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 -

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0. ACRONYMS

AR	Androgen Receptor
BPA	Bisphenol A
CERI	Chemicals Evaluation and Research Institute (Japan)
CV	Coefficient of Variation
DCC-FBS	Dextran-Coated Charcoal-treated Fetal Bovine Serum
DIP	Data Interpretation Procedure
DMSO	Dimethylsulfoxide
E2	17β-Estradiol
EC	European Commission
EC50	The molar concentration of a compound which produces 50% of the maximum possible response for that compound
ECVAM	European Centre for the Validation of Alternative Methods
EDCs	Endocrine Disrupting Chemicals
EDTA	(OECD) Task Force on Endocrine Disruptor Testing and Assessment
EMEM	Eagle's Minimum Essential Medium
ER	Estrogen Receptor
ERE	Estrogen Responsive Element
EU	European Union
GD 34	OECD Guidance Document 34 "Guidance document on the validation and international acceptance of new or updated test methods for hazard assessment"
GLP	Good Laboratory Practice
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods (U.S.)
JaCVAM	Japanese Centre for the Validation of Alternative Methods

NICEATM	National Toxicology Program (NTP) Interagency Centre for the Evaluation of Alternative Toxicological Methods (U.S.)
NIEHS	National Institute of Environment and Health Sciences (U.S.)
NIHS	National Institute of Health Sciences (Japan)
NP	Nonylphenol
OECD	Organisation for Economic Co-operation and Development
PC50/PC10	The concentration of chemical estimated to cause 50% or 10%, respectively, of activity of the positive control response on a plate by plate basis.
PM	Prediction Model
PVAP	The Preliminary Validation Assessment Panel of the 'Japanese multi-laboratories validation study of a stably transfected ER alpha mediated reporter gene assay in Japan'
QA	Quality Assurance
SD	Standard Deviation
SE	Standard Error
SOP	Standard Operating Procedure
SPSF	Standard Project Submission Form
TA	Transcriptional Activation
TS	Testosterone
US EPA	United Sates Environmental Protection Agency
VMG	Validation Management Group
VMG-NA	Validation Management Group for Non –Animal Testing
WNT	(OECD) Working Group of the National Coordinators for the Test Guidelines Programme

1. SUMMARY ASSESSMENT

1 Numerous chemicals found in the environment, as well as some synthetic chemicals may disrupt the endocrine functions of wildlife and humans. At the present time, there is global concern regarding endocrine disruption effects resulting from chemical exposure, particularly those mediated by the estrogen receptor (ER).

2 Some *in vitro* assays, such as the transcriptional activation (TA) assays and receptor binding assays, have been proposed and incorporated into the “OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals” as “Level 2” *in vitro* assays to provide mechanistic information for prioritization purposes.

3 Several *in vitro* TA and receptor binding assay methods are currently at, or will soon begin validation at national, European and international levels, but are not yet close to completion and full assessment of their validation status. Although the need is urgent, at the present time there are no *in vitro* screening assays for estrogenic activity that have been peer reviewed for potential test guideline development, to enable use for OECD regulatory purposes.

4 Recognizing this urgency, Japan has made an extensive effort to establish and domestically validate a new *in vitro* pre-screening procedure, the **Stably Transfected Transcriptional Activation (TA) Assay** using the hER-HeLa-9903 cell line for detecting the estrogenic activity of chemicals mediated by the human estrogen receptor α (hERα) for a level 2 screening test in the OECD Conceptual Framework for the Testing and Assessment of endocrine disrupting chemicals (EDCs).

5 Under the agreement of the 1st OECD validation management group for non-animal testing (VMG-NA) meeting that Japan would take lead in this assay, validation work on the hERα mediated stably transfected TA assay conducted in Japan consisted of both pre-validation and inter-laboratory validation. The pre-validation work was conducted in the Chemicals Evaluation and Research Institute (CERI), Japan and the inter-laboratory validation study was conducted within four Japanese domestic laboratories upon the initiative of CERI.

