DETAILS OF LEAD COUNTRY/CONSORTIUM

Country /Organisation:	Japan
Agency/ministry/Other:	Lead Institute: Japanese Centre for the Validation of Alternative Methods (JaCVAM), National Institute of Health Sciences (NIHS) Supporting Ministry: Ministry of Health, Labour and Welfare (MHLW), Japan
Mail Address:	National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-5801, Japan
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PROJECT OUTCOMES

- ☐ New Test Guideline
- ☒ Revised Test Guideline
- ☐ Deletion of an existing Test Guideline
- ☐ Guidance document
- ☐ Detailed Review Paper
- ☐ Other, please specify below

PROPOSED WORK PLAN and RESOURCE NEEDS:

1. Draft workplan for development of the proposal, including any need to establish Ad Hoc Expert Group and mode of meetings (face-to-face, teleconference; electronic discussion group). Indicate key milestones, including first and subsequent drafts of documents and timing of meetings.

At a meeting, held on April, 2007 at the European Centre for the Validation of Alternative Methods (ECVAM), in Ispar, Italy, the non-Commission members of the ECVAM Scientific Advisory Committee (ESAC) unanimously endorsed the following statement: there is strong evidence that the human reconstituted epidermis (HRE) EPISKIN method is a relevant stand alone test for predicting rabbit skin irritation and a possible replacement for the Draize skin irritation test. Furthermore, the non-Commission members of ESAC endorsed the same statement for other HREs as EpiDerm and SkinEthics at a meeting, held on November, 2008 in Brussels.

The Standard Project Submission Forms (SPSF) for these methods were proposed to the OECD secretary by the EU coordinator. At the OECD expert consultation meeting, held in October, 2008 at BfR in Berlin, Germany, these test methods for a new Test Guideline were investigated by other member countries. We have heard that the next expert consultation meeting will be held in June, 2009 at Washington D.C., USA.

Considering the EPISKIN statement and its protocol, the Japanese Centre for the Validation of Alternative Methods (JaCVAM) and the Japanese Society for Alternatives to Animal Experiments (JSAAE) created a program to validate the usefulness, reproducibility (including intra- and inter-laboratory variability and transferability), and relevance of HREs using a Japanese model (LabCyte EPI-MODEL24) as attached at attachment No. 1 to 3. This model is Japanese HRE and commercially available in Japan as attached at attachment No. 4 and 5. This validation study provided strong evidence that the *in vitro* skin irritation method is reliable. JaCVAM will evaluate this method according to the JaCVAM independent peer review system and propose to publish an additional information on this method in a new draft of the skin irritation Test Guideline.

2. Will additional information, including generation or collection of data, be required? If yes, please describe the anticipated process and timelines.

We will submit a report of this validation study by the 21st meeting of National Coordinators of the Test Guidelines Programme in March, 2009, at Paris, France. We will be able to introduce the independent peer review results on this method to expert consultation members and the OECD secretary in June, 2009.

3. Indicate the estimated overall resource need (time/money) for member country / consortium and Secretariat

We will submit the independent peer review report on this method to the OECD secretary by this summer.

4. Is this proposal intended to replace an existing Test Guideline or lead to the deletion of an existing Test Guideline?

No. These are additional test methods for a draft of the Test Guideline.

ESSENTIAL INFORMATION

In this section, please provide the information required by the Working Group of National Coordinators of the Test Guidelines Programme to assess the suitability of the project for the workplan of the Test Guidelines Programme

1. What is the existing or expected regulatory need/data requirement that will be met by the proposed outcome of the project? Please provide details below or as an attachment.

The proposed Test Guideline will be used to meet the regulatory measures that are necessary to evaluate skin irritation caused by chemicals.

or as attachment No.__

2. How will the work contribute to further international harmonisation of hazard and risk assessment? Please provide details below or as an attachment.

This test method allows the hazard identification of irritant substances to be identified in accordance with UN GHS category 2.

or as attachment No.__

3. How will the proposed project address issues and /or endpoints which are of major human health or environmental concerns? Please provide details below or as an attachment.

This test method will provide a measure of the ability to screen and identify skin irritants.

or as attachment No.__

4. Will the project have general support from OECD member countries or is the outcome relevant for just one or a few member countries / stakeholders? Provide details of the countries and the rationale for this view below.

