

Draft Proposed Performance Standards for Luciferase-Based Stably Transfected Transcriptional Activation In Vitro Assays to Detect Estrogenic Agonist Activity of Chemicals

INTRODUCTION

1. Prior to the acceptance of a new test method for regulatory testing applications, validation studies are conducted using scientifically sound principles to establish its reliability (i.e., the extent of intra- and interlaboratory reproducibility over time when performed using the standardized protocol), and its relevance (i.e., the ability of the test method to correctly predict or measure the biological effect of interest)[1,2,3,4]. The purpose of performance standards is to communicate the basis by which new proprietary (i.e., copyrighted, trademarked, registered) or nonproprietary test methods have been determined to have sufficient accuracy (i.e., agreement between a test method result and an accepted reference value), reliability, and relevance for a specific testing purpose.

2. Performance standards are based on an adequately validated test method and provide a basis for evaluating the comparability of a proposed test method that is mechanistically and functionally similar [2]. The three elements of performance standards are:

- Essential test method components: These consist of essential structural, functional, and procedural elements of a validated test method that should be included in the protocol of a proposed test method that is considered to be mechanistically and functionally similar to the validated method. Essential test method components include unique characteristics of the test method, critical procedural details, and quality control measures.

- A minimum list of reference substances: Reference substances are used to assess the accuracy and reliability of a proposed mechanistically and functionally similar test method. These substances are a representative subset of those used to demonstrate the reliability and the accuracy of the validated test method, and are the minimum number that should be used to evaluate the performance of a proposed mechanistically and functionally similar test method.

- Accuracy and reliability performance values: These are the standards for accuracy (i.e., sensitivity, specificity, false positive/negative rates) and reliability (i.e., degree to which the test method can be performed reproducibly within and among laboratories over time) that the proposed test method should meet or exceed when evaluated using the minimum list of reference substances.

ESSENTIAL TEST METHOD COMPONENTS AND OTHER VALIDATION CONSIDERATIONS

3. Certain principles are important in delineating the essential test method components that determine whether transcriptional activation (TA) tests are functionally and mechanistically similar. *In vitro* estrogen receptor (ER) TA assays are designed to identify substances that might interfere with ER-mediated cellular processes *in vivo*. The interaction of estrogens with cellular ER initiates a cascade of events leading to the expression of specific genes in multiple target tissues. This process can be evaluated using a number of *in vitro* endpoints including receptor binding, cellular proliferation, and transcriptional activation (TA, reporter gene) assays.

4. Essential test method components for *in vitro* ER TA protocols should include:

- The use of a strong reference estrogen, preferably 17 β -estradiol, to demonstrate the adequacy of the method for detecting ER agonists;

- A weak positive control with a maximum TA response two to three orders of magnitude lower than the reference estrogen should be included to provide another quality control measure by which to judge the acceptability of the method for detecting a weak agonist, and by which to evaluate the reproducibility of the test method.

- A solvent (e.g., DMSO, EtOH, or H₂O) that is miscible with cell culture media at concentrations that are not cytotoxic and do not otherwise interfere with the test system.

- A minimum limit concentration and number of concentrations to be tested. In the absence of solubility or cytotoxicity restraints, the limit concentration should be 1 mM. At least seven concentrations spaced at logarithmic (log 10) intervals should be tested up to the limit concentration.

- An evaluation of cytotoxicity and how it is applied to the test method should be included in each study. Concentrations of test substances that reduce viability by greater than 20% should not be considered in the analysis of the data.

- All concentrations of the control (i.e., solvent, weak positive), the reference estrogen, and the test substance should be tested in more than one replicate well.

5. No standardized statistical methods for analyzing data obtained from *in vitro* ER TA agonist assays have been developed. Each test method should establish a well-defined method for classifying a positive and a negative response. Where possible, positive results should be characterized by both the magnitude of the effect and the concentration at which the effect occurs (e.g., an EC₅₀, PC₅₀, % max, etc.).

6. To ensure that a proposed *in vitro* ER TA test method possess characteristics similar to other validated test methods, at a minimum, the reference substances for testing ER agonists listed in Table 1 should be used.