6 The overall goal of the validation efforts for the stably transfected TA assay using hER-HeLa-9903 cell line as reported herein is to develop and validate a test method and protocol that will support the development of test guidelines for the detection of chemicals potentially possessing estrogenic activity through hERα.

7 In the pre-validation study, the mean Log_{10}[EC50 (M)] for 17β estradiol (E2), the positive reference chemical, in 13 runs showed acceptable and normal variation observed for such assays.

8 As for the results of the inter-laboratory validation study, statistical analysis using nine coded test chemicals revealed that the reproducibility within four participating laboratories of this assay system appeared to have acceptably low between-lab variation. The results showed that the test system is highly reliable and that the test protocol used in this study is adequately transferable for practical use.

9 Log_{10}[EC50 (M)] obtained with the proposed assay system showed high consistency with the data obtained by the ER-CALUX, HELN-ERα and LUMI-CELL™ assay systems at. R^2=0.987 (n=8), R^2=0.937 (n=7) and R^2=0.922 (n=7), respectively. Moreover, Log_{10}[EC50 (M)] values obtained in the proposed stably transfected TA assay for several known estrogenic positive chemicals are consistent and correlate well with those listed in ICCVAM report (2003) R^2=0.802 (n=20).

10 The results obtained by the stably transfected TA assay and the information given in the ICCVAM report (2003) were compared with regard to 46 chemicals. The collected information listed in the ICCVAM report is based on several different *in vitro* assay systems to detect estrogenic activities, and the assay performance parameters for the stably transfected TA assay, concordance, sensitivity and specificity, were 80%, 79% and 82%, respectively.

11 So as to provide supplemental information, the results obtained from the receptor binding assay using hERα and the stably transfected TA assay were compared with regard to 48 chemicals. The concordance, sensitivity and specificity, were 77%, 71% and 83%, respectively.

12 Furthermore, as a part of supplemental information, the results obtained by the uterotrophic assay and the stably transfected TA assay were also compared with regard to 48 chemicals, and the concordance, sensitivity and specificity, were 90%, 91% and 88%, respectively.

13 The comparative results between the endpoints of the stably transfected ER TA assay and data provided in the ICCVAM report (2003), ER binding assays and the immature rat uterotrophic assay indicate high concordance and therefore suggest that the outcomes of

the stably transfected ER TA assay can provide reliable information about the biological effect of chemicals mediated by ER-ligand interactions.

14 Accordingly, the overall assay performance of the stably transfected TA assay system using hER-HeLa-9903 was deemed satisfactory for practical use, and in accordance with OECD Guidance Document No.34 (GD 34).

15 A Japanese human ERα mediated stably transfected TA assay system using hER-HeLa-9903 cell line is well-established and has been shown to be a well-validated assay for the development of an OECD test guideline for the detection of chemicals possessing potential estrogenic activity mediated through hERα. The assay is a therefore a promising method to use in the prescreening process of an endocrine disruptor screening strategy.

2. INTRODUCTION

16 A number of chemicals found in the environment, as well as some synthetic chemicals, may disrupt the endocrine functions of wildlife and humans. At the present time, there is global concern regarding endocrine disruption effects resulting from chemical exposure, particularly those mediated by the ER. To ensure chemical safety, an effective screening method for chemicals to detect endocrine modulating potencies has been sought by regulatory agencies in several countries, including the United States Environment Protection Agency (US-EPA), Japan and Europe (EDSTAC, 1998; OECD, 2001, ECB, 2006). The US-EPA developed a chemical screening and testing program consisting of a tiered system to evaluate the endocrine disrupting effects of chemicals (Earl-Gray L. Jr., 1998). In this program, the hormone receptor mediated reporter gene assay system is proposed for pre-screening and the Tier 1 screening battery.