☒ Many countries ☐ A few countries ☐ Only for the submitting country

The other HREs have already been discussed in many countries as methods to assess hemical safety. We hope this test method will also be investigated with other HREs for a new test guideline at the OECD expert consultation meeting.

5. If the Test Guideline is not intended for general use, indicate if the Test Guideline would be intended for:

☐ Specific (limited) applications such as pesticide usage, or

☐ for specific classes of chemicals (e.g. surfactants) rather than for chemicals in general.

6. If the expected outcome of this proposal is a Test Guideline or a Guidance Document, provide information on the intended use, applicability and limitations of the test method.

The method is rather simple and does not require any sophisticated equipments.

7. Provide supporting information on the validation status (i.e. relevance and reliability) of the method. Principles for validation of test methods for OECD Test Guidelines are described in Guidance Document 34.

Provide justification and rationale for the test, including data.

If there are no or limited data available to support the reliability and relevance of the proposed test, indicate if validation work is included in the project.

If there is no need for validation provide a detailed justification.

JaCVAM and JSAAE managed this validation study and the validation study was conducted in accordance with the OECD Guidance Document 34.

ADDITIONAL INFORMATION

In this section please provide further information to allow the Working Group of National Coordinators of the Test Guidelines Programme to assess the suitability of the project for the workplan of the Test Guidelines Programme

1. If the expected outcome of the project proposal is a Test Guideline and is based on existing, regional or international documents such as guidelines, protocols or guidance material, please provide that information here or as an attachment.

The project plan and standardized operation protocol for the validation study of LabCyte EPI-MODEL24 proposed by the validation management team are provided as attachment No. 1 and No.2.

Introduction materials of this HRE model are provided the basic data with LabCyte EPI-MODEL24 according the ECVAM performance standards, morphological characterizations of LabCyte EPI-MODEL24 for an alternative to *in vivo* model and company profile of Japan Tissue Engineering Co., Ltd., who produced LabCyte EPI-MODEL24 as attachment No.3 to 5.

or as attachment No. 1 to 5

2. If Animal Welfare considerations are addressed in the project proposal, provide details below or as an attachment. Explain if the project is aimed at refining, reducing and/or replacing the use of animals.

If the project is not specifically developed for animal welfare purposes, indicate if the animal welfare considerations have been a component of the project proposal.

Indicate if animal welfare considerations are irrelevant to the project, for example for physico-chemical properties.

No.

or as attachment No.__

3. Provide information on expected or possible resource savings in member countries as a result of this project.

The cost of conducting a GLP-compliant this test method in accordance with GLP is similar to conducting a GLP-compliant *in vivo* skin test method with three animals. However, the duration of this test method is considerably shorter than that of the *in vivo* skin test method.

4. If the expected outcome of the proposed project is a Guidance Document or Detailed Review Paper, will it be directly linked to the development of a particular Test Guideline or a series of Test Guidelines?

☐ Yes, it is the initial step in the development of a new or revision of existing Guidelines.

☒ Yes, additional guidance is needed for the most appropriate selection of the Guidelines on the subject.

☐ No, the guidance is on issues related to testing or the development of Test Guidelines in general.

There are _5_ attachments added to this form.

ASSESSMENT OF PROJECT PROPOSAL

(To be completed by all member countries /stakeholders except the submitter)

Country / Organisation:	

Representative: (Preferably NC):	

Taking into account the project information, requested above, does this project meet the needs of the member countries for addition to the workplan of the Test Guidelines Programme

☐ Yes ☐ No ☐ Further information needed

If the response is "No" or "Further information needed", please provide justification:

Remarks as appropriate, including further information needs, if any:

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Organisation de Coopération et de Développement Économiques
Organisation for Economic Co-operation and Development 12-Feb-2009

English - Or. English

ENVIRONMENT DIRECTORATE
JOINT MEETING OF THE CHEMICALS COMMITTEE AND
THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY

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Test Guidelines Programme

DRAFT TEST GUIDELINE 455: THE STABLY TRANSFECTED HUMAN ESTROGEN RECEPTOR-α TRANSCRIPTIONAL ACTIVATION ASSAY FOR DETECTION OF ESTROGENIC AGONIST ACTIVITY OF CHEMICALS

21st Meeting of the Working Group of National Coordinators of the Test Guidelines Programme

31st March-2nd April 2009, OECD Headquarters, Paris, France

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JT03259611

English - Or. English

This document includes a proposal for a new Test Guideline 455 on "*The Stably Transfected Human Estrogen Receptor-α Transcriptional Activation Assay for Detection of Estrogenic Agonist Activity of Chemicals – STTA*".

