	160	1.0E-05	
P1	B8	2	Test Chemical 1	TC1	3	2.00E-05	40	0	40	80	160	1.0E-05	
P1	B9	3	Test Chemical 1	TC1	3	2.00E-05	40	0	40	80	160	1.0E-05	
P1	B10	1	Test Chemical 1	TC1	4	2.00E-06	40	0	40	80	160	1.0E-06	
P1	B11	2	Test Chemical 1	TC1	4	2.00E-06	40	0	40	80	160	1.0E-06	
P1	B12	3	Test Chemical 1	TC1	4	2.00E-06	40	0	40	80	160	1.0E-06	
P1	C1	1	Test Chemical 1	TC1	5	2.00E-07	40	0	40	80	160	1.0E-07	
P1	C2	2	Test Chemical 1	TC1	5	2.00E-07	40	0	40	80	160	1.0E-07	
P1	C3	3	Test Chemical 1	TC1	5	2.00E-07	40	0	40	80	160	1.0E-07	
P1	C4	1	Test Chemical 1	TC1	6	2.00E-08	40	0	40	80	160	1.0E-08	
P1	C5	2	Test Chemical 1	TC1	6	2.00E-08	40	0	40	80	160	1.0E-08	
P1	C6	3	Test Chemical 1	TC1	6	2.00E-08	40	0	40	80	160	1.0E-08	
P1	C7	1	Test Chemical 1	TC1	7	2.00E-09	40	0	40	80	160	1.0E-09	
P1	C8	2	Test Chemical 1	TC1	7	2.00E-09	40	0	40	80	160	1.0E-09	
P1	C9	3	Test Chemical 1	TC1	7	2.00E-09	40	0	40	80	160	1.0E-09	
P1	C10	1	Test Chemical 1	TC1	8	2.00E-10	40	0	40	80	160	1.0E-10	
P1	C11	2	Test Chemical 1	TC1	8	2.00E-10	40	0	40	80	160	1.0E-10	
P1	C12	3	Test Chemical 1	TC1	8	2.00E-10	40	0	40	80	160	1.0E-10	
P1	D1	1	Test Chemical 2	TC2	1	2.00E-03	40	0	40	80	160	1.0E-03	
P1	D2	2	Test Chemical 2	TC2	1	2.00E-03	40	0	40	80	160	1.0E-03	
P1	D3	3	Test Chemical 2	TC2	1	2.00E-03	40	0	40	80	160	1.0E-03	
P1	D4	1	Test Chemical 2	TC2	2	2.00E-04	40	0	40	80	160	1.0E-04	
P1	D5	2	Test Chemical 2	TC2	2	2.00E-04	40	0	40	80	160	1.0E-04	
P1	D6	3	Test Chemical 2	TC2	2	2.00E-04	40	0	40	80	160	1.0E-04	
P1	D7	1	Test Chemical 2	TC2	3	2.00E-05	40	0	40	80	160	1.0E-05	
P1	D8	2	Test Chemical 2	TC2	3	2.00E-05	40	0	40	80	160	1.0E-05	
P1	D9	3	Test Chemical 2	TC2	3	2.00E-05	40	0	40	80	160	1.0E-05	
P1	D10	1	Test Chemical 2	TC2	4	2.00E-06	40	0	40	80	160	1.0E-06	
P1	D11	2	Test Chemical 2	TC2	4	2.00E-06	40	0	40	80	160	1.0E-06	
P1	D12	3	Test Chemical 2	TC2	4	2.00E-06	40	0	40	80	160	1.0E-06	
P1	E1	1	Test Chemical 2	TC2	5	2.00E-07	40	0	40	80	160	1.0E-07	
P1	E2	2	Test Chemical 2	TC2	5	2.00E-07	40	0	40	80	160	1.0E-07	
P1	E3	3	Test Chemical 2	TC2	5	2.00E-07	40	0	40	80	160	1.0E-07	

Competitive Binding Assay Well Layout												
Plate	Position	Replicate	Well type	Well Code	Concentration Code	Competitor Initial Concentration (M)	hrER stock (μL)	Buffer Volume (μL)	Tracer (Hot E2) Volume (μL)	Volume from dilution plate (μL)	Final Volume (μL)	Competitor Final Concentration (M)
P1	E4	1	Test Chemical 2	TC2	6	-	40	0	40	80	160	1.0E-08
P1	E5	2	Test Chemical 2	TC2	6	-	40	0	40	80	160	1.0E-08
P1	E6	3	Test Chemical 2	TC2	6	-	40	0	40	80	160	1.0E-08
P1	E7	1	Test Chemical 2	TC2	7	2.00E-06	40	0	40	80	160	1.0E-09
P1	E8	2	Test Chemical 2	TC2	7	2.00E-06	40	0	40	80	160	1.0E-09
P1	E9	3	Test Chemical 2	TC2	7	2.00E-06	40	0	40	80	160	1.0E-09
P1	E10	1	Test Chemical 2	TC2	8	2.00E-06	40	0	40	80	160	1.0E-10
P1	E11	2	Test Chemical 2	TC2	8	2.00E-06	40	0	40	80	160	1.0E-10
P1	E12	3	Test Chemical 2	TC2	8	2.00E-06	40	0	40	80	160	1.0E-10
P1	F1	1	Test Chemical 3	TC3	1	2.00E-03	40	0	40	80	160	1.0E-03
P1	F2	2	Test Chemical 3	TC3	1	2.00E-03	40	0	40	80	160	1.0E-03
P1	F3	3	Test Chemical 3	TC3	1	2.00E-03	40	0	40	80	160	1.0E-03
P1	F4	1	Test Chemical 3	TC3	2	2.00E-04	40	0	40	80	160	1.0E-04
P1	F5	2	Test Chemical 3	TC3	2	2.00E-04	40	0	40	80	160	1.0E-04
P1	F6	3	Test Chemical 3	TC3	2	2.00E-04	40	0	40	80	160	1.0E-04
P1	F7	1	Test Chemical 3	TC3	3	2.00E-05	40	0	40	80	160	1.0E-05
P1	F8	2	Test Chemical 3	TC3	3	2.00E-05	40	0	40	80	160	1.0E-05
P1	F9	3	Test Chemical 3	TC3	3	2.00E-05	40	0	40	80	160	1.0E-05
P1	F10	1	Test Chemical 3	TC3	4	2.00E-06	40	0	40	80	160	1.0E-06
P1	F11	2	Test Chemical 3	TC3	4	2.00E-06	40	0	40	80	160	1.0E-06
P1	F12	3	Test Chemical 3	TC3	4	2.00E-06	40	0	40	80	160	1.0E-06
P1	G1	1	Test Chemical 3	TC3	5	2.00E-07	40	0	40	80	160	1.0E-07
P1	G2	2	Test Chemical 3	TC3	5	2.00E-07	40	0	40	80	160	1.0E-07
P1	G3	3	Test Chemical 3	TC3	5	2.00E-07	40	0	40	80	160	1.0E-07
P1	G4	1	Test Chemical 3	TC3	6	2.00E-08	40	0	40	80	160	1.0E-08
P1	G5	2	Test Chemical 3	TC3	6	2.00E-08	40	0	40	80	160	1.0E-08
P1	G6	3	Test Chemical 3	TC3	6	2.00E-08	40	0	40	80	160	1.0E-08
P1	G7	1	Test Chemical 3	TC3	7	2.00E-09	40	0	40	80	160	1.0E-09
P1	G8	2	Test Chemical 3	TC3	7	2.00E-09	40	0	40	80	160	1.0E-09
P1	G9	3	Test Chemical 3	TC3	7	2.00E-09	40	0	40	80	160	1.0E-09
P1	G10	1	Test Chemical 3	TC3	8	2.00E-10	40	0	40	80	160	1.0E-10
P1	G11	2	Test Chemical 3	TC3	8	2.00E-10	40	0	40	80	160	1.0E-10
P1	G12	3	Test Chemical 3	TC3	8	2.00E-10	40	0	40	80	160	1.0E-10
P1	H1	1	norethynodrel	NE		IC50	40	0	40	80	160	
P1	H2	2	norethynodrel	NE		IC50	40	0	40	80	160	
P1	H3	3	norethynodrel	NE		IC50	40	0	40	80	160	
P1	H4	1	norethynodrel	NE		1.00E-4.5	40	0	40	80	160	
P1	H5	2	norethynodrel	NE		1.00E-4.5	40	0	40	80	160	
P1	H6	3	norethynodrel	NE		1.00E-4.5	40	0	40	80	160	
P1	H7	1	cold E2 S			IC50	40	0	40	80	160	
P1	H8	2	cold E2 S			IC50	40	0	40	80	160	
P1	H9	3	cold E2 S			IC50	40	0	40	80	160	
P1	H10	1	cold E2 S			1.00E-7	40	0	40	80	160	
P1	H11	2	cold E2 S			1.00E-7	40	0	40	80	160	
P1	H12	3	cold E2 S			1.00E-7	40	0	40	80	160	

Appendix 3

THE CHEMICAL EVALUATION AND RESEARCH INSTITUTE (CERI) *IN VITRO* ESTROGEN RECEPTOR BINDING ASSAY USING A HUMAN RECOMBINANT ERA LIGAND BINDING DOMAIN PROTEIN

INITIAL CONSIDERATIONS AND LIMITATIONS (SEE ALSO GENERAL INTRODUCTION)

1. This *in vitro* Estrogen Receptor (ER α) saturation and competitive binding assay uses a ligand binding domain (LBD) of the human ER α (hrER α). This protein construct was produced by the Chemicals Evaluation Research Institute (CERI), Japan, and exists as a glutathione-S-transferase (GST) fusion protein, and is expressed in *E. coli*. The CERI protocol underwent an international multi-laboratory validation study (2) which has demonstrated its relevance and reliability for the intended purpose of the assay.
2. This assay is a screening procedure for identifying substances that can bind to the hrER α . It is used to determine the ability of a test chemical to compete with 17 β -estradiol for binding to hrER α -LBD. Quantitative assay results may include the IC₅₀ (a measure of the concentration of test chemical needed to displace half of the [³H]-17 β -estradiol from the hrER α) and the relative binding affinities of test chemicals for the hrER α compared to 17 β -estradiol. For chemical screening purposes, acceptable qualitative assay results may include classifications of test chemicals as either hrER α binders, non-binders, or equivocal based upon criteria described for the binding curves.
3. The assay uses a radioactive ligand that requires a radioactive materials license for the laboratory. All procedures with radioisotopes and hazardous chemicals should follow the regulations and procedures as described by national legislation.
4. The “GENERAL INTRODUCTION” and “hrER BINDING ASSAY COMPONENTS” should be read before using this assay for regulatory purposes. Definitions and abbreviations used in this TG are described in Appendix 1.