17 The endocrine disrupter testing and assessment task force (EDTA) was established in 1997 as a special activity under the OECD test guideline program: (1) to investigate regulatory requirements and needs in member countries for endocrine disrupting chemicals (EDCs); (2) to try to develop harmonized assessment practices in member countries for EDCs; and (3) to develop test guidelines for EDCs. Under the EDTA's supervision, the validation management groups for mammalian (VMG-mammalian) and for ecotoxicity (VMG-eco) tests were established in 1999 and 2001, respectively.

18 The 6th EDTA meeting held in Tokyo in 2002 confirmed the urgent need for cost-efficient

and quick screening test methods not requiring animals, and therefore agreed to establish the validation management groups for non-animal testing (VMG-NA). The OECD conceptual framework for testing and assessment of potential endocrine disrupting chemicals from both new and existing substances, including such different chemical sectors as pharmaceuticals, industrial chemicals and pesticides, was also agreed upon at this meeting (OECD, 2002). This framework is not a testing scheme but rather a toolbox that contains various tests, each of which can contribute information about detecting the hazards of endocrine disruption. Within this toolbox framework, there are five levels, each level corresponding to a different level of biological complexity.

19 Some *in vitro* assays, such as the transcriptional activation (TA) and receptor binding assays, have been proposed and incorporated into the "OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals" as "Level 2" *in vitro* assays to provide mechanistic information for prioritization purposes.

20 A main mechanism of action of hormones is via binding with their specific receptors after secretion from endocrine glands. Hormone receptors are distributed in the cell-membrane or inner-nucleus. The action of hydrophilic ligands, such as growth hormone and insulin, are known to be mediated through membrane receptors, and the hydrophobic ligands, such as steroid and thyroid hormones, act through nuclear receptors after penetration into the nucleus.

21 Nuclear receptors, such as steroid hormone receptors and thyroid hormone receptors, are known to be one of the main effector sites of endocrine disruptors, and the signal transduction through these nuclear receptors would be a starting point for the harmful effects of endocrine disruptors. The estrogen receptor is well characterized and well known as a major mediator of estrogenic effects. Estrogenic effects may be observed at very low concentrations; therefore a highly sensitive assay method is necessary for hazard assessment.

22 Nuclear receptors play important roles in the regulation of target gene expression. In this regard, the reporter gene assay technique that has long been used to evaluate specific gene expression would be applicable for evaluation of the hormonal activities of chemicals.

23 Generally, transcription regulatory sequences are located either upstream or downstream of the structural gene. Expression of the hormone responsive gene is regulated through the binding of receptors with their ligands; the hormonal activity will be presented by transcriptional activation induced by the binding of receptor-ligand complex to the

cis-region of the target gene.

24 In reporter gene assays, a reporter gene, which is not expressed in host cells such as a firefly luciferase gene or a β-galactosidase gene, is used to visualize the gene expression induced by receptor-ligand interaction.

25 Thus, the reporter gene assay technique may be suitable for detecting the hormonal activities of chemicals because this technique has long been used to detect the enhancers and promoter activity of genes. The reporter gene assay system may also provide a powerful tool for screening endocrine disrupting chemicals (Takeyoshi et al., 2002; Yamasaki et al., 2002).

26 Several *in vitro* TA and ER binding assay methods are currently or will soon begin validation at national, European and international levels, but are not yet close to completion and full assessment of their validation status. Although the need is urgent, at the present time there are no *in vitro* screening assays for estrogenic activity that have been peer reviewed for potential test guideline development, to enable use for OECD regulatory purposes.

27 Recognizing this urgency, Japan has made an extensive effort to establish and domestically validate a new *in vitro* pre-screening procedure, the **Stably Transfected Transcriptional Activation (TA) Assay** using hER-HeLa-9903 cell line for detecting the estrogenic activity of chemicals for a level 2 screening test in the OECD Conceptual Framework for the Testing and Assessment of EDCs under the agreement of the 1st OECD VMG-NA meeting that Japan would take lead in this assay.