The STTA Test Guideline was drafted by Japan and was first circulated to member countries for comments on December 19, 2007. The draft Test Guideline was provisionally approved by the WNT 20, pending three specific issues needing further attention by the STTA Expert Working Group prior to final adoption. These issues included:

- The 3 proposed challenging chemicals
- The use of PC10/PC50 and the possibility of including PCmax in the Data Interpretation Criteria
- The inclusion of weak positives to ensure specificity of the test

After WNT 20, the STTA Expert Working Group addressed the specific issues raised by the WNT 20 and a revised proposal that was sent to WNT for approval in June 2008. Since UK, Greece and BIAC still had comments on the revised version, it was not possible to submit the STTA TG to the Joint Meeting for endorsement without informing the WNT of the comments and amendments of the draft STTA TG.

The STTA Expert Working Group continued their work which was presented and further discussed at the 6th VMG NA meeting in November 2008. A revised STTA TG was circulated to the WNT for approval by written procedure on 24 November 2008 with a deadline of 9 January 2009. Comments were received from Denmark, USA, EC and BIAC, the UK stated they had no further comments. The final draft (version 4) has been prepared based on the comments received after the last round of circulation to the WNT, in addition to some minor editorial corrections by the Secretariat.

ACTION REQUIRED: ***The Working Group of National Coordinators of the Test Guidelines Programme is invited to approve the draft STTA Test Guideline, revised as appropriate.***

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

DRAFT PROPOSAL FOR A NEW GUIDELINE 455

THE STABLY TRANSFECTED HUMAN ESTROGEN RECEPTOR-A TRANSCRIPTIONAL ACTIVATION ASSAY FOR DETECTION OF ESTROGENIC AGONIST-ACTIVITY OF CHEMICALS

INTRODUCTION

1. 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 for testing and assessment of potential endocrine disrupting chemicals comprises five levels, each level corresponding to a different level of biological complexity (1). The Transcriptional Activation (TA) assay described in this Test Guideline is a level 2 "*in vitro* assay, providing mechanistic information". The validation study of the Stably Transfected Transactivation Assay (STTA) by the Japanese Chemicals Evaluation and Research Institute (CERI) using the hERα-HeLa-9903 cell line to detect estrogenic agonist activity mediated through human estrogen receptor alpha (hERα) demonstrated the relevance and reliability of the assay for its intended purpose (2).

2. *In vitro* TA assays are based upon the production of a reporter gene product induced by a chemical, following binding of the chemical to a specific receptor and subsequent downstream transcriptional activation. TA assays using activation of reporter genes are screening assays that have long been used to evaluate the specific gene expression regulated by specific nuclear receptors, such as the estrogen receptors (ERs) (3)(4)(5)(6). They have been proposed for the detection of estrogenic transactivation regulated by the ER (7)(8)(9). The nuclear ERs exist as at least two subtypes, termed α and β, encoded by distinct genes and with different tissue distribution, relative ligand binding affinities and biological functions. Nuclear ERα mediates the classic estrogenic response, therefore models currently being developed to measure ER activation mainly relate to ERα. The aim of this TA assay is to evaluate the ability of a chemical to function as an ERα ligand and activate an agonist response, for screening and prioritisation purposes but can also provide mechanistic information that can be used in a weight of evidence approach.

3. Definitions and abbreviations used in this Test Guidelines are described in Annex 1.

INITIAL CONSIDERATIONS AND LIMITATIONS

4. Estrogen agonists act as ligands for ERs, and may activate the transcription of estrogen responsive genes. This interaction may have the potential to trigger adverse health effects by disrupting estrogen-regulated systems. This Test Guideline describes an assay that evaluates TA mediated by the hERα. This process is considered to be one of the key mechanisms of possible endocrine disruption related health hazards, although there are also other important endocrine disruption mechanisms. These include (i) actions mediated via other nuclear receptors linked to the endocrine system and interactions with steroidogenic enzymes, (ii) metabolic activation or deactivation of hormones, (iii) distribution of hormones to target tissues, and (iv) clearance of hormones from the body. This Test Guideline exclusively addresses TA of an estrogen-regulated reporter gene by agonist binding to the hERα, and therefore it should not be directly extrapolated to the complex *in vivo* situation of estrogen regulation of cellular processes. Furthermore, this Test Guideline does not address antagonist interaction with the hERα and subsequent effect on transcription.