PRINCIPLES OF THE ASSAY (SEE ALSO GENERAL INTRODUCTION)

5. The hrER α binding assay measures the ability of a radiolabelled ligand ([³H]17 β -estradiol) to bind with the ER in the presence of increasing concentrations of a test chemical (i.e. competitor). Test chemicals that possess a high affinity for the ER compete with the radiolabelled ligand at a lower concentration as compared with those chemicals with lower affinity for the receptor.
6. This assay consists of two major components: a saturation binding experiment to characterise receptor-ligand interaction parameters, followed by a competitive binding experiment that characterises the competition between a test chemical and a radiolabelled ligand for binding to the ER.

7. The purpose of the saturation binding experiment is to characterise a particular batch of receptors for binding affinity and number in preparation for the competitive binding experiment. The saturation binding experiment measures, under equilibrium conditions, the affinity of a fixed concentration of the estrogen receptor for its natural ligand (represented by the dissociation constant, K_d), and the concentration of active receptor sites (B_{max}).
8. The competitive binding experiment measures the affinity of a substance to compete with [^3H]17 β -estradiol for binding to the ER. The affinity is quantified by the concentration of test chemical that, at equilibrium, inhibits 50% of the specific binding of the [^3H]17 β -estradiol (termed the “inhibitory concentration 50%” or IC_{50}). This can also be evaluated using the relative binding affinity (RBA, relative to the IC_{50} of estradiol measured separately in the same run). The competitive binding experiment measures the binding of [^3H]17 β -estradiol at a fixed concentration in the presence of a wide range (eight orders of magnitude) of test chemical concentrations. The data are then fit, where possible, to a form of the Hill equation (Hill, 1910) that describes the displacement of the radioligand by a one-site competitive binder. The extent of displacement of the radiolabelled estradiol at equilibrium is used to characterise the test chemical as a binder, non-binder, or generating an equivocal response.

PROCEDURE

Demonstration of Acceptable hrER α Protein Performance

9. Prior to routinely conducting the saturation and competitive binding assays, each new batch of hrER α should be shown to be performing correctly in the laboratory in which it will be used. A two-step process should be used to demonstrate performance. These steps are the following:
 - Conduct a saturation [^3H]-17 β -estradiol binding assay to demonstrate hrER α specificity and saturation. Nonlinear regression analysis of these data (e.g. BioSoft; McPherson, 1985; Motulsky, 1995) and the subsequent Scatchard plot should document hrER α binding affinity of the [^3H]-17 β -estradiol (K_d) and the number of receptors (B_{max}) for a particular batch of hrER α .
 - Conduct a competitive binding assay using the control substances (reference estrogen (17 β -estradiol), a weak binder (e.g. norethynodrel or norethindrone), and a non-binder (octyltriethoxysilane, OTES). Each laboratory should establish an historical database to document the consistency of IC_{50} and the relevant values for the reference estrogen and weak binder among experiments and different batches of hrER α . In addition, the parameters of the competitive binding curves for the control substances should be within the limits of the 95% confidence interval (see Table 1) that were developed using data from laboratories that participated in the validation study for this assay (2).

Table 1: Performance criteria developed for the reference estrogen and weak binder, CERI hrER Binding Assay.

Substance	Parameter	Mean ^a	Standard Deviation(n)	95% Confidence Intervals ^b	
				Lower Limit	Upper Limit
17 β -estradiol	Top	104.74	13.12 (70)	101.6	107.9
	Bottom	0.85	2.41 (70)	0.28	1.43
	HillSlope	-1.22	0.20 (70)	-1.27	-1.17
	LogIC ₅₀	-8.93	0.23 (70)	-8.98	-8.87
Norethynodrel	Top	101.31	10.55 (68)	98.76	103.90
	Bottom	2.39	5.01 (68)	1.18	3.60
	HillSlope	-1.04	0.21 (68)	-1.09	-0.99
	LogIC ₅₀	-6.19	0.40 (68)	-6.29	-6.10
Norethindrone ^c	Top	92.27	7.79 (23)	88.90	95.63
	Bottom	16.52	10.59 (23)	11.94	21.10
	Hill Slope	-1.18	0.32 (23)	-1.31	-1.04
	LogIC ₅₀	-6.01	0.54 (23)	-6.25	-5.78

^a Mean $\pm$ Standard Deviation (SD) with (sample size (n) were calculated using curve fit estimates (4-parameter Hill equation) for control runs conducted in four laboratories during the validation study (see Annex N of reference 2).

^b The 95% confidence are provided as a guide for acceptability criteria.

^c Testing of norethindrone was optional for Subtask 4 during validation study (see Reference 2, see Subtask 4). Thus, the mean $\pm$ SD (n) were calculated using curve fit estimates (4-parameter Hill equation) for control runs conducted in two laboratories.

The range for the IC₅₀ will be dependent upon the K_d of the receptor preparation and concentration of radiolabelled ligand used within each laboratory. Appropriate adjustment for the range of the IC₅₀ based upon the conditions used to conduct the assay will be acceptable.

Demonstration of laboratory proficiency

- See paragraphs 17 and 18 and Table 2 in “**hrER BINDING ASSAY COMPONENTS**” of this test method. Each assay (saturation and competitive binding) should consist of three independent runs (i.e. with fresh dilutions of receptor, chemicals, and reagents) on different days, and each run should contain three replicates.

Determination of Receptor (hrER α) Concentration

- The concentration of active receptor varies slightly by batch and storage conditions. For this reason, the concentration of active receptor as received from the supplier should be determined. This will yield the appropriate concentration of active receptor at the time of the run.
- Under conditions corresponding to competitive binding (i.e. 0.5 nM [³H]-estradiol), nominal concentrations of 0.1, 0.2, 0.4 and 0.6 nM receptor should be incubated in the absence (total binding) and presence (non-specific binding) of 1 μ M unlabelled estradiol. Specific binding, calculated as the difference of total and non-specific binding, is plotted against the nominal receptor concentration. The concentration of receptor that gives

specific binding values corresponding to 40% of added radiolabel is related to the corresponding receptor concentration, and this receptor concentration should be used for saturation and competitive binding experiments. Frequently, a final hrER concentration of 0.2 nM will comply with this condition.

13. If the 40% criterion repeatedly cannot be met, the experimental set up should be checked for potential errors. Failure to achieve the 40% criterion may indicate that there is very little active receptor in the recombinant batch, and the use of another receptor batch should then be considered.

Saturation assay

14. Eight increasing concentrations of [^3H]17 β -estradiol should be evaluated in triplicate, under the following three conditions (see Table 2):
 - a. In the absence of unlabelled 17 β -estradiol and presence of ER. This is the determination of total binding by measure of the radioactivity in the wells that have only [^3H]17 β -estradiol.
 - b. In the presence of a 2000- fold excess concentration of unlabelled 17 β -estradiol over labelled 17 β -estradiol and presence of ER. The intent of this condition is to saturate the active binding sites with unlabelled 17 β -estradiol, and by measuring the radioactivity in the wells, determine the non-specific binding. Any remaining hot estradiol that can bind to the receptor is considered to be binding at a non-specific site as the cold estradiol should be at such a high concentration that it is bound to all of the available specific sites on the receptor.
 - c. In the absence of unlabelled 17 β -estradiol and absence of ER (determination of total radioactivity)

Preparation of [^3H]-17 β -estradiol, unlabelled 17 β -estradiol solutions and hrER α

15. A 40 nM solution of [^3H]-17 β -estradiol should be prepared from a 1 μM stock solution of [^3H]-17 β -estradiol in DMSO, by adding DMSO (to prepare 200 nM) and assay buffer at room temperature (to prepare 40 nM). Using this 40 nM solution, the series of [^3H]-17 β -estradiol dilutions prepared, ranging from 0.313 nM to 40 nM with assay buffer at room temperature (as represented in lane 12 of Table 2). The final assay concentrations, ranging from 0.0313 to 4.0 nM, will be obtained by adding 10 μl of these solutions to the respective assay wells of a 96-well microtiter plate (see Tables 2 and 3). Preparation of assay buffer, calculation of the original [^3H]-17 β -estradiol stock solution based on its specific activity, preparation of dilutions and determination of the concentrations are described in depth in the CERI protocol (2).
16. Dilutions of unlabelled 17 β -estradiol solutions should be prepared from a 1 nM 17 β -estradiol stock solution by adding assay buffer to achieve eight increasing concentrations initially ranging from 0.625 μM to 80 μM . The final assay concentrations, ranging from 0.0625 to 8 μM , will be obtained by adding 10 μl of these solutions to the respective assay

wells of a 96-well microtiter plate dedicated to the measurement of non-specific binding (see Tables 2 and 3). Preparation of unlabelled 17 β -estradiol dilutions is described in depth in the CERI protocol (2).

17. The concentration of receptor that gives 40 $\pm$ 10% specific binding should be used (see paragraphs 12-13). The hrER α solution should be prepared with ice-cold assay buffer immediately prior to use, i.e. after all wells for total binding, non-specific binding and hot ligand alone have been prepared.

18. The 96-well microtiter plates are prepared as illustrated in Table 2, with 3 replicates per [^{3} H]-17 β -estradiol concentration. Volume assignment of [^{3} H]-17 β -estradiol, unlabelled 17 β -estradiol, buffer and receptor are provided in Table 3.