28 Japan endorses the OECD Guidance Document 34 (GD 34), and this validation report therefore adheres to the internationally agreed OECD guidance on validation and international acceptance of new or updated test methods for hazard assessment.

3. OBJECTIVES

29 The overall goal of the validation efforts for the stably transfected TA assay using hER-HeLa-9903 cell line as reported herein is to develop and validate a test method and protocol that will support the development of test guidelines for the detection of chemicals potentially possessing estrogenic activity through hERα.

30 The data obtained from TA assays are typically analyzed to derive the EC50 value as a biological parameter. This parameter (EC50) is calculated by applying an appropriate model equation, such as a logistic equation. For the use of such model equations to calculate the EC50 value, the full-dose response curve is required. However, the full-dose response curve cannot always be obtained, due to the solubility of a test chemical in the assay media or the cytotoxicity of a test chemical. In such cases, the quantitative evaluation of the test chemical through use of the EC50 is not possible. The quantitative explanation is important for providing information about the strength of the potential activity of a test chemical. Therefore, such new reliable and relevant parameters other than EC50 were also investigated within this validation work.

31 This study report will provide information on: (1) reliability; (2) relevance; (3) transferability of a protocol; (4) identification of the acceptable variations of protocols; (5) limitations of the test method; and (6) possible reliable and relevant parameters other than the EC50.

4. VALIDATION DESIGN

32 The work of validating the stably transfected TA assay using hER-HeLa-9903 cell line to detect estrogenic activity consisted of both pre-validation and inter-laboratory validations. The pre-validation work was conducted at the Chemicals Evaluation and Research Institute (CERI), Japan, and the domestic inter-laboratory validation study was conducted by four Japanese laboratories, including CERI, on the initiative of CERI. All the processes of the validation work were financially supported by the Ministry of Economy Trade and Industry (METI) and the Ministry of Health Labor and Welfare (MHLW), Japan.

33 The overall validation design is shown in Fig. 1. This approach is also presented in Fig. 2, which shows how the assessment process of the relevance and reliability of a test method can be undertaken in a stepwise, yet flexible, manner while still providing the information necessary to address the 1996 Solna criteria and principles for validation.

