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APPENDIX 1

DEFINITIONS AND ABBREVIATIONS

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

Accuracy: (a) The closeness of agreement between a test method result and an accepted reference value. (b) The proportion of correct outcomes of a test method.

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

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

BG-1: An immortalized human ovarian adenocarcinoma cells that endogenously express estrogen receptors alpha and beta.

BG-1Luc4E2: The BG-1Luc4E2 cell line was derived from BG-1 immortalized adenocarcinoma cells that endogenously express both forms of the estrogen receptor (ERα and ERβ) and have been stably transfected with the plasmid pGudLucERE. 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.

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 cellular morphology.

CF: The OECD Conceptual Framework for the Screening and Testing of Endocrine Disrupting Chemicals.

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.

Cytotoxicity: The adverse effects resulting from interference with structures and/or processes essential for cell survival, proliferation, and/or function. For most substances, toxicity is a consequence of non-specific alternations in "basal cell functions" (i.e., via mitochondria, plasma membrane integrity, etc.).

DMEM: Dulbecco's Modification of Eagle's Medium

DMSO: Dimethyl sulfoxide

E2: 17β-estradiol

EC_{50}: The half maximal effective concentration of a test substance.

ED: Endocrine disruption

EE: 17α-ethynyl estradiol

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

FBS: Fetal bovine serum

hERα: Human estrogen receptor alpha

hERß: Human estrogen receptor beta

IC_{50}: The half maximal effective concentration of an inhibitory test substance.

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

MMTV: Mouse Mammary Tumor Virus

Proficiency: The demonstrated ability to properly conduct a test method prior to testing unknown substances.

Proficiency Chemicals: A list of substances that can be used by laboratories to demonstrate technical competence with a standardized 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.

Ral: raloxifene HCl

Ral/E2: The antagonist reference standard, which is a combination of raloxifene HCl (Ral) and 17β-estradiol (E2).

Reference standard: a reference substance used to demonstrate the adequacy of a test method. 17β-estradiol is the estrogenic reference standard and Raloxifene HCl the anti-estrogenic reference standard for the BG1Luc ER TA.

Reliability: A measure of the degree to which a test method can be performed reproducibly within and among laboratories over time.

RLU: Relative Light Units

RNA: Ribonucleic Acid

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

SD: Standard deviation

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).

TG: Test Guideline

Transcription: mRNA synthesis

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

Validation: The process by which the reliability and accuracy of a procedure are established for a specific purpose.

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

22 December 2011

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

DRAFT PROPOSAL FOR AN UPDATED TG 455

Performance-Based Test Guideline for Stably Transfected Transactivation *In Vitro* Assays to Detect Estrogen Receptor Agonists

GENERAL INTRODUCTION

Performance-Based Test Guideline

1. This Performance-Based Test Guideline (PBTG) comprises several mechanistic and functionally similar test methods for the identification of estrogen receptor (i.e, ERα, and/or ERβ) agonists 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 (GD) on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment (1). The fully validated reference test methods (Annex 2 and Annex 3) that provide the basis for this PBTG are:

- The Stably Transfected TA assay (STTA) using the (h) ERα-HeLa-9903 cell line (2) and
- The BG1Luc ER TA assay (3) using the BG1Luc-4E2 cell line which predominately expresses hERα with some contribution from hERβ (4) (5).

Performance standards (PS) (6) are available to facilitate the development and validation of similar test methods for the same hazard endpoint and allow for timely amendment of this PBTG so that new similar test methods can be added to an updated PBTG only after review and agreement that performance standards are met.

Background and principles of the test methods included in the PBTG

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 2011. The original and revised CFs are included as Annexes in the Guidance Document on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption (7). The revised CF comprises five levels, each level corresponding to a difference level of biological complexity. The ER Transactivation (TA) assays described in this PBTG are level 2, which includes "*in vitro assays providing data about selected endocrine mechanism(s)/pathway(s)*. This PBTG is for *in vitro* Transactivation (TA) test methods designed to identify estrogen receptor (ER) agonists.