Table 2: Saturation Binding Assay Microtiter Plate Layout

	1*	2*	3*	4*	5*	6*	7*	8*	9*	10	11**	12**
	For measurement of TB			For measurement of NSB			For determination of hot ligand alone				Unlabelled E2 dilutions for plate column 4-6	[^{3} H]E2 dilutions for plate column 1-9
A	0.0313 nM [^{3} H] E2 + ER			0.0313 nM [^{3} H] E2 + 0.0625 μ M E2 + ER			0.0313 nM				0.625 μ M	0.313 nM
B	0.0625 nM [^{3} H] E2 + ER			0.0625 nM [^{3} H] E2 + 0.125 μ M E2 + ER			0.0625 nM				1.25 μ M	0.625 nM
C	0.125 nM [^{3} H] E2 + ER			0.125 nM [^{3} H] E2 + 0.25 μ M E2 + ER			0.125 nM				2.5 μ M	1.25 nM
D	0.250 nM [^{3} H] E2 + ER			0.250 nM [^{3} H] E2 + 0.5 μ M E2 + ER			0.250 nM				5 μ M	2.5 nM
E	0.50 nM [^{3} H] E2 + ER			0.50 nM [^{3} H] E2 + 1 μ M E2 + ER			0.50 nM				10 μ M	5 nM
F	1.00 nM [^{3} H] E2 + ER			1.00 nM [^{3} H] E2 + 2 μ M E2 + ER			1.00 nM				20 μ M	10 nM
G	2.00 nM [^{3} H] E2 + ER			2.00 nM [^{3} H] E2 + 4 μ M E2 + ER			2.00 nM				40 μ M	20 nM
H	4.00 nM [^{3} H] E2 + ER			4.00 nM [^{3} H] E2 + 8 μ M E2 + ER			4.00 nM				80 μ M	40 nM

TB: total binding,

NSB: non-specific binding

[^{3} H] E2: [^{3} H]17 β -estradiol

E2: unlabelled 17 β -estradiol

*The indicated concentrations here are the final concentrations in each well.

**The dilutions of unlabelled E2 and [^{3} H]E2 can be prepared in a different plate.

Table 3: Reagent Volumes for Saturation Microtiter Plate

Lane Number		1	2	3	4	5	6	7*	8*	9*
Preparation Steps		TB Wells			NSB Wells			Hot Ligand Alone		
Volume of components for reaction wells above and order to add	Buffer	60 µl			50 µl			90 µl		
	unlabelled E2 from lane 11 in Table2	-			10 µl			-		
	[3H]E2 from lane12 in Table2	10 µl			10 µl			10 µl		
	hrERα	30 µl			30 µl			-		
Total reaction volume		100 µl			100 µl			100 µl		
Incubation		FOLLOWING 2 HOUR INCUBATION REACTION						Quantification of the radioactivity just after the preparation. No incubation		
Treatment with 0.4% DCC		Yes			Yes			No		
Volume of 0.4% DCC		100 µl			100 µl			-		
Filtration		Yes			Yes			No		
MEASURING THE DPMS										
Quantification volume added to scintillation cocktail		100 µl**			100 µl**			50 µl		

* If an LSC for microplates is used for measuring dpms, the preparation of hot ligand alone in the same assay plate of TB and NSB wells is not appropriate. The hot ligand alone should be prepared in a different plate.

** If centrifugation is used to separate DCC, the 50 μ l of supernatant should be measured by LSC in order to avoid contamination of DCC.

19. Assay microtiter plates for the determination of total binding and non-specific binding should be incubated at room temperature (22°C to 28°C) for two hours.

Measurement of [^{3} H]-17 β -Estradiol bound to hrER α

20. Following the two hour incubation period, [^{3} H]-17 β -Estradiol bound to hrER α should be separated from free [^{3} H]-17 β -Estradiol by adding 100 μ l an ice cold 0.4% DCC suspension to the wells. The plates should then be placed on ice for 10 minutes and the reaction mixture and DCC suspension should be filtered, by transfer to a microtiter plate filter, to remove DCC. A 100 μ l of the filtrate should then be added to scintillation fluid in LSC vials for determination of disintegration per minute (dpms) per vial by liquid scintillation counting.

21. Alternatively, if a microplate filter is not available, removal of DCC can be obtained by centrifugation. A 50 µl of supernatant containing the hrERα-bound [³H]-17β-estradiol should then be taken with extreme care, to avoid any contamination of the wells by touching DCC, and should be used for scintillation counting.
22. The hot ligand alone condition is used for determining the disintegration per minute (dpm) of [³H]-17β-estradiol added to the assay wells. The radioactivity should be quantified just after preparation. These wells should not be incubated and should not be treated with DCC suspension but their content should be delivered directly into the scintillation fluid. These measures demonstrate how much [³H]-17β-estradiol in dpms was added to each set of wells for the total binding and non-specific binding.

Competitive binding assay

23. The competitive binding assay measures the binding of a single concentration of [³H]-17β-estradiol in the presence of increasing concentrations of a test chemical. Three concurrent replicates should be used at each concentration within one run. In addition, three non-concurrent runs should be performed for each chemical tested. The assay should be set up in one or more 96-well microtiter plates.

Controls

24. When performing the assay, concurrent solvent and controls (i.e. reference estrogen, weak binder, and non-binder) should be included in each experiment. Full concentration curves for the reference estrogen and controls (i.e. weak binder and non-binder) should be used in one plate during each run. All other plates should contain (i) a high- (maximum displacement i.e. approximately full displacement of radiolabelled ligand) and medium- (approximately, the IC₅₀) concentration of E2 and weak binder in triplicate; (ii) solvent control and non-specific binding, each in triplicate. Procedures for the preparation of assay buffer, [³H]-17β-estradiol, hrERα and test chemical solutions are described in depth in the CERI protocol (2).

Solvent control:

25. The solvent control indicates that the solvent does not interact with the test system and also measures total binding (TB). DMSO is the preferred solvent. Alternatively, if the highest concentration of the test chemical is not soluble in DMSO, ethanol may be used. The concentration of DMSO in the final assay wells should be 2.05% and could be increased up to 2.5% in case of lack of solubility of the test chemical. Concentrations of DMSO above 2.5% should not be used because of interference of higher solvent concentrations with the assay. For test chemicals that are not soluble in DMSO, but are soluble in ethanol, a maximum of 2% ethanol may be used in the assay without interference.

Buffer control:

26. The buffer control (BC) should contain neither solvent nor test chemical, but all of the other components of the assay. The results of the buffer control are compared to the solvent control to verify that the solvent used does not affect the assay system.

Strong binder (reference estrogen)

27. 17 β -estradiol (CAS 50-28-2) is the endogenous ligand and binds with high affinity to the ER, α subtype. A standard curve using unlabelled 17 β -estradiol should be prepared for each hrER α competitive binding assay, to allow for an assessment of variability when conducting the assay over time within the same laboratory. Eight solutions of unlabelled 17 β -estradiol should be prepared in DMSO and assay buffer, with final concentrations in the assay wells to be used for the standard curve spaced as follows: 10^{-6} , 10^{-7} , 10^{-8} , $10^{-8.5}$, 10^{-9} , $10^{-9.5}$, 10^{-10} , 10^{-11} M. The highest concentration of unlabelled 17 β -estradiol (1 μ M) should serve as the non-specific binding indicator. This concentration is distinguished by the label “NSB” in Table 4 even though it is also part of the standard curve.

Weak binder

28. A weak binder (norethynodrel (CAS68-23-5), or alternate, norethindrone (CAS 68-22-4)) should be included to demonstrate the sensitivity of each experiment and to allow an assessment of variability when conducting the assay over time. Eight solutions of the weak binder should be prepared in DMSO and assay buffer, with final concentrations in the assay wells as follows: $10^{-4.5}$, $10^{-5.5}$, 10^{-6} , $10^{-6.5}$, 10^{-7} , $10^{-7.5}$, 10^{-8} and 10^{-9} M.

Non binder

29. Octytriethoxysilane (OTES, CAS 2943-75-1) should be used as the negative control (non-binder). It provides assurance that the assay as run, will detect test chemicals that do not bind to the hrER α . Eight solutions of the non-binder should be prepared in DMSO and assay buffer, with final concentrations in the assay wells as follows: 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} , 10^{-8} , 10^{-9} , 10^{-10} M. Di-n-butyl phthalate (DBP, CAS 84-72-2) can be used as an alternative non-binder, but only tested up to 10^{-4} M. The maximum solubility of DBP in the assay has been demonstrated to be 10^{-4} M.

hrER α concentration

30. The amount of receptor that gives specific binding of $40 \pm 10\%$ should be used (see paragraphs 12-13 of Appendix 3). The hrER α solution should be prepared by dilution of the functional hrER α into ice cold assay buffer, immediately prior to use.

[^{3} H]-17 β -estradiol

31. The final concentration of [^{3} H]-17 β -estradiol in the assay wells should be of 0.5 nM.

Test Chemicals

32. In the first instance, it is necessary to conduct a solubility test to determine the limit of solubility for each test chemical and to identify the appropriate concentration range to use when conducting the test protocol. The limit of solubility of each test chemical is to be initially determined in the solvent and then further confirmed under assay conditions. The

final concentration tested in the assay should not exceed 1mM. Range finder testing includes a solvent control along with at least 8 log serial dilutions, starting at maximum acceptable concentration (e.g. 1 mM or lower, based upon the limit of solubility), and the presence of cloudiness or precipitate noted (see also paragraph 35 of Appendix 3). Once the concentration range for testing has been determined, a test chemical should be tested using 8 log concentrations spaced appropriately as defined in the preceding range finding test. Concentrations tested in the second and third experiments should be further adjusted as appropriate to better characterise the concentration response curve, if necessary.

33. Dilutions of the test chemical should be prepared in the appropriate solvent (see paragraph 25 of Appendix 3). If the highest concentration of the test chemical is not soluble in either DMSO or ethanol, and adding more solvent would cause the solvent concentration in the final tube to be greater than the acceptable limit, the highest concentration may be reduced to the next lower concentration. In this case, an additional concentration may be added at the low end of the concentration series. Other concentrations in the series should remain unchanged.
34. The test chemical solutions should be closely monitored when added to the assay well, as the test chemical may precipitate upon addition to the assay well. The data for all wells that contain precipitate should be excluded from curve-fitting, and the reason for exclusion of the data noted.
35. If there is prior existing information from other sources that provide a $\log(\text{IC}_{50})$ of a test chemical, it may be appropriate to geometrically space the dilutions more closely around the expected $\log(\text{IC}_{50})$ (i.e. 0.5 log units). The final results should show enough sufficient spread of concentrations on either side of the $\log(\text{IC}_{50})$, including the “top” and “bottom”, such that the binding curve can be adequately characterised.