Table 1. 29 Reference Substances for Evaluation of ER Agonist Accuracy

Substance	CASRN	Expected Response ¹	Bg1Luc EC ₅₀ Value (M) ²	STTA ER TA Results ^{3,4}	STTA PC ₅₀ Value (M) ⁵
Ethyl paraben	120-47-8	POS	2.48×10^{-5}	POS	Not reported
Kaempferol	520-18-3	POS	3.99×10^{-6}	POS	Not reported
Methyl testosterone	58-18-4	POS	3.29×10^{-6}	POS	?
<i>p</i> -n-Nonylphenol	104-40-5	POS	3.06×10^{-6}	NEG	-
Butylbenzyl phthalate	85-68-7	POS	1.98×10^{-6}	POS	Not reported
<i>p,p'</i> -Methoxychlor	72-43-5	POS	1.92×10^{-6}	NEG	-
Apigenin	520-36-5	POS	1.85×10^{-6}	POS	Not reported
19-Nortestosterone	434-22-0	POS	1.80×10^{-6}	POS	Not reported
Daidzein	486-66-8	POS	8.71×10^{-7}	POS	1.51×10^{-7}
Bisphenol A	80-05-7	POS	5.33×10^{-7}	POS	1.52×10^{-7}
Kepone	143-50-0	POS	4.91×10^{-7}	POS	2.94×10^{-7}
<i>o,p'</i> -DDT	789-02-6	POS	3.94×10^{-7}	POS	Not reported
4-Cumylphenol	599-64-4	POS	3.20×10^{-7}	POS	1.60×10^{-6}
Genistein	446-72-0	POS	2.71×10^{-7}	POS	2.45×10^{-8}
Bisphenol B	77-40-7	POS	1.67×10^{-7}	POS	1.58×10^{-7}
Coumestrol	479-13-0	POS	8.77×10^{-8}	POS	Not reported
4- <i>tert</i> -Octylphenol	140-66-9	POS	3.19×10^{-8}	POS	7.37×10^{-8}
17 α -Estradiol	57-91-0	POS	1.54×10^{-9}	POS	6.44×10^{-10}
Norethynodrel	68-23-5	POS	9.39×10^{-10}	POS	Not reported
Estrone	53-16-7	POS	2.57×10^{-10}	POS	5.88×10^{-10}
Diethylstilbestrol	56-53-1	POS	3.34×10^{-11}	POS	Not reported
<i>meso</i> -Hexestrol	84-16-2	POS	1.65×10^{-11}	POS	Not reported
17 β -Estradiol	50-28-2	POS	8.37×10^{-12}	POS	Not applicable
17 α -Ethinyl estradiol	57-63-6	POS	7.31×10^{-12}	POS	$>1.0 \times 10^{-11}$
Atrazine	1912-24-9	NEG	-	NEG	-
Corticosterone	50-22-6	NEG	-	NEG	-
Linuron	330-55-2	NEG	-	NEG	-
Phenobarbital	50-06-6	NEG	-	NEG	-
Spirolactone	52-01-7	NEG	-	NEG	-

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; EC₅₀ – half maximal effective concentration; NEG = negative; PC₅₀ - the concentration of a test chemical which induces a response which is 50% of the maximal positive control response; POS = positive.

¹Expected responses and BG1Luc ER TA data compiled and reported in 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 [6].

² Mean EC₅₀ values were calculated with values reported by the laboratories of the BG1Luc ER TA validation study (XDS, ECVAM, and Hiyoshi).

³See Annex 2, Table 5 for definitions of positive and negative.

⁴Data extracted from Table 12 of the 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 [7].

⁵PC₅₀ 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 [7]

7. All substances should be tested in a coded/blinded manner. When evaluated using these reference substances, the reliability and accuracy (i.e. sensitivity, specificity, false positive rates, and false negative rates) of the proposed ER TA test method should approximate the following:

- An overall accuracy of 100%
- Sensitivity of 100 %
- Specificity of 100 %

- False positive rate of 0%

- False negative rate of 0%

8. Although it is not realistic to expect test methods to perform identically, discordant results should be discussed in terms of the ability of the test method to detect a similar range of potencies and chemical/product classes.

LITERATURE

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