5. This test method is specifically designed to detect hERα-mediated TA by measuring chemiluminescence as the endpoint. However, non-receptor-mediated luminescence signals have been reported at phytoestrogen concentrations higher than 1 μM due to the over-activation of the luciferase reporter gene (10)(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 (Annex 2).

6. It is recognized that this assay using the hERα-HeLa-9903 cell line is only one of several ER transcriptional activation assays currently being developed and validated. It is, therefore the intention that a generic performance based Test Guideline will replace this Test Guideline as soon as such guideline is developed and approved.

PRINCIPLE OF THE TEST

7. The TA assay using a reporter gene technique is an *in vitro* tool that provides mechanistic data. 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.

8. The test system provided in this guideline utilises the hERα-HeLa-9903 cell line, which is derived from a human cervical tumor, with two stably inserted constructs: (i) the hERα 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 hERα-HeLa-9903 cell line can measure the ability of a test chemical to induce hERα-mediated transactivation of luciferase gene expression.

9. Data interpretation for this assay 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) 17β estradiol (E2) (*i.e.*, the PC10). Data analysis and interpretation are discussed in greater detail in paragraphs 34- 45.

PROCEDURE

Cell Lines

10. The stably transfected hERα-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 Bank[1].

11. 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 mycoplasm infection (12)(13)(14).

Stability of the cell line

[1] JCRB Cell Bank : National Institute of Biomedical Innovation, 7-6-8 Asagi Saito, Ibaraki-shi, Osaka 567-0085, Japan Fax: +81-72-641-9812

12. To monitor the stability of the cell line, E2, 17α-estradiol, 17α-methyltestosterone, and corticosterone should be used as the reference chemicals and a complete concentration response curve in the test concentration range provided in Table 1 should be measured at least ones each time the assay is performed, and the results should be in agreement with the results provided in Table 1.

Cell Culture and Plating Conditions

13. 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 CO_2 incubator (5% CO_2) at 37±1°C. Upon reaching 75-90% confluency, cells can be subcultured at 10 mL of $0.4 \times 10^5 - 1 \times 10^5$ 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 1×10^4 cells/100 µL/well. Next, the cells should be pre-incubated in a 5% CO_2 incubator at 37°±1°C for 3 hours before the chemical exposure. The plastic-ware should be free of estrogenic activity.

14. 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.

15. The DCC-FBS can be prepared as described in Annex 3, or obtained from commercial sources.

Acceptability Criteria

Positive and Negative Reference Chemicals

16. 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 compound (corticosterone). Acceptable range values derived from the validation study are given in Table 1 (2). These 4 concurrent reference chemicals 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 chemicals, 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 chemicals should fall within the acceptable range, and the PC50s and EC50s where they can be calculated (see Table 1).

Table 1. Acceptable range values of the 4 reference chemicals for the STTA assay (means ± 2 standard deviations).

Name	logPC50	logPC10	logEC50	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	$10^{-14} \sim 10^{-8}$ M
17α-Estradiol CAS No: 57-91-0	-9.6 ~ -8.1	-10.7 ~ -9.3	-9.6 ~ -8.4	0.9 ~ 2.0	$10^{-12} \sim 10^{-6}$ M
Corticosterone CAS No: 50-22-6	–	–	–	–	$10^{-10} \sim 10^{-4}$M
17α-Methyltestosterone CAS No: 58-18-4	-6.0 ~ -5.1	-8.0 ~ -6.2	–	–	$10^{-11} \sim 10^{-5}$ M

Positive and Vehicle Controls

17. The positive control (PC) (1 nM of E2) 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 vehicle control, if the PC uses a different vehicle than the test chemical, another vehicle control should be tested at least in triplicate on the same plate with the PC.

Fold-induction

18. The mean luciferase activity of the PC (1 nM E2) should be at least 4-fold that of the mean vehicle control 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).

