DRAFT OECD GUIDELINE

FOR THE TESTING OF CHEMICALS

Stably Transfected Transcriptional Activativation (TA) Assay for Detecting Estrogenic Activity of Chemicals

- The Human Estrogen Receptor Alpha Mediated Reporter Gene Assay
Using HeLa-hER-9903 Cell Line -

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INTRODUCTION

1. The OECD endocrine disrupter testing and assessment task force (EDTA) initiated a high-priority activity in 1998 to revise existing guidelines and to develop new guidelines for the screening and testing of potential endocrine disrupters (1)(2). In 2002, EDTA established the OECD conceptual framework for testing and assessment of potential endocrine disrupting chemicals containing five levels, each level corresponding to a different level of biological complexity (3). Transcriptional Activation (TA) assay described in this test guideline has been set in "level 2" in this framework as an *in vitro* assay to provide mechanistic information. The validation work consisted of intra- and inter-laboratory studies for Stably Transfected Transactivation Assay using hER-*HeLa*-9903 cell line to detect estrogenic activity mediated through human estrogen receptor alpha (hERα) has demonstrated the sufficient relevance and reproducibility of the assay with a reference compound (17β-estradiol, natural ligand of estrogen receptors), weak to strong estrogenic compounds, and negative reference compounds (4). This Test Guideline XXX is the outcome of the experiences and observations from the validation studies and the results obtained thereby.

2. The transcriptional activation assay using reporter gene technique is a *in vitro* screening assay that has long been used to evaluate the specific gene expression regulated by specific nuclear receptors, such as ER and androgen receptor (5)(6)(7)(8), and was proposed to detect estrogenic transactivation regulated by ER (3)(9)(10)(11). It is based on the production of reporter gene product induced by a chemical following the ligand-receptor binding followed by a transcriptional activation. Therefore, it can evaluate the ability of a chemical to activate estrogenic responses.

DEFINITIONS

3. Definitions used in this Test Guideline are provided in ANNEX 1.

INITIAL CONSIDERATIONS AND LIMITATIONS

4. Estrogen agonists and antagonists act as ligands for estrogen receptors, and may activate or inhibit the transcription process of estrogen reactive genes. This may have the potential to trigger adverse health hazards by disrupting endocrine systems. The transcriptional activation assay in this test guideline can address one of the key mechanisms of possible health hazards based on the transcriptional activation through human estrogen α (hERα), not including the actions mediated through other nuclear receptors, the entire metabolic activation or deactivation, distribution to target tissues, and clearance from the body. Therefore, it has not been directly extrapolated to the complex *in vivo* estrogenic situation.

5. This test method is specifically designed to detect hER mediated transcriptional activation. However, non-receptor mediated luminescence signals have been reported at concentrations higher than 1 μM of the phytoestrogens showing an over activation of the luciferase reporter gene (12)(13). This information indicates that luciferase expression obtained at high concentrations of phytoestrogens needs to be examined carefully in such stably transfected TA assay systems.

PRINCIPLE OF THE TEST

6. The transcriptional activation assay using reporter gene technique is an *in vitro* tool that allows the identification of promoters and enhancers together with an assessment of the correlations between their activities and conformations by measurement of the reporter proteins that are expressed from reporter genes. The promoters and the enhancers, which are upstream of all protein coding regions on the genome, adjust the activity and enhancement of the expression of the proteins. Because the reporter genes that code useful proteins that become indicators later in the target cells are artificially built downstream of the promoters and enhancers, reporter genes have become a focus of investigations. In the case of firefly luciferase (a gene from the firefly), if a substrate is added to the cells expressing this enzyme, bioluminescence is observed so the expression from the reporter gene is detected visually and can also be measured quantitatively.

7. The test system provided in this test guideline uses the human cervical tumor cells host cell line HeLa cell line with an inserted construct: Human ERα expression vector (full-length) with a firefly luciferase reporter construct bearing five tandem repeats of a vitellogenin estrogen-responsive element (ERE) driven by a mouse metallothionein promoter TATA element. Accordingly, the transcriptional activation assay using this stably transfected cell line, hER-*HeLa*-9903, can provide the hERα mediated transcriptional activation of a test chemical.

PROCEDURE

Regulatory compliance and laboratory verification

8. The assay should be conducted in conformity with the OECD Good Laboratory Practice and Quality Assurance Procedures (14).