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 (8) (9) (10). 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 chemical 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 (11) (12) (13) (14) (15). They have been proposed for the detection of estrogenic transactivation regulated by the ER (16) (17) (18). There are at least two subtypes of nuclear ERs, termed α and β, which are encoded by distinct genes and with different tissue distributions, relative ligand binding affinities and biological functions (19) (20) (21) (22) (23) (24) (25). Nuclear ERα mediates the classic estrogenic response (26) (27) (28) (29), and therefore most models currently being developed to measure ER activation are specific to ERα. The assays are used to identify chemicals that activate 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 test methods. 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 BG1Luc 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 ovarian cells lines are included in ICCVAM Test Method Evaluation Report The LUMI-CELL® ER (BG1Luc ER TA) Test Method: An *In Vitro* Assay for Identifying Human Estrogen Receptor Agonist and Antagonist Activity of Chemicals (8). These performance standards have been modified to be applicable to both the STTA and BG1 methods (2).

6. Definitions and abbreviations used in this Test Guideline are described in Annex 1.

Scope and limitations related to the TA assays

7. These test methods 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 signaling 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 test methods under this PBTG addresses these modes of action.

9. This PBTG addresses the ability of chemicals to activate (*i.e.* act as agonists) but not to suppress ER-dependent transcription (*i.e.* act as antagonists). Therefore, chemicals that are negative in these test methods should be evaluated in an ER binding assay or in an assay known to detect ER antagonists before concluding that the chemical does not bind to the receptor. In addition, the assay is only likely to inform on the agonist 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.

10. For informational purposes Table 1 provides the test results for the 34 chemicals that were tested in both of the fully validated test methods described in this PBTG. Of these chemicals, 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 (3) (18) (30) (32) (33) (34) (35). There was 100% agreement between the two test methods on the classifications of all the chemicals, and each chemical was correctly classified as an ER agonist or negative. Supplementary information on this group of chemicals as well as additional chemicals tested in the STTA and BG1 Luc ER TA test methods during the validation studies is provided in the Performance Standards for the ER TA (2), Annex 2 (Tables 1, 2 and 3).

Table 1: Comparison of Results from STTA and BG1Luc ER TA Assays for Chemicals Tested in Both Assays and Classified as ER Agonists or Negatives

Chemical	CASRN	STTA ER TA[1]			BG1Luc ER TA[2]		Data Source For Classification[4]		
		ER TA Activity	PC_{10} Value (M)	PC_{50} Value[b] (M)	ER TA Activity	EC_{50} Value[b,3] (M)	Other ER TAs[c]	ER Binding	Uterotrophic
17-ß 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
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
17-α Ethinyl estradiol[a]	57-63-6	POS	$<1.00 \times 10^{-11}$	$<1.00 \times 10^{-11}$	POS	4.20×10^{-8}	POS(22/22)	POS	POS
17-β-Trenbolone	10161-33-8	POS	1.78×10^{-8}	2.73×10^{-7}	POS	7.31×10^{-12}	POS (2/2)	NT	NT
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
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
4-*tert*-Octylphenol[a]	140-66-9	POS	1.85×10^{-9}	7.37×10^{-8}	POS	3.19×10^{-8}	POS(21/24)	POS	POS
Apigenin[a]	520-36-5	POS	1.31×10^{-7}	5.71×10^{-7}	POS	1.60×10^{-6}	POS(26/26)	POS	NT
Atrazine[a]	1912-24-9	NEG	-	-	NEG	-	NEG (30/30)	NEG	NT
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
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
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
Corticosterone[a]	50-22-6	NEG	-	-	NEG	-	NEG(6/6)	NEG	NT
Coumestrol[a]	479-13-0	POS	1.23×10^{-9}	2.00×10^{-8}	POS	1.32×10^{-7}	POS(30/30)	POS	NT
Daidzein[a]	486-66-8	POS	1.76×10^{-8}	1.51×10^{-7}	POS	7.95×10^{-7}	POS(39/39)	POS	POS
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
Di-n-butyl phthalate	84-74-2	POS	4.09×10^{-6}		POS	4.09×10^{-6}	POS(6/11)	POS	NEG
Ethyl paraben	120-47-8	POS	5.00×10^{-6}	(no PC_{50})	POS	2.48×10^{-5}	POS		NT
Estrone[a]	53-16-7	POS	3.02×10^{-11}	5.88×10^{-10}	POS	2.34×10^{-10}	POS(26/28)	POS	POS
Genistein[a]	446-72-0	POS	2.24×10^{-9}	2.45×10^{-8}	POS	2.71×10^{-7}	POS(100/102)	POS	POS
Haloperidol	52-86-8	NEG	-	-	NEG	-	NEG (2/2)	NEG	NT
Kaempferol[a]	520-18-3	POS	1.36×10^{-7}	1.21×10^{-6}	POS	3.99×10^{-6}	POS(23/23)	POS	NT
Kepone[a]	143-50-0	POS	7.11×10^{-7}	7.68×10^{-6}	POS	4.91×10^{-7}	POS(14/18)	POS	NT
Ketoconazole	65277-42-1	NEG	-	-	NEG	-	NEG (2/2)	NEG	NT
Linuron[a]	330-55-2	NEG	-	-	NEG	-	NEG (8/8)	NEG	NT
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
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
Morin	480-16-0	POS	5.43×10^{-7}	4.16×10^{-6}	POS	2.37×10^{-6}	POS(2/2)	POS	NT
Norethynodrel[a]	68-23-5	POS	1.11×10^{-11}	1.50×10^{-9}	POS	9.39×10^{-10}	POS(5/5)	POS	NT
p,p'-Methoxychlor[a]	72-43-5	POS	1.23×10^{-6}	(no PC_{50})[b]	POS	1.92×10^{-6}	POS(24/27)	POS	POS
Phenobarbital[a]	57-30-7	NEG	-	-	NEG	-	NEG(2/2)	NEG	NT
Reserpine	50-55-5	NEG	-	-	NEG	-	NEG(4/4)	NEG	NT
Spironolactone[a]	52-01-7	NEG	-	-	NEG	-	NEG(4/4)	NEG	NT
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; EC_{50} = half maximal effective concentration of test chemical; NEG = negative; POS = positive; PC_{10} (and PC_{50}) = the concentration of a test chemical at which the response is 10% (or 50 % for PC_{50}) of the response induced by the positive control (E2, 1nM) in each plate.