Assay plate organisation

36. Labelled microtiter plates should be prepared using sextuple incubations for the solvent control, the highest concentration the reference estrogen (E2) which also serves as the non-specific binding (NSB) indicator, the buffer control, and triplicate incubations for each of the eight concentrations of the non-binding control (octyltriethoxysilane), the seven lower concentrations for the reference estrogen (E2), the eight concentrations of the weak binder (norethynodrel or norethindrone), and the eight concentrations of each test chemical (TC). An example layout of the plate layout diagram for the full concentration curves for the reference estrogen and controls is given below in Table 4. Additional microtiter plates are used for the test chemical and should contain plate controls (i.e. (i) a high- (maximum displacement) and medium- (approximately, the IC_{50}) concentration of E2 and weak binder in triplicate; (ii) solvent control (as total binding) and non-specific binding, each in sextuple (Table 5). An example of a competitive assay microtiter plate layout worksheet using three unknown test chemicals is provided in Appendix 3.3. The concentrations indicated in the worksheet as well as in Tables 4 and 5 refer to the final concentrations used in each assay well. The maximum concentration for E2 should be 1×10^{-7} M and for the weak binder, the

highest concentration used for the weak binder on plate 1 should be used. The IC_{50} concentration has to be determined by the laboratory based on their historical control database. The expectation is that this value would be similar to that observed in the validation studies (see table 1).

Table 4: Competitive Binding Assay Microtiter Plate Layout^{1,2}, Full Concentration Curves for Reference Estrogen and Controls (Plate 1)

	<i>1</i>	<i>2</i>	<i>3</i>	<i>4</i>	<i>5</i>	<i>6</i>	<i>7</i>	<i>8</i>	<i>9</i>	<i>10</i>	<i>11</i>	<i>12</i>
	Buffer Control and Positive Control (E2)			Weak Positive (Norethynodrel)			Negative Control (OTES)			TB and NSB		
<i>A</i>	Blank*			1×10 ⁻⁹ M			1×10 ⁻¹⁰ M			TB (solvent control) (2.05% DMSO)		
<i>B</i>	E2 (1×10 ⁻¹¹ M)			1×10 ⁻⁸ M			1×10 ⁻⁹ M					
<i>C</i>	E2 (1×10 ⁻¹⁰ M)			1×10 ^{-7.5} M			1×10 ⁻⁸ M			NSB (10 ⁻⁶ M E2)		
<i>D</i>	E2 (1×10 ^{-9.5} M)			1×10 ⁻⁷ M			1×10 ⁻⁷ M					
<i>E</i>	E2 (1×10 ⁻⁹ M)			1×10 ^{-6.5} M			1×10 ⁻⁶ M			Buffer control		
<i>F</i>	E2 (1×10 ^{-8.5} M)			1×10 ⁻⁶ M			1×10 ⁻⁵ M					
<i>G</i>	E2 (1×10 ⁻⁸ M)			1×10 ^{-5.5} M			1×10 ⁻⁴ M			Blank (for hot)**		
<i>H</i>	E2 (1×10 ⁻⁷ M)			1×10 ^{-4.5} M			1×10 ⁻³ M					

¹ Sample set up for the standards microtiter plate to be run with each experiment.

² Note that this microtiter plate is made using the dilutions made in the dilution plate described for the standards in the previous sections.

In this example, the weak binder is norethinodrel (NE)

* real blank, well not used

** blank, not used during the incubation, but used to confirm the total radioactivity added.

Table 5: Competitive Binding Assay Microtiter Plate Layout, Additional Plates for Test Chemicals (TC) and Plate Controls.

	<i>1</i>	<i>2</i>	<i>3</i>	<i>4</i>	<i>5</i>	<i>6</i>	<i>7</i>	<i>8</i>	<i>9</i>	<i>10</i>	<i>11</i>	<i>12</i>
	Test Chemical-1 (TC-1)			Test Chemical-2 (TC-2)			Test Chemical-3 (TC-3)			Controls		
<i>A</i>	TC-1 (1×10^{-10} M)			TC-2 (1×10^{-10} M)			TC-3 (1×10^{-10} M)			E2 (1×10^{-7} M)		
<i>B</i>	TC-1 (1×10^{-9} M)			TC-2 (1×10^{-9} M)			TC-3 (1×10^{-9} M)			E2 (IC_{50})		
<i>C</i>	TC-1 (1×10^{-8} M)			TC-2 (1×10^{-8} M)			TC-3 (1×10^{-8} M)			NE ($1 \times 10^{-4.5}$ M)		

D	TC-1 (1×10^{-7} M)	TC-2 (1×10^{-7} M)	TC-3 (1×10^{-7} M)	NE (IC_{50})
E	TC-1 (1×10^{-6} M)	TC-2 (1×10^{-6} M)	TC-3 (1×10^{-6} M)	NSB (10^{-6} M E ₂)
F	TC-1 (1×10^{-5} M)	TC-2 (1×10^{-5} M)	TC-3 (1×10^{-5} M)	
G	TC-1 (1×10^{-4} M)	TC-2 (1×10^{-4} M)	TC-3 (1×10^{-4} M)	TB (Solvent control)
H	TC-1 (1×10^{-3} M)	TC-2 (1×10^{-3} M)	TC-3 (1×10^{-3} M)	

In this example, the weak binder is norethinodrel (NE)

Completion of competitive binding assay

37. Excepting wells for total binding and blanks (for hot), as shown in Table 6, 50 μ l of the assay buffer should be placed in each well, and should be mixed with 10 μ l of the solvent control, reference estrogen (E₂), weak binder, non-binder, and test chemicals, respectively, 10 μ l of a 5 nM [³H]-17 β -estradiol solution. Then, 30 μ l of ice cold receptor solution was added to each plate and mixed gently. The hrER α solution should be the last reagent to be added. Assay microtiter plates should be incubated at room temperature (22° to 28°C) for 2 hours.

Table 6: Volume of Assay Components for hrER Competitive Binding Assay, Microtiter Plates

<i>Preparation Steps</i>		Other than TB wells	TB wells	Blank (for hot)
Volume of components for reaction wells above and order to add	Room Temperature assay Buffer	50 μ l	60 μ l	90 μ l
	Unlabelled E ₂ , weak binder, non-binder, solvent and test chemicals*	10 μ l	-	-
	[³ H]-17 β -estradiol to yield final concentration of 0.5 nM (i.e. 5 nM)	10 μ l	10 μ l	10 μ l
	hrER α concentration as determined (see paragraphs 12-13)	30 μ l	30 μ l	-
Total volume in each assay well		100 μ l	100 μ l	100 μ l

*properly prepared to obtain final concentration within the acceptable solvent concentration

38. The quantification of [³H]-17 β -Estradiol bound to hrER α , following separation of [³H]-17 β -Estradiol bound to hrER α from free [³H]-17 β -Estradiol by adding 100 μ l of ice-cold DCC suspension to each well, should then be performed as described in paragraphs 21-23 of Appendix 3 for the saturation binding assay.

39. Wells G10-12 and H10-12 (identified as blank (for hot) in Table 4) represent the dpms of the [³H]-labelled-estradiol in 10 μ l. The 10 μ l aliquot should be delivered directly into the scintillation fluid.

Acceptability criteria

Saturation binding assay

40. The specific binding curve should reach a plateau as increasing concentrations of [³H]-17 β -estradiol were used, indicating saturation of hrER α with ligand.
41. The specific binding at 0.5 nM of [³H]-17 β -estradiol should be inside the acceptable range 30% to 50% of the average measured total radioactivity added across runs. Occasional slight excursions outside of this range are acceptable, but if runs are consistently outside this range or a particular run is significantly outside this range, the protein concentration should be adjusted and the saturation assay repeated.
42. The data should produce a linear Scatchard plot.
43. The non-specific binding should not be excessive. The value for non-specific binding should typically be <35% of the total binding. However, the ratio might occasionally exceed this limit when measuring very low dpm for the lowest concentration of radiolabelled 17 β -estradiol tested.

Competitive binding assay

44. Increasing concentrations of unlabelled 17 β -estradiol should displace [³H]-17 β -estradiol from the receptor in a manner consistent with a one-site competitive binding.
45. The IC₅₀ value for the reference estrogen (i.e. 17 β -estradiol) should be approximately equal to the molar concentration of [³H]-17 β -estradiol plus the K_d determined from the saturation binding assay.
46. The total specific binding should be consistently within the acceptable range of 40 $\pm$ 10 % when the average measured concentration of total radioactivity added to each well was 0.5 nM across runs. Occasional slight excursions outside of this range are acceptable, but if runs are consistently outside this range or a particular run is significantly outside this range, the protein concentration should be adjusted.
47. The solvent should not alter the sensitivity or reproducibility of the assay. The results of the solvent control (TB wells) are compared to the buffer control to verify that the solvent used does not affect the assay system. The results of the TB and Buffer control should be comparable if there is no effect of the solvent on the assay.
48. The non-binder should not displace more than 25% of the [³H]-17 β -estradiol from the hrER α when tested up to 10⁻³ M (OTES) or 10⁻⁴ M (DBP).
49. Performance criteria were developed for the reference estrogen and two weak binders (e.g. norethynodrel, norethindrone) using data from the validation study for the CERI hrER Binding Assay (Annex N of reference 2). 95% confidence intervals are provided for the mean $\pm$ SD (n) of all control runs across four laboratories that participated in the validation study. 95% confidence intervals were calculated for the curve fit parameters (i.e. top, bottom, Hillslope and Log IC₅₀) for the reference estrogen and weak binders, and the Log₁₀RBA of the weak binders relative to the reference estrogen. Table 1 provides expected ranges for the curve fit parameters that can be used as performance criteria. In practice, the

range of the IC_{50} may vary slightly based upon the experimentally derived K_d of the receptor preparation and ligand concentration used for the assay.

50. No performance criteria were developed for curve fit parameters for the test chemicals because of the wide array of existing potential test chemicals and variation in potential affinities and outcomes (e.g. Full curve, partial curve, no curve fit). However, professional judgment should be applied when reviewing results from each run for a test chemical. A sufficient range of concentrations of the test chemical should be used to clearly define the top (e.g. 90 - 100% of binding) of the competitive curve. Variability among replicates at each concentration of test chemical as well as among the 3 non-concurrent runs should be reasonable and scientifically defensible. Controls from each run for a test chemical should approach the measures of performance reported for this CERI assay and be consistent historical control data from each respective laboratory.