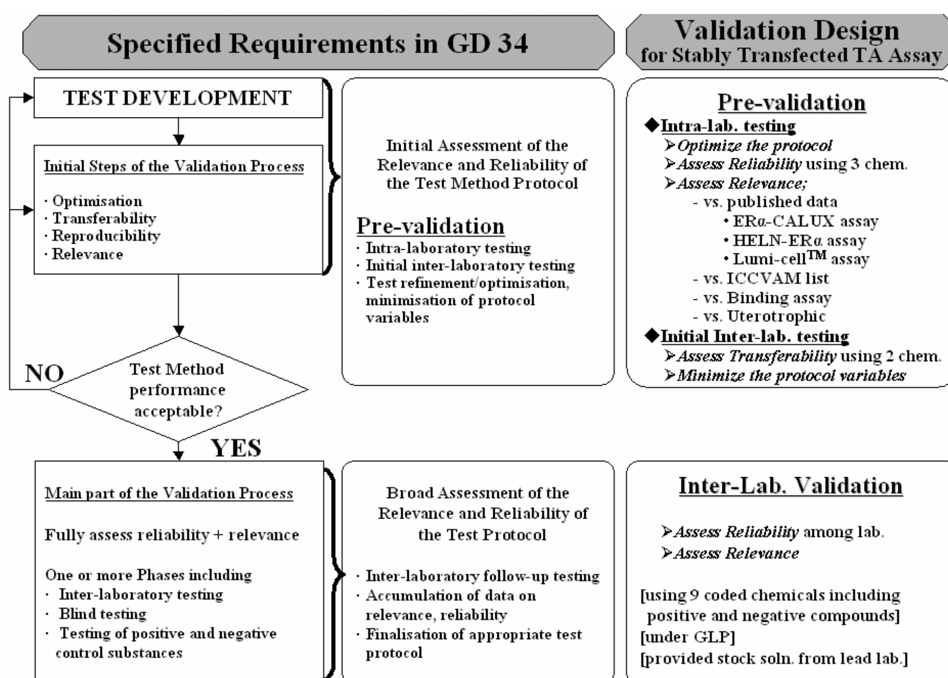

Fig. 1 Validation Design Scheme According to GD 34 Specified Requirements

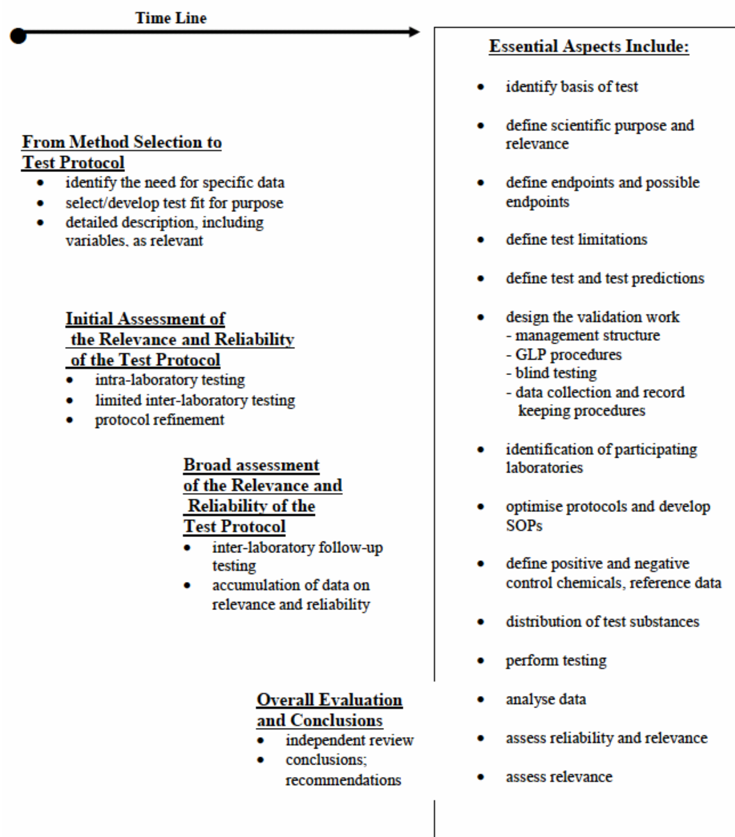

Fig. 2 Assessment Process of the Relevance and Reliability of New or Significantly Revised Testing Methods for Hazard Characterization Specified in GD 34

34 The pre-validation study of stably transfected TA assays using hER-HeLa-9903 cell line conducted by CERI was designed to identify both the reliability and relevance of the testing system. In order to demonstrate the relevance, the test results obtained were compared to the published data that used other stably transfected cell lines (ERα-CALUX, HELN-ERα, LUMI-CELL^{TM}) to detect estrogenic activity (Sonneveld et al; 2006; Escade et al, 2006; Jefferson et al, 2002). Also, further comparisons were made with the results available in the ICCVAM list of Recommended Substances for Validation of *In Vitro* Estrogen Receptor Transcriptional Activation Assays (ICCVAM, 2003). Furthermore, to provide supportive information demonstrating the ability to detect estrogenic activity, the results obtained from the relevant assays (receptor binding assay using hERα and uterotorophic assay) were compared to those obtained using the proposed assay system.

35 The inter-laboratory validation study was planned by CERI and conducted at CERI's initiative with three other participating laboratories (APPENDIX 1). Before starting the inter-laboratory study, the laboratory, the assay skills, and implementation structures of each laboratory were assessed by laboratory inspections and audits conducted on an independent basis by the CERI supervised study director and Quality Assurance (QA) manager, under the standard GLP organizational structure as shown in Fig. 3.