19. 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 (standard deviations) 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.

Chemicals to Demonstrate Laboratory Proficiency

20. Prior to testing unknown chemicals in the STTA assay, the responsiveness of the test system should be confirmed by each laboratory, at least once for each newly prepared batch of cell stocks taken from the frozen stock by independent testing of the 11 proficiency chemicals listed in Table 2. This should be done at least in duplicate, on different days, and the results should be comparable to Table 2 and any deviations should be justified.

Table 2. List of Proficiency Chemicals

Compound	CAS No.	Class[2]	Test concentration range	Note
Diethylstilbestrol (DES)	56-53-1	Positive	10^{-14} - 10^{-8} M	
17α-Ethynyl estradiol (EE)	57-63-6	Positive	10^{-14} - 10^{-8} M	
Hexestrol	84-16-2	Positive	10^{-13} - 10^{-7} M	
Genistein	446-72-0	Positive	10^{-12} – 10^{-5} M	Cytotoxic at (0.01)[4], 0.1 and 1 mM
Estrone	53-16-7	Positive	10^{-12} - 10^{-6} M	
Butyl paraben	94-26-8	Positive	10^{-11} – 10^{-4} M	Cytotoxic at (0.1)[4] and 1 mM
4-*n*-Nonylphenol	104-40-5	Positive	10^{-12} – 10^{-5} M	Cytotoxic at 0.1 and 1 mM
1,3,5-Tris(4hydroxyphenyl)benzene[1]	15797-52-1	Positive	10^{-12} - 10^{-5} M	Cytotoxic at 100 µM. PCmax approx 15% of PC Binds to hERα and has ER antagonist activity
Dibutyl phthalate (DBP)	84-74-2	Negative[3]	10^{-11} – 10^{-4} M	Cytotoxic at 1 mM
Atrazine	1912-24-9	Negative	10^{-11} – 10^{-4} M	Cytotoxic [4] at 1 mM
Corticosterone	50-22-6	Negative	10^{-10} – 10^{-4} M	If not cytotoxic at 1 mM, then that should be the highest tested concentration

[1] Compound selected to challenge solubility and cytotoxicity.
[2] See Table 5 for definitions of positive and negative.
[3] Negative for ERα mediated transcriptional activation but may not be negative for non-ERα mediated transcriptional activation. Thus a positive result in this assay with DBP would indicate that the system is detecting other than pure ERα mediated activity and is therefore unacceptable.
[4] Cytotoxicity is close to 80%.

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 vehicle control. Test substances should be dissolved in a solvent that solubilizes that test substance 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, 100 µM, 10 µM, 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, 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 substance has reduced the cell number by 20% or more, this concentration is 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, 1 µM, 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 concentration 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 chemicals can be assigned as shown in Table 3.

Table 3.: Example of plate concentration assignment of the reference chemicals in the assay plate

Row	17α-Methyltestosterone			Corticosterone			17α-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	→	→	→	→	→

Plate controls = VC: Vehicle control (DMSO); PC: Positive control (1 nM E2)

27. The reference chemicals (E2, 17α-Estradiol, 17α-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 chemicals should be tested for each cell source.

Table 4.: Example of plate concentration assignment of test and plate control chemicals in the assay plate

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	→	→	→	→	→

28. 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.

29. After adding the chemicals, the assay plates should be incubated in a 5% CO_2 incubator at 37±1°C for 20-24 hours to induce the reporter gene products.

30. 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.

31. Repeat definitive tests for the same chemical should be conducted on different days, to ensure independence.

Luciferase assay

32. A commercial luciferase assay reagent [*e.g.*. Steady-Glo® Luciferase Assay System (Promega, E2510, or equivalents)] or a standard luciferase assay system (Promega, E1500, or equivalents) can be used for the assay, as long as the acceptability criteria is 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 (Promega, E1531, or equivalents) should be used before adding the substrate. The luciferase reagent should be applied following the manufacturers' instructions.

ANALYSIS OF DATA

33. 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 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

34. 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).