ANALYSIS OF DATA

Saturation binding assay

51. Both total and non-specific binding are measured. From these values, specific binding of increasing concentrations of [^3H]-17 β -estradiol under equilibrium conditions is calculated by subtracting non-specific from total. A graph of specific binding versus [^3H]-17 β -estradiol concentration should reach a plateau for maximum specific binding indicative of saturation of the hrER α with the [^3H]-17 β -estradiol. In addition, analysis of the data should document the binding of the [^3H]-17 β - estradiol to a single, high-affinity binding site. Non-specific, total, and specific binding should be displayed on a saturation binding curve. Further analysis of these data should use a non-linear regression analysis (e.g. BioSoft; McPherson, 1985; Motulsky, 1995) with a final display of the data as a Scatchard plot.
52. The data analysis should determine B_{max} and K_d from the total binding data alone, using the assumption that non-specific binding is linear, unless justification is given for using a different method. In addition, robust regression should be used when determining the best fit unless justification is given. The method chosen for robust regression should be stated. Correction for ligand depletion (e.g. using the method of Swillens 1995) should always be used when determining B_{max} and K_d from saturation binding data.

Competitive binding assay

53. The competitive binding curve is plotted as specific [^3H]-17 β - estradiol binding versus the concentration ($\log_{10}$ units) of the competitor. The concentration of the test chemical that inhibits 50% of the maximum specific [^3H]-17 β -estradiol binding is the IC_{50} value.
54. Estimates of $\log(IC_{50})$ values for the positive controls (e.g. reference estrogen and weak binder) should be determined using an appropriate nonlinear curve fitting software to fit a four parameter Hill equation (e.g. BioSoft; McPherson, 1985; Motulsky, 1995). The top, bottom, slope, and $\log(IC_{50})$ should generally be left unconstrained when fitting these

curves. Robust regression should be used when determining the best fit unless justification is given. Correction for ligand depletion should not be used. Following the initial analysis, each binding curve should be reviewed to ensure appropriate fit to the model. The relative binding affinity (RBA) for the weak binder should be calculated as a percent of the log (IC₅₀) for the weak binder relative to the log (IC₅₀) for 17β-estradiol. Results from the positive controls and the non-binder control should be evaluated using the measures of the assay performance in paragraphs 44-49 of this Appendix 3.

55. Data for all test chemicals should be analysed using a step-wise approach to ensure that data are appropriately analysed and that each competitive binding curve is properly classified. It is recommended that each run for a test chemical initially undergo a standardised data analysis that is identical to that used for the reference estrogen and weak binder controls (see paragraph 54 of this Appendix 3). Once completed, a technical review of the curve fit parameters as well as a visual review of how well the data fit the generated competitive binding curve for each run should be conducted. During this technical review, the observations of a concentration dependent decrease in the percent [³H]-17β-estradiol specifically bound, low variability among the technical replicates at each test chemical concentration, and consistency in fit parameters among the three runs are a good indication that the assay and data analyses were conducted appropriately.

Data interpretation

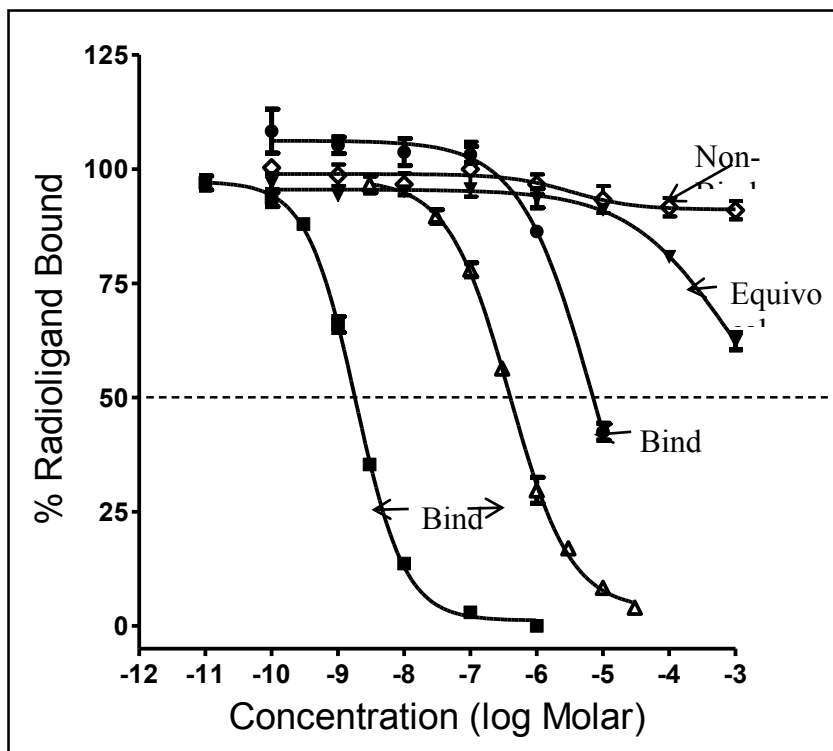
56. Providing that all acceptability criteria are fulfilled, a test chemical is considered to be a binder for the hrERα if a binding curve can be fit and the lowest point on the response curve within the range of the data is less than 50% (Figure 1).
57. Providing that all acceptability criteria are fulfilled, a test chemical is considered to be a non-binder for the hrERα if:
- A binding curve can be fit and the lowest point on the fitted response curve within the range of the data is above 75%, or
 - A binding curve cannot be fit and the lowest unsmoothed average percent binding among the concentration groups in the data is above 75%.
58. Test chemicals are considered equivocal if none of the above conditions are met (e.g. the lowest point on the fitted response curve is between 76 – 51%).

Table 7: Criteria for assigning classification based upon competitive binding curve for a test chemical.

Classification	Criteria
Binder ^a	A binding curve can be fit. • The lowest point on the response curve within the range of the data is less than 50%.
Non-binder ^b	If a binding curve can be fit, • the lowest point on the fitted response curve within the range of the

	data is above 75%. If a binding curve cannot be fit, • the lowest unsmoothed average percent binding among the concentration groups in the data is above 75%.
Equivocal ^c	Any testable run that is neither a binder nor a non-binder (e.g. The lowest point on the fitted response curve is between 76 – 51%).

Figure 1: Examples of test chemical classification using competitive binding curve.



59. Multiple runs conducted within a laboratory for a test chemical are combined by assigning numeric values to each run and averaging across the runs as shown in Table 8. Results for the combined runs within each laboratory are compared with the expected classification for each test chemical.

Table 8: Method for classification of test chemical using multiple runs within a laboratory

To assign value to each run:	
Classification	Numeric Value
Binder	2
Equivocal	1
Non-binder	0
To classify average of numeric value across runs:	
Classification	Numeric Value
Binder	Average ≥ 1.5

Equivocal	$0.5 \leq \text{Average} < 1.5$
Non-binder	$\text{Average} < 0.5$

TEST REPORT

60. See paragraph 24 of “hrER BINDING ASSAY COMPONENTS” of this test method.

Appendix 3.1

LIST OF TERMS

[³H]E2: 17 β -Estradiol radiolabelled with tritium

DCC: Dextran-coated charcoal

E2: Unlabelled 17 β -estradiol (inert)

Assay buffer: 10 mM Tris-HCl, pH 7.4, containing 1 mM EDTA, 1mM EGTA, 1 mM NaVO₃, 10 % Glycerol, 0.2 mM Leupeptin, 1 mM Dithiothreitol and 10 mg/ml Bovine Serum Albumin

hrER α : Human recombinant estrogen receptor alpha (ligand binding domain)

Replicate: One of multiple wells that contain the same contents at the same concentrations and are assayed concurrently within a single run. In this protocol, each concentration of test chemical is tested in triplicate; that is, there are three replicates that are assayed simultaneously at each concentration of test chemical.

Run: A complete set of concurrently-run microtiter plate assay wells that provides all the information necessary to characterise binding of a test chemical to the hrER α (viz., total [³H]-17 β -estradiol added to the assay well, maximum binding of [³H]-17 β -estradiol to the hrER α , nonspecific binding, and total binding at various concentrations of test chemical). A run could consist of as few as one assay well (i.e. replicate) per concentration, but since this protocol requires assaying in triplicate, one run consists of three assay wells per concentration. In addition, this protocol requires three independent (i.e. non-concurrent) runs per chemical.