36 Although the pre-validation study was conducted without GLP compliance, the inter-laboratory validation study was conducted with GLP compliance and managed by CERI's QA audit system.

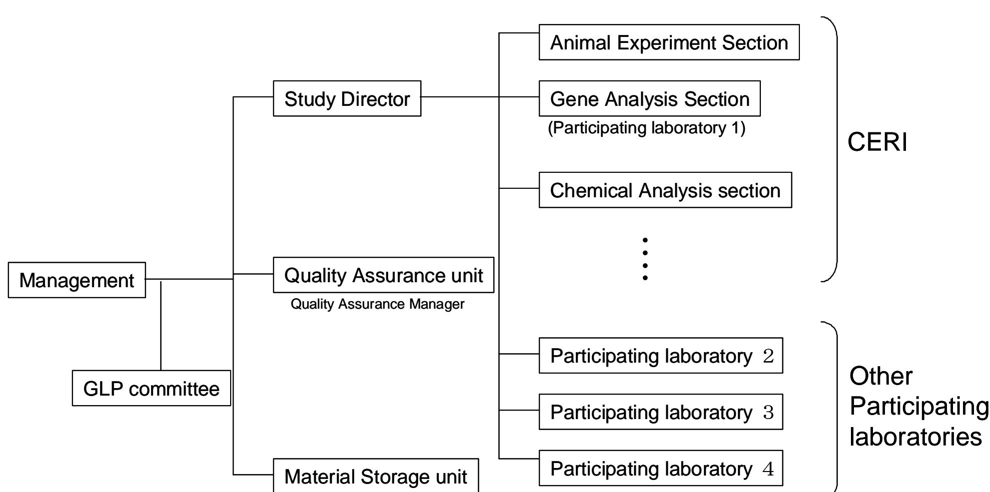

Fig. 3 Organization Schema of CERI GLP System Employed for the Inter-laboratory Validation Study.

37 The inter-laboratory validation study of the stably transfected TA assay using hER-HeLa-9903 cell line was designed to:

- Assess the intra- and inter-laboratory variability and reproducibility of the protocol among the investigated endpoints;
- Assess the relevance of the proposed test method to detect a range of estrogenic activity;
- Identify acceptable variations of the test protocol;
- Identify limitations of the test method; and
- Provide possible reliable and relevant parameters other than the EC50.

38 In order to assess both the reliability of the assay protocol and the protocol transferability, the inter-laboratory validation study consisted of assays repeated three times using nine coded test chemicals with or without estrogenic activity and one reference chemical (17β-Estradiol, E2), in each laboratory. Assay data were gathered in CERI and were analyzed with regard to reproducibility of the analytical parameters calculated as EC50, PC50 and PC10. These PC50 and PC10 values are defined as the concentration of chemical estimated to cause 50% or 10%, respectively, of an activity in the positive control response. The details of PC50 and PC10 are described in the section entitled "Data Recording and Analyses (p.23)".

5. TEST METHOD USED

5.1 Test protocol

39 The standard operating procedure (SOP) used for the pre-validation study and the protocol used for inter-laboratory validation study are attached in APPENDIX 2 and APPENDIX 3, respectively. The support protocols for the preparation of mediums, reconstitution of frozen stock cells, propagation, preparation of frozen stock, preparation of assay plates and chemiluminescence detection are included in the Appendices. The assay methodology used in the pre-validation study was substantially identical to that of the inter-laboratory validation study. The summary of the protocols is shown in Table 1.

Table 1 Summary of the protocol

Factors	
Cell line	hERα-HeLa-9903 stable cell line
Cell medium	Eagle's Minimum Essential Medium (EMEM) without phenol red with 10% dextran-coated charcoal