[a]Common chemicals tested in the STTA ER TA and BG1Luc ER TA that were designated as ER Agonists or negatives and used to evaluate accuracy in the BG1 Luc ER TA validation study (ICCVAM BG1Luc ER TA Evaluation Report, Table 4-1 (3).

[b]Maximum concentration tested in the absence of limitations due to cytotoxicity or insolubility was 1×10^{-5} M (STTA ER TA) and 1×10^{-3} M (BG1Luc ER TA).

[c]Number in parenthesis represents the test results classified as positive (POS) or negative (NEG) over the total number of referenced studies.

[1]Values 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)

[2]ICCVAM Test Method Evaluation Report on the LUMI-CELL® ER (BG1Luc ER TA) Test Method: An *In Vitro* Method for Identifying ER Agonists and Antagonists (3)

[3]Mean EC_{50} values were calculated with values reported by the laboratories of the BG1Luc ER TA validation study (XDS, ECVAM, and Hiyoshi) (3).

[4]Classification 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 (3) (18) (30) (32) (33) (34) (35).

COMMON ELEMENTS FOR ALL TEST METHODS

Estrogen TA Assay Test Method Components

11. This PBTG applies to methods using a stably transfected or endogenous ERα receptor and stably transfected reporter gene construct under the control of one or more estrogen response elements; however, other receptors such as ERβ may be present. These are *invariable* test method components.

Control substances

12. The basis for the proposed concurrent reference estrogen and controls should be described. Concurrent controls (negative, solvent, and positive), as appropriate, serve as an indication that the test method is operative under the test conditions and provide a basis for experiment-to-experiment comparisons; they are usually part of the acceptability critera 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, 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 contaminates (e.g. fungi, yeast and viruses).

Demonstratation of Laboratory Proficiency

14. Prior to testing unknown chemicals with any of the test methods under this PBTG, the responsiveness of the test system should be confirmed by each laboratory with independent testing of the 14 proficiency chemicals listed in Table 2. This list is a subset of the Reference Chemicals provided in the Performance Standards for the ER TA (6). These chemicals are commercially available, represent the classes of chemicals commonly associated with ER agonist activity, exhibit a suitable range of potency expected for ER agonists (i.e., strong to weak) and negatives. Testing of these chemicals should be replicated at least twice, on different days. Proficiency is demonstrated by correct classification (positive/negative) of each proficiency chemical. Proficiency testing should be repeated by each technician when learning the test methods.