Appendix 3.2

COMPETITIVE BINDING ASSAY WELL LAYOUT

Plate	Position	Replicate	Well type	Well Code	Concentration Code	Competitor Initial Concentration (M)	hrER stock (μl)	Buffer Volume (μl)	Tracer (Hot E2) Volume (μL)	Volume from dilution plate (μL)	Final Volume (μl)	Competitor Final Concentration (M)
S	A1	1	Blank	BK	BK1	—	—	—	—	—	—	—
S	A2	2	Blank	BK	BK2	—	—	—	—	—	—	—
S	A3	3	Blank	BK	BK3	—	—	—	—	—	—	—
S	B1	1	cold E2	S	S1	1.00E-10	30	50	10	10	100	1.0E-11
S	B2	2	cold E2	S	S1	1.00E-10	30	50	10	10	100	1.0E-11
S	B3	3	cold E2	S	S1	1.00E-10	30	50	10	10	100	1.0E-11
S	C1	1	cold E2	S	S2	1.00E-09	30	50	10	10	100	1.0E-10
S	C2	2	cold E2	S	S2	1.00E-09	30	50	10	10	100	1.0E-10
S	C3	3	cold E2	S	S2	1.00E-09	30	50	10	10	100	1.0E-10
S	D1	1	cold E2	S	S3	3.16E-09	30	50	10	10	100	3.2E-10
S	D2	2	cold E2	S	S3	3.16E-09	30	50	10	10	100	3.2E-10
S	D3	3	cold E2	S	S3	3.16E-09	30	50	10	10	100	3.2E-10
S	E1	1	cold E2	S	S4	1.00E-08	30	50	10	10	100	1.0E-09
S	E2	2	cold E2	S	S4	1.00E-08	30	50	10	10	100	1.0E-09
S	E3	3	cold E2	S	S4	1.00E-08	30	50	10	10	100	1.0E-09
S	F1	1	cold E2	S	S5	3.16E-08	30	50	10	10	100	3.2E-09
S	F2	2	cold E2	S	S5	3.16E-08	30	50	10	10	100	3.2E-09
S	F3	3	cold E2	S	S5	3.16E-08	30	50	10	10	100	3.2E-09
S	G1	1	cold E2	S	S6	1.00E-07	30	50	10	10	100	1.0E-08
S	G2	2	cold E2	S	S6	1.00E-07	30	50	10	10	100	1.0E-08
S	G3	3	cold E2	S	S6	1.00E-07	30	50	10	10	100	1.0E-08
S	H1	1	cold E2	S	S7	1.00E-06	30	50	10	10	100	1.0E-07
S	H2	2	cold E2	S	S7	1.00E-06	30	50	10	10	100	1.0E-07
S	H3	3	cold E2	S	S7	1.00E-06	30	50	10	10	100	1.0E-07
S	A4	1	norethynodrel	NE	WP1	1.00E-08	30	50	10	10	100	1.0E-09
S	A5	2	norethynodrel	NE	WP1	1.00E-08	30	50	10	10	100	1.0E-09
S	A6	3	norethynodrel	NE	WP1	1.00E-08	30	50	10	10	100	1.0E-09
S	B4	1	norethynodrel	NE	WP2	1.00E-07	30	50	10	10	100	1.0E-08
S	B5	2	norethynodrel	NE	WP2	1.00E-07	30	50	10	10	100	1.0E-08
S	B6	3	norethynodrel	NE	WP2	1.00E-07	30	50	10	10	100	1.0E-08
S	C4	1	norethynodrel	NE	WP3	3.16E-07	30	50	10	10	100	3.2E-08
S	C5	2	norethynodrel	NE	WP3	3.16E-07	30	50	10	10	100	3.2E-08
S	C6	3	norethynodrel	NE	WP3	3.16E-07	30	50	10	10	100	3.2E-08
S	D4	1	norethynodrel	NE	WP4	1.00E-06	30	50	10	10	100	1.0E-07
S	D5	2	norethynodrel	NE	WP4	1.00E-06	30	50	10	10	100	1.0E-07
S	D6	3	norethynodrel	NE	WP4	1.00E-06	30	50	10	10	100	1.0E-07
S	E4	1	norethynodrel	NE	WP5	3.16E-06	30	50	10	10	100	3.2E-07
S	E5	2	norethynodrel	NE	WP5	3.16E-06	30	50	10	10	100	3.2E-07
S	E6	3	norethynodrel	NE	WP5	3.16E-06	30	50	10	10	100	3.2E-07
S	F4	1	norethynodrel	NE	WP6	1.00E-05	30	50	10	10	100	1.0E-06
S	F5	2	norethynodrel	NE	WP6	1.00E-05	30	50	10	10	100	1.0E-06
S	F6	3	norethynodrel	NE	WP6	1.00E-05	30	50	10	10	100	1.0E-06
S	G4	1	norethynodrel	NE	WP7	3.16E-05	30	50	10	10	100	3.2E-06
S	G5	2	norethynodrel	NE	WP7	3.16E-05	30	50	10	10	100	3.2E-06
S	G6	3	norethynodrel	NE	WP7	3.16E-05	30	50	10	10	100	3.2E-06

Competitive Binding Assay Well Layout												
Plate	Position	Replicate	Well type	Well Code	Concentration Code	Competitor Initial Concentration (M)	hrER stock (μl)	Buffer Volume (μl)	Tracer (Hot E2) Volume (μL)	Volume from dilution plate (μL)	Final Volume (μl)	Competitor Final Concentration (M)
S	H4	1	norethynodrel	NE	WP8	3.16E-04	30	50	10	10	100	3.2E-05
S	H5	2	norethynodrel	NE	WP8	3.16E-04	30	50	10	10	100	3.2E-05
S	H6	3	norethynodrel	NE	WP8	3.16E-04	30	50	10	10	100	3.2E-05
S	A7	1	OTES	N	OTES1	1.00E-09	30	50	10	10	100	1.0E-10
S	A8	2	OTES	N	OTES1	1.00E-09	30	50	10	10	100	1.0E-10
S	A9	3	OTES	N	OTES1	1.00E-09	30	50	10	10	100	1.0E-10
S	B7	1	OTES	N	OTES2	1.00E-08	30	50	10	10	100	1.0E-09
S	B8	2	OTES	N	OTES2	1.00E-08	30	50	10	10	100	1.0E-09
S	B9	3	OTES	N	OTES2	1.00E-08	30	50	10	10	100	1.0E-09
S	C7	1	OTES	N	OTES3	1.00E-07	30	50	10	10	100	1.0E-08
S	C8	2	OTES	N	OTES3	1.00E-07	30	50	10	10	100	1.0E-08
S	C9	3	OTES	N	OTES3	1.00E-07	30	50	10	10	100	1.0E-08
S	D7	1	OTES	N	OTES4	1.00E-06	30	50	10	10	100	1.0E-07
S	D8	2	OTES	N	OTES4	1.00E-06	30	50	10	10	100	1.0E-07
S	D9	3	OTES	N	OTES4	1.00E-06	30	50	10	10	100	1.0E-07
S	E7	1	OTES	N	OTES5	1.00E-05	30	50	10	10	100	1.0E-06
S	E8	2	OTES	N	OTES5	1.00E-05	30	50	10	10	100	1.0E-06
S	E9	3	OTES	N	OTES5	1.00E-05	30	50	10	10	100	1.0E-06
S	F7	1	OTES	N	OTES6	1.00E-04	30	50	10	10	100	1.0E-05
S	F8	2	OTES	N	OTES6	1.00E-04	30	50	10	10	100	1.0E-05
S	F9	3	OTES	N	OTES6	1.00E-04	30	50	10	10	100	1.0E-05
S	G7	1	OTES	N	OTES7	1.00E-03	30	50	10	10	100	1.0E-04
S	G8	2	OTES	N	OTES7	1.00E-03	30	50	10	10	100	1.0E-04
S	G9	3	OTES	N	OTES7	1.00E-03	30	50	10	10	100	1.0E-04
S	H7	1	OTES	N	OTES8DBP7	1.00E-02	30	50	10	10	100	1.0E-03
S	H8	2	OTES	N	OTES88	1.00E-02	30	50	10	10	100	1.0E-03
S	H9	3	OTES	N	OTES8	1.00E-02	30	50	10	10	100	1.0E-03
S	A10	1	total binding	TB	TB1	-	30	60	10	-	100	-
S	A11	2	total binding	TB	TB2	-	30	60	10	-	100	-
S	A12	3	total binding	TB	TB3	-	30	60	10	-	100	-
S	B10	4	total binding	TB	TB4	-	30	60	10	-	100	-
S	B11	5	total binding	TB	TB5	-	30	60	10	-	100	-
S	B12	6	total binding	TB	TB6	-	30	60	10	-	100	-
S	C10	1	cold E2 (high)	NSB	S1	1.00E-05	30	50	10	10	100	1.0E-06
S	C11	2	cold E2 (high)	NSB	S2	1.00E-05	30	50	10	10	100	1.0E-06
S	C12	3	cold E2 (high)	NSB	S3	1.00E-05	30	50	10	10	100	1.0E-06
S	D10	4	cold E2 (high)	NSB	S4	1.00E-05	30	50	10	10	100	1.0E-06
S	D11	5	cold E2 (high)	NSB	S5	1.00E-05	30	50	10	10	100	1.0E-06
S	D12	6	cold E2 (high)	NSB	S6	1.00E-05	30	50	10	10	100	1.0E-06
S	E10	1	Buffer control	BC	BC1	-	-	100	-	-	100	-
S	E11	2	Buffer control	BC	BC2	-	-	100	-	-	100	-
S	E12	3	Buffer control	BC	BC3	-	-	100	-	-	100	-
S	F10	4	Buffer control	BC	BC4	-	-	100	-	-	100	-
S	F11	5	Buffer control	BC	BC5	-	-	100	-	-	100	-
S	F12	6	Buffer control	BC	BC6	-	-	100	-	-	100	-
S	G10*	1	Blank (for hot)	Hot	H1	-	90	-	10	-	100	-
S	G11*	2	Blank (for hot)	Hot	H2	-	90	-	10	-	100	-
S	G12*	3	Blank (for hot)	Hot	H3	-	90	-	10	-	100	-
S	H10*	4	Blank (for hot)	Hot	H4	-	90	-	10	-	100	-
S	H11*	5	Blank (for hot)	Hot	H5	-	90	-	10	-	100	-
S	H12	6	Blank (for hot)	Hot	H6	-	90	-	10	-	100	-

*: Note that the "hot" wells are empty during incubation. The 10 μl are added only for scintillation counting.