9. Prior to the study, the responsiveness of the test system should have been verified using appropriate dose(s) of a reference estrogen: 17βestradiol (CAS No. 50-28-2) (E2) to examine whether a desired dose-response can be observed in the range of acceptable variation. If this is not the case the experimental design should be modified.

Cell lines

10. The hERα-*HeLa*-9903 stable cell line should be used for the assay. The cell line can be obtained from Sumitomo Chemicals Co.

Cell culture and preparation of assay plate

11. Cells should be maintained in Eagle's Minimum Essential Medium (EMEM) without phenol red, supplemented with a 10% dextran-coated-charcoal (DCC)-treated fetal bovine serum (DCC-FBS), in a CO_2 incubator (5% CO_2) at 37˚C. Cells should be suspended with 10%FBS-EMEM and plated into a well of microplate at a density of 1 x 10^4 cells/well. Then the cells should be pre-incubated in a 5% CO_2 incubator at 37˚C for 3-h before the chemical exposure.

Vehicle

12. Dimethylsulfoxide (DMSO) can be used as a vehicle. The other vehicles (e.g. ethanol, water) can also be used after confirmation of no effects on the test results.

Preparation of chemical

13. Chemical should be dissolved in dimethylsulfoxide (DMSO) at a concentration of 10 mM, and this solution should be serially diluted with the same solvent at a common ratio of 1:10 in order to prepare stock solutions with concentrations of 1 mM, 100 µM, 10 µM, 1 µM, 100 nM and 10 nM.

Control substances

14. 17β-estradiol (E2) should be used as the positive control substance. In this case, stock solutions should be prepared at concentrations of 100 µM, 10 µM, 1 µM, 100 nM, 10 nM, 1 nM and 100 pM with DMSO. DMSO should be used as concurrent vehicle control.

Reagents for the luciferase assay

15. A commercial luciferase assay reagent can be used for the assay, e.g. Steady-Glo Luciferase Assay System (Promega, E2510) or a standard luciferase assay system (Promega, E1500). The assay reagents should be selected based on the sensitivity of luminometer to be used in the assay. When using the standard luciferase assay system, Cell Culture Lysis Reagent (Promega, E1531) should be used before adding the substrate.

Chemical exposure

16. Each test chemical diluted in DMSO should be added to the wells to achieve final concentrations of 10 μM, 1 μM, 100 nM, 10 nM, 1 nM, 100 pM, and 10 pM (10^{-11}-10^{-5}M) for testing in triplicate. Test samples and control substances can be assigned according to the assignment table shown below;

Plate dose assignment of test chemicals in the assay plate

	Test Chemical 1			Test Chemical 2			Test Chemical 3			E2		
	1	2	3	4	5	6	7	8	9	10	11	12
A	10 μM	→	→	→	→	→	→	→	→	100 nM	→	→
B	1 μM	→	→	→	→	→	→	→	→	10 nM	→	→
C	100 nM	→	→	→	→	→	→	→	→	1 nM	→	→
D	10 nM	→	→	→	→	→	→	→	→	100 pM	→	→
E	1 nM	→	→	→	→	→	→	→	→	10 pM	→	→
F	100 pM	→	→	→	→	→	→	→	→	1 pM	→	→
G	10 pM	→	→	→	→	→	→	→	→	0.1 pM	→	→
H	VC	→	→		→	→	PC	→	→	→	→	→

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

17. Positive control wells treated with 1 nM of E2 and vehicle control wells treated with DMSO alone should be prepared on every assay plate.

18. After adding the chemicals, the assay plates should be incubated in a CO_2 incubator for 20-24 hours to induce the reporter gene products.

Luciferase assay

19. Luciferase activity should be measured with the luciferase assay reagent and a luminometer according to the manufacturer's instructions. In the case using Steady-Glo Luciferase Assay System (Promega, E2510), add equal volume of the assay substrate to the medium remained in the well, and read the plates on a luminometer at least 5 minutes after the adding of substrate.