Table 2: List of (14) Proficiency Chemicals

Chemical Name	CASRN	Expected Response[1]	STTA ER TA			BG1Luc ER TA		MeSH Chemical Class[5]	Product Class[6]
			PC10 Value (M)[2]	PC_{50} Value (M)[2]	Test concentration range (M)	Bg1Luc EC_{50} Value (M)[3]	Highest Concentration for Range Finder (M)[4]		
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
17∝-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
meso-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
4-*tert*-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
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
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
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
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	Plasticizer, Industrial Chemical
p,p'-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
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
Atrazine	1912-24-9	NEG	-	-	$10^{-10} - 10^{-4}$	-	4.64×10^{-4}	Heterocyclic Compound	Herbicide
Spironolactone	52-01-7	NEG	-	-	$10^{-11} - 10^{-5}$	-	2.40×10^{-3}	Lactone, Steroid	Pharmaceutical
Ketoconazole	65277-42-1	NEG	-	-	$10^{-11} - 10^{-5}$	-	9.41×10^{-5}	Heterocyclic	Pharmaceutical

								Compound	
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; EC_{50} = half maximal effective concentration of test chemical; NEG = negative; POS = positive; PC_{10} (and PC_{50}) = the concentration of a test chemical at which the response is 10% (or 50 % for PC_{50}) of the response induced by the positive control (E2, 1nM) in each plate.

[1]Classification as positive or negative for ER agonist activity was based upon the ICCVAM Background Review Documents (BRD) for ER Binding and TA test methods (31) (32) as well as empirical data and other information obtained from referenced studies published and reviewed after the completion of the ICCVAM BRDs (3) (18) (30) (31) (32) (33) (34) (35).

[2]Values 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).

[3]Mean EC_{50} values were calculated with values reported by the laboratories of the BG1Luc ER TA validation study (XDS, ECVAM, and Hiyoshi) (3).

[4]Concentrations reported were the highest concentrations tested (range finder) during the validation of the BG1Luc ER TA. 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 (BG1Luc ER TA) Test Method: An *In Vitro* Assay for Identifying Human Estrogen Receptor Agonist and Antagonist Activity of Chemicals (3).

[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 recognized standardized 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)

Test Run Acceptability Criteria

15. Acceptance or rejection of a test run is based on the evaluation of results obtained for the reference estrogen and controls used for each experiment. Values for the PC_{50} or EC_{50} values for the reference estrogen should meet the acceptable criteria as provided for the selected test method (e.g, STTA (Annex 2) or BG1Luc ER TA (Annex 3)), and all positive/negative controls should be correctly classified for each accepted experiment. The ability to consistently conduct the test method 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 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 (i.e., efficacy and potency).
- The mean reporter activity of the reference concentration of estrogne should be at least the minimum specified in the test methods relative to that of the vehicle (solvent) control to ensure adequate sensitivity. For the STTA and BG1Luc ER TA test methods, 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 chemical and not demonstrate cytotoxicity.

Analysis of data

16. Each test method should establish a well-defined method for classifying a positive and negative response.

17. Meeting the acceptability criteria (paragraph 15) indicates the assay system is operating properly, but it does not ensure that any particular test will produce accurate data. Replicating the results of the first test is the best indication that accurate data were produced. If two tests give reproducible results (e.g., both test results indicate a substance is positive), it is not necessary to conduct a third test.

18. If two results do not give reproducible results (e.g., a substance is positive in one test and negative in the other test), or if a higher degree of certainty is required regarding the outcome of this assay, at least three independent tests should be conducted.

General Data Interpretation Criteria

19. 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) assessments of ER-mediated activity should be based on empirical data and sound scientific judgement. 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 EC_{50}, PC_{50}, RPCMax , etc.).

Test Report

20. The test report should include the following information:

Test method:

- Test method used;

Test substance:

- identification data and Chemical Abstracts Service Registry Number (CAS RN), 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:
  - Is ER endogenously expressed? If not, which receptor(s) were Transfected;
  - Species of origin of the receptorReporter 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, CO_2 concentration;
- concentration of test substance;
- volume of vehicle and test substance added;
- incubation temperature and humidity;
- duration of treatment;
- cell density at the start of - and during treatment;
- positive and negative reference chemicals;
- duration of treatment period;
- reporter reagents (Product name, supplier and lot);
- criteria for considering tests as positive, negative or equivocal;

Reliability check:

- fold inductions for each assay plate and whether they meet the minimum required by the test method based on historical controls;
- actual $\log_{10}EC_{50}$, $\log_{10}PC_{50}$, and Hillslope values for concurrent positive controls/reference substances;

Results:

- raw and normalised data;
- the maximum fold induction level;
- cytotoxicity data;
- if it exists, the lowest effective concentration (LEC);
- PRCMax, PCMax, PC_{50} and/or EC_{50} values, as appropriate;
- concentration-response relationship, where possible;
- 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

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