35. If the Hill's logistic equation is applicable to the concentration response data, the EC50 should be calculated by the following equation (15):

Y=Bottom + (Top-Bottom) / (1+10 exp ((log EC50 -X) x Hilllope))
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:

(i) The RPCMax 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 PCMax (concentration associated with the RPCMax); and

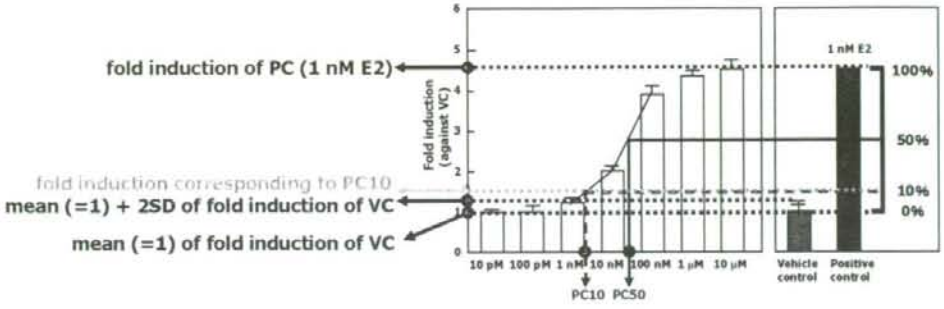
(ii) For positive chemicals, the concentrations that induce the PC10 and, if appropriate, the PC50.

37. The PCx value can be calculated by interpolating between 2 points on the X-Y coordinate, one immediately above and one immediately below a PCx value. Where the data points lying immediately above and below the PCx value have the coordinates (a,b) and (c,d) respectively, then the PCx value may be calculated using the following equation:

$$\log[PCx] = \log[c]+(x-d)/(d-b)$$

38. Descriptions of PC values are provided in Figure 1 below.

Figure 1.: Example of how to derive PC-values. The PC (Positive control; 1 nM of E2) is included on each assay plate



39. 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 performance standard requirements;
  - The mean luciferase activity of the positive controls (1 nM E2) should be at least 4-fold that of the mean vehicle control on each plate
  - 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 VC (vehicle control).
  - The results of 4 reference chemicals should be within the acceptable range (Table 1).
- Be reproducible.

Data Interpretation Criteria

Table 5. : Positive and negative decision criteria

Positive	If the RPCMax 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 RPCMax fails to achieve at least 10% of the response of the positive control in two of two or two of three runs.

40. Data interpretation criteria are shown in Table 5. 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% (PC50) or 10% (PC10) of positive control values are reached 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 positive control 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.

41. The calculations of PC10, PC50 and PCMax can be made by using a spreadsheet available with the Test Guideline on the OECD public website[2].

42. It should be sufficient to obtain PC10 or PC50 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%.

43. 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, see paragraphs 41 and 42.

44. Where more information is required in addition to the screening and prioritisation purposes of this TG for positive test compounds, 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 ERα-specific response, using an ERα antagonist (see Annex 3).

TEST REPORT

45. The test report should include the following information:

Test substance:

- identification data and CAS Number, if known;
- physical nature and purity;
- physicochemical properties relevant to the conduct of the study;
- stability of the test substance.

Solvent/Vehicle:

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

Cells:

- type and source of cells;
- number of cell passages;
- methods for maintenance of cell cultures.

Test conditions:

cytotoxicity data (and justifications for the method of choice) and solubility limitations should be reported, as well as:

- composition of media, CO_2 concentration;
- concentration of test chemical;
- volume of vehicle and test substance added;
- incubation temperature and humidity;

[2] [http://www.oecd.org/document/55/0,3343,en_2649_34377_2349687_1_1_1_1,00.html]

- duration of treatment;
- cell density during treatment;
- positive and negative reference chemicals;
- duration of treatment period;
- Luciferase assay reagents (Product name, supplier and lot);
- acceptability and data interpretation criteria.

Reliability check:

- Fold inductions for each assay plate.
- Actual logEC50, logPC50, logPC10 and Hill slope values for concurrent reference chemicals.

Results:

- Raw and normalised data of luminescent signals;
- Concentration-response relationship, where possible;
- RPCMax, PMax, PC50 and/or PC10 values, as appropriate;
- EC50 values, if appropriate;
- Statistical analyses, if any, together with a measure of error (*e.g.*, SEM, SD, CV or 95% CI) and a description of how these values were obtained.

Discussion of the results

Conclusion

LITERATURE

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