Analysis of data

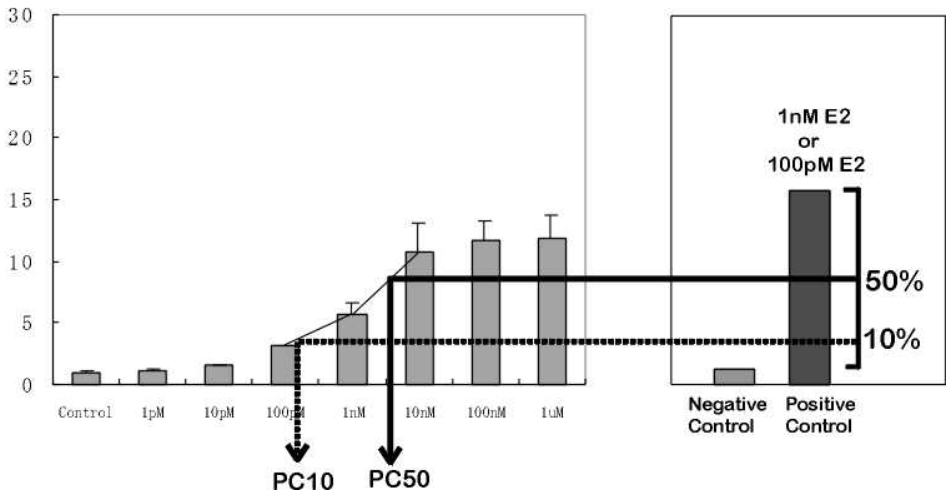
20. The luminescence signal data should be processed and the average for the concurrent vehicle control wells should be calculated. The value for each test well should be divided by the average value of the vehicle control wells to obtain individual relative

transcriptional activity. Then the average transcriptional activity should be calculated for each concentration of the test chemical. The PC50 and PC10 values should be calculated for each test chemical. These PC values are defined as the concentration of chemical estimated to cause 50% or 10%, respectively, of activity of the positive control response. The calculations described above will be made in the common spreadsheet. If Hill's logistic equation is applicable to dose response data, EC50 should be calculated by the following equation:

Y=Bottom + (Top-Bottom)/(1+10^((LogEC50-X)*HillSlope))

*Where X is the logarithm of concentration, Y is the response and Y starts at the Bottom and goes to the Top with a sigmoid shape.

Descriptions of PC values are provided in Figure below;



Description of PC10 and PC50

Considerations for range finding

21. If necessary, a preliminary test can be carried out to determine appropriate concentration range of chemical to be tested in which the chemical causes any cytotoxicity and solubility problems.

Test report

22. The test report must include the following information:

Testing facility:

- Responsible personnel and their study responsibilities.

Test substance:

- identification data and CAS no., if known;
- physical nature and purity;
- physicochemical properties relevant to the conduct of the study;
- stability of the test substance.

Solvent/Vehicle:

- characterization (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 cultures;
- number of cell passages, if applicable;
- methods for maintenance of cell cultures, if applicable.

Test conditions:

- cytotoxicity data and solubility limitations, if available;
- composition of media, CO_2 concentration;
- concentration of test substance;
- volume of vehicle and test substance added;
- incubation temperature;
- duration of treatment;
- cell density during treatment;

- positive and negative controls;
- length of expression period;
- Luciferase assay reagents (Product name, supplier and lot.);
- criteria for considering tests as positive, negative or equivocal.

Results:

- Raw data of luminescent signals;
- dose-response relationship, where possible;
- statistical analyses, if any.

Discussion of the results

Conclusion

GUIDANCE FOR THE INTERPRETATION AND ACCEPTANCE OF THE RESULTS

23. In general, when PC50 can be calculated, a test chemical is considered as positive in hERα mediated transcriptional activation. In this case, dose responsiveness should be considered.

ANNEX 1

DEFINITIONS

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

Dose is the concentration of test substance applied. For the Transcriptional Activation assay, "mol" unit is used as the concentration in the reaction medium.

Estrogenic activity is the capability of a chemical to act like estradiol 17β-estradiol mediated though estrogen receptors.

Validation is a scientific process designed to characterize the operational requirements and limitations of a test method and to demonstrate its reliability and relevance for a particular purpose according to the OECD Guidance Document No.34 (i).

(i) OECD (2005) OECD Environment, Health and Safety Publications Series on Testing and Assessment No.34. Guidance document on the validation and international acceptance or new or updated test methods for hazard assessment.

204 **ANNEX 2**

205 OECD EDTA Conceptual Framework

206 [Available on http://www.oecd.org/dataoecd/17/33/23652447.doc]

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209 **LITERATURE**

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