Competitive Binding Assay Well Layout												
Plate	Position	Replicate	Well type	Well Code	Concentration Code	Competitor Initial Concentration (M)	hrER stock (μl)	Buffer Volume (μl)	Tracer (Hot E2) Volume (μL)	Volume from dilution plate (μL)	Final Volume (μl)	Competitor Final Concentration (M)
P1	A1	1	Unknown 1	U1	1	1.00E-09	30	50	10	10	100	1.0E-10
P1	A2	2	Unknown 1	U1	1	1.00E-09	30	50	10	10	100	1.0E-10
P1	A3	3	Unknown 1	U1	1	1.00E-09	30	50	10	10	100	1.0E-10
P1	B1	1	Unknown 1	U1	2	1.00E-08	30	50	10	10	100	1.0E-09
P1	B2	2	Unknown 1	U1	2	1.00E-08	30	50	10	10	100	1.0E-09
P1	B3	3	Unknown 1	U1	2	1.00E-08	30	50	10	10	100	1.0E-09
P1	C1	1	Unknown 1	U1	3	1.00E-07	30	50	10	10	100	1.0E-08
P1	C2	2	Unknown 1	U1	3	1.00E-07	30	50	10	10	100	1.0E-08
P1	C3	3	Unknown 1	U1	3	1.00E-07	30	50	10	10	100	1.0E-08
P1	D1	1	Unknown 1	U1	4	1.00E-06	30	50	10	10	100	1.0E-07
P1	D2	2	Unknown 1	U1	4	1.00E-06	30	50	10	10	100	1.0E-07
P1	D3	3	Unknown 1	U1	4	1.00E-06	30	50	10	10	100	1.0E-07
P1	E1	1	Unknown 1	U1	5	1.00E-05	30	50	10	10	100	1.0E-06
P1	E2	2	Unknown 1	U1	5	1.00E-05	30	50	10	10	100	1.0E-06
P1	E3	3	Unknown 1	U1	5	1.00E-05	30	50	10	10	100	1.0E-06
P1	F1	1	Unknown 1	U1	6	1.00E-04	30	50	10	10	100	1.0E-05
P1	F2	2	Unknown 1	U1	6	1.00E-04	30	50	10	10	100	1.0E-05
P1	F3	3	Unknown 1	U1	6	1.00E-04	30	50	10	10	100	1.0E-05
P1	G1	1	Unknown 1	U1	7	1.00E-03	30	50	10	10	100	1.0E-04
P1	G2	2	Unknown 1	U1	7	1.00E-03	30	50	10	10	100	1.0E-04
P1	G3	3	Unknown 1	U1	7	1.00E-03	30	50	10	10	100	1.0E-04
P1	H1	1	Unknown 1	U1	8	1.00E-02	30	50	10	10	100	1.0E-03
P1	H2	2	Unknown 1	U1	8	1.00E-02	30	50	10	10	100	1.0E-03
P1	H3	3	Unknown 1	U1	8	1.00E-02	30	50	10	10	100	1.0E-03
P1	A4	1	Unknown 2	U2	1	1.00E-09	30	50	10	10	100	1.0E-10
P1	A5	2	Unknown 2	U2	1	1.00E-09	30	50	10	10	100	1.0E-10
P1	A6	3	Unknown 2	U2	1	1.00E-09	30	50	10	10	100	1.0E-10
P1	B4	1	Unknown 2	U2	2	1.00E-08	30	50	10	10	100	1.0E-09
P1	B5	2	Unknown 2	U2	2	1.00E-08	30	50	10	10	100	1.0E-09
P1	B6	3	Unknown 2	U2	2	1.00E-08	30	50	10	10	100	1.0E-09
P1	C4	1	Unknown 2	U2	3	1.00E-07	30	50	10	10	100	1.0E-08
P1	C5	2	Unknown 2	U2	3	1.00E-07	30	50	10	10	100	1.0E-08
P1	C6	3	Unknown 2	U2	3	1.00E-07	30	50	10	10	100	1.0E-08
P1	D4	1	Unknown 2	U2	4	1.00E-06	30	50	10	10	100	1.0E-07
P1	D5	2	Unknown 2	U2	4	1.00E-06	30	50	10	10	100	1.0E-07
P1	D6	3	Unknown 2	U2	4	1.00E-06	30	50	10	10	100	1.0E-07
P1	E4	1	Unknown 2	U2	5	1.00E-05	30	50	10	10	100	1.0E-06
P1	E5	2	Unknown 2	U2	5	1.00E-05	30	50	10	10	100	1.0E-06
P1	E6	3	Unknown 2	U2	5	1.00E-05	30	50	10	10	100	1.0E-06
P1	F4	1	Unknown 2	U2	6	1.00E-04	30	50	10	10	100	1.0E-05
P1	F5	2	Unknown 2	U2	6	1.00E-04	30	50	10	10	100	1.0E-05
P1	F6	3	Unknown 2	U2	6	1.00E-04	30	50	10	10	100	1.0E-05
P1	G4	1	Unknown 2	U2	7	1.00E-03	30	50	10	10	100	1.0E-04
P1	G5	2	Unknown 2	U2	7	1.00E-03	30	50	10	10	100	1.0E-04
P1	G6	3	Unknown 2	U2	7	1.00E-03	30	50	10	10	100	1.0E-04
P1	H4	1	Unknown 2	U2	8	1.00E-02	30	50	10	10	100	1.0E-03
P1	H5	2	Unknown 2	U2	8	1.00E-02	30	50	10	10	100	1.0E-03
P1	H6	3	Unknown 2	U2	8	1.00E-02	30	50	10	10	100	1.0E-03

Competitive Binding Assay Well Layout												
Plate	Position	Replicate	Well type	Well Code	Concentration Code	Competitor Initial Concentration (M)	hrER stock (μl)	Buffer Volume (μl)	Tracer (Hot E2) Volume (μL)	Volume from dilution plate (μL)	Final Volume (μl)	Competitor Final Concentration (M)
P1	A7	1	Unknown 3	U3	1	1.00E-09	30	50	10	10	100	1.0E-10
P1	A8	2	Unknown 3	U3	1	1.00E-09	30	50	10	10	100	1.0E-10
P1	A9	3	Unknown 3	U3	1	1.00E-09	30	50	10	10	100	1.0E-10
P1	B7	1	Unknown 3	U3	2	1.00E-08	30	50	10	10	100	1.0E-09
P1	B8	2	Unknown 3	U3	2	1.00E-08	30	50	10	10	100	1.0E-09
P1	B9	3	Unknown 3	U3	2	1.00E-08	30	50	10	10	100	1.0E-09
P1	C7	1	Unknown 3	U3	3	1.00E-07	30	50	10	10	100	1.0E-08
P1	C8	2	Unknown 3	U3	3	1.00E-07	30	50	10	10	100	1.0E-08
P1	C9	3	Unknown 3	U3	3	1.00E-07	30	50	10	10	100	1.0E-08
P1	D7	1	Unknown 3	U3	4	1.00E-06	30	50	10	10	100	1.0E-07
P1	D8	2	Unknown 3	U3	4	1.00E-06	30	50	10	10	100	1.0E-07
P1	D9	3	Unknown 3	U3	4	1.00E-06	30	50	10	10	100	1.0E-07
P1	E7	1	Unknown 3	U3	5	1.00E-05	30	50	10	10	100	1.0E-06
P1	E8	2	Unknown 3	U3	5	1.00E-05	30	50	10	10	100	1.0E-06
P1	E9	3	Unknown 3	U3	5	1.00E-05	30	50	10	10	100	1.0E-06
P1	F7	1	Unknown 3	U3	6	1.00E-04	30	50	10	10	100	1.0E-05
P1	F8	2	Unknown 3	U3	6	1.00E-04	30	50	10	10	100	1.0E-05
P1	F9	3	Unknown 3	U3	6	1.00E-04	30	50	10	10	100	1.0E-05
P1	G7	1	Unknown 3	U3	7	1.00E-03	30	50	10	10	100	1.0E-04
P1	G8	2	Unknown 3	U3	7	1.00E-03	30	50	10	10	100	1.0E-04
P1	G9	3	Unknown 3	U3	7	1.00E-03	30	50	10	10	100	1.0E-04
P1	H7	1	Unknown 3	U3	8	1.00E-02	30	50	10	10	100	1.0E-03
P1	H8	2	Unknown 3	U3	8	1.00E-02	30	50	10	10	100	1.0E-03
P1	H9	3	Unknown 3	U3	8	1.00E-02	30	50	10	10	100	1.0E-03
P1	A10	1	Control E2 (max)	S	E2max1	1.00E-06	30	50	10	10	100	1.00E-07
P1	A11	2	Control E2 (max)	S	E2max2	1.00E-06	30	50	10	10	100	1.00E-07
P1	A12	3	Control E2 (max)	S	E2max3	1.00E-06	30	50	10	10	100	1.00E-07
P1	B10	1	Control E2 (IC50)	S	E2IC501	E2IC50x10	30	50	10	10	100	E2IC50
P1	B11	2	Control E2 (IC50)	S	E2IC502	E2IC50x10	30	50	10	10	100	E2IC50
P1	B12	3	Control E2 (IC50)	S	E2IC503	E2IC50x10	30	50	10	10	100	E2IC50
P1	C10	1	Control NE (max)	S	Nemax1	1.00E-3.5	30	50	10	10	100	1.00E-4.5
P1	C11	2	Control NE (max)	S	Nemax2	1.00E-3.5	30	50	10	10	100	1.00E-4.5
P1	C12	3	Control NE (max)	S	Nemax3	1.00E-3.5	30	50	10	10	100	1.00E-4.5
P1	D10	1	Control NE	S	NEIC501	NEIC50 x10	30	50	10	10	100	NEIC50
P1	D11	2	Control NE	S	NEIC502	NEIC50 x10	30	50	10	10	100	NEIC50
P1	D12	3	Control NE	S	NEIC503	NEIC50 x10	30	50	10	10	100	NEIC50
P1	E10	1	cold E2 (high)	NSB	S1	1.00E-05	30	50	10	10	100	1.0E-06
P1	E11	2	cold E2 (high)	NSB	S2	1.00E-05	30	50	10	10	100	1.0E-06
P1	E12	3	cold E2 (high)	NSB	S3	1.00E-05	30	50	10	10	100	1.0E-06
P1	F10	4	cold E2 (high)	NSB	S4	1.00E-05	30	50	10	10	100	1.0E-06
P1	F11	5	cold E2 (high)	NSB	S5	1.00E-05	30	50	10	10	100	1.0E-06
P1	F12	6	cold E2 (high)	NSB	S6	1.00E-05	30	50	10	10	100	1.0E-06
P1	G10	1	total binding	TB	TB1	-	30	60	10	-	100	-
P1	G11	2	total binding	TB	TB2	-	30	60	10	-	100	-
P1	G12	3	total binding	TB	TB3	-	30	60	10	-	100	-
P1	H10	4	total binding	TB	TB4	-	30	60	10	-	100	-
P1	H11	5	total binding	TB	TB5	-	30	60	10	-	100	-
P1	H12	6	total binding	TB	TB6	-	30	60	10	-	100	-

Appendix 4

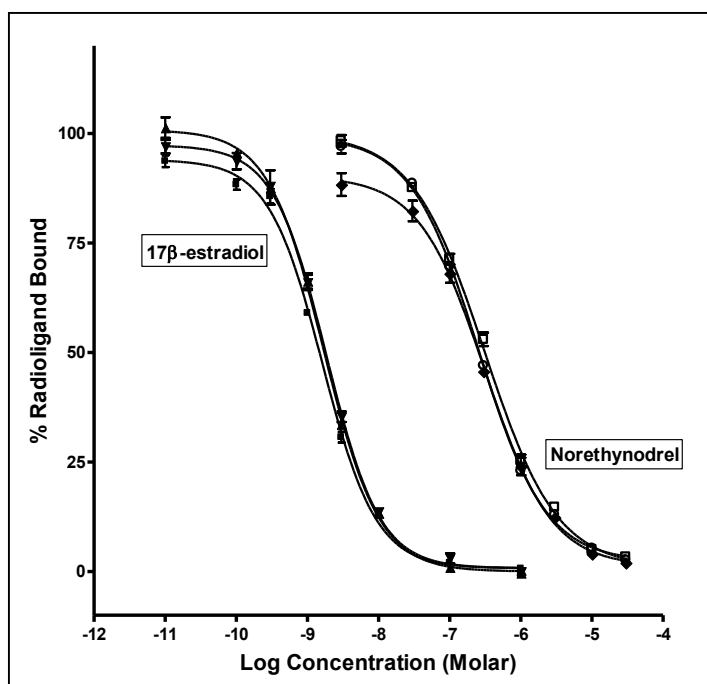
CONSIDERATIONS FOR THE ANALYSIS OF DATA FROM THE HRER COMPETITIVE BINDING ASSAY

1. The hrER α competitive binding assay measures the binding of a single concentration of [^{3} H]-17 β -estradiol in the presence of increasing concentrations of a test chemical. The competitive binding curve is plotted as specific [^{3} H]-17 β - estradiol binding versus the concentration (log10 units) of the competitor. The concentration of the test chemical that inhibits 50% of the maximum specific [^{3} H]-17 β -estradiol binding is the IC₅₀.

Data Analysis for the Reference Estrogen and Weak Binder (1)

2. Data from the control runs are transformed (i.e. percent [^{3} H]-17 β -estradiol specific binding and the log concentration of the control chemical) for further analysis. Estimates of log(IC₅₀) values for the positive controls (e.g. reference estrogen and weak binder) should be determined using an appropriate nonlinear curve fitting software to fit a four parameter Hill equation(e.g. BioSoft; GraphPad Prism) (2). The top, bottom, slope, and log(IC₅₀) can typically be left unconstrained when fitting these curves. Robust regression should be used when determining the best fit unless justification is given. The method chosen for robust regression should be stated. Correction for ligand depletion was not needed for the FW or CERI hrER assays, but may be considered if needed. Following the initial analysis, each binding curve should be reviewed to ensure an appropriate fit to the model. The relative binding affinity (RBA) for the weak binder can be calculated as a percent of the log (IC₅₀) for the weak binder relative to the log (IC₅₀) for 17 β -estradiol. Results for the positive controls and the non-binder control should be evaluated using measures of assay performance and acceptability criteria as described in this test method (paragraph 20), Appendix 2 (FW Assay, paragraphs 41-51) and Appendix 3 (CERI Assay, paragraphs 41-51). Examples of 3 runs for the reference estrogen and weak binder are shown in Figure 1.

Figure 1. Examples of the competitive binding curves for the reference estrogen and the control weak binder.

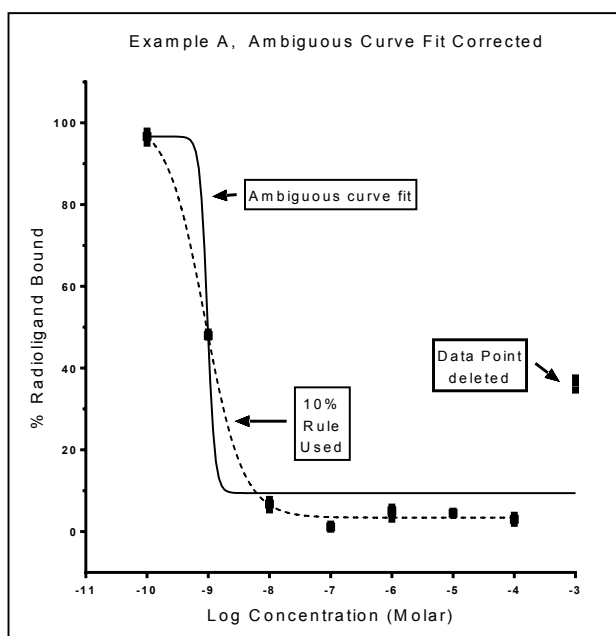


Data Analysis for Test Chemicals

3. Data for all test chemicals should be analysed using a step-wise approach to ensure that data are appropriately analysed and that each competitive binding curve is properly classified. Each run for a test chemical should initially undergo a standardised data analysis that is identical to that used for the reference estrogen and weak binder controls. Once completed, a technical review of the curve fit parameters as well as a visual review of how well the data fit the generated competitive binding curve for each run should be conducted. During this technical review, the observations of a concentration dependent decrease in the percent [^3H]-17 β -estradiol specifically bound, low variability among the technical replicates at each chemical concentration, and consistency in fit parameters among the three runs are a good indication that the assay and data analyses were conducted appropriately. Professional judgment should be applied when reviewing results from each run for a test chemical, and the data used to classify each test chemical as a binder or non-binder should be scientifically defensible.
4. Occasionally, there may be examples of data that require additional attention in order to appropriately analyse and interpret the hrER binding data. Previous studies had shown cases where the analysis and interpretation of competitive receptor binding data can be complicated by an upturn of the percent specific binding when testing chemicals at the highest concentrations (Figure 2). This is a well-known issue that has been encountered when using protocols for a number of competitive receptor binding assays (3). In these cases, a concentration dependent response is observed at lower concentrations, but as the

concentration of the test chemical approaches the limit of solubility, the displacement of [^3H]17 β -estradiol no longer decreases. In these cases, data for the higher concentrations indicate that the biological limit of the assay has been reached. For example, this phenomenon is many times associated with chemical insolubility and precipitation at high concentrations, or may also be a reflection of exceeding the capacity of the dextran-coated charcoal to trap the unbound radiolabelled ligand during the separation procedure at the highest chemical concentrations. Leaving such data points in when fitting competitive binding data to a sigmoid curve can sometimes lead to a misclassification of the ER binding potential for a test chemical (Figure 2). To avoid this, the protocol for the FW and CERI hrER binding assays includes an option to exclude from the analyses data points where the mean of the replicates for the percent [^3H]17 β -estradiol specific bound is 10% or more above the mean observed at a lower concentration (i.e. This is commonly referred to as the 10% rule). This rule can only be used once for a given curve, and there must be data remaining for at least 6 concentrations such that the curve can be correctly classified.

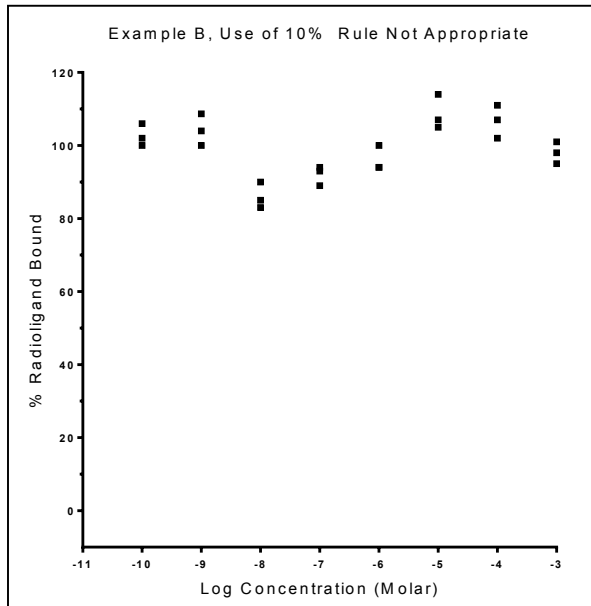
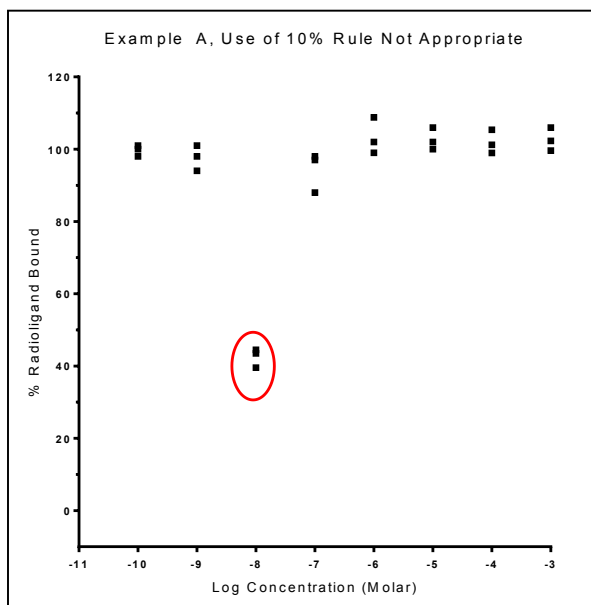
Figure 2: Examples, Competitive Binding Curves with and without Use of the 10% Rule.



5. The appropriate use of the 10% rule to correct these curves should be carefully considered and reserved for those cases where there is a strong indication of a hrER binder. During the conduct of experiments for the validation study of the FW hrER Binding Assay, it was observed that the 10% rule sometimes had an unintended and unforeseen consequence. Chemicals that did not interact with the receptor (i.e. true non-binders) often showed variability around 100% radioligand binding that was greater than 10% across the range of concentrations tested. If the lowest value happened to be at a low concentration, the data from all higher concentrations could potentially be deleted from the analysis by using the

10% rule, even though those concentrations could be useful in establishing that the chemical is a non-binder. Figure 3 show examples where the use of the 10% rule is not appropriate.

Figure 3: Examples, Competitive Binding Data Where Use of the 10% Rule is Not Appropriate.



References

- (1) OECD (2015). *Integrated Summary Report: Validation of Two Binding Assays Using Human Recombinant Estrogen Receptor Alpha (hrER α)*, Health and Safety Publications, Series on Testing and Assessment (No 226), Organisation for Economic Cooperation and Development, Paris.
- (2) Motulsky H. and Christopoulos A. (2003). *The law of mass action, In Fitting Models to Biological Data Using Linear and Non-linear Regression*. GraphPad Software Inc., San Diego, CA, pp 187-191. www.graphpad.com/manuals/Prism4/RegressionBook.pdf
- (3) Laws SC, Yavanhxay S, Cooper RL, Eldridge JC. (2006). *Nature of the Binding Interaction for 50 Structurally Diverse Chemicals with Rat Estrogen Receptors*. Toxicological Sci. 94(1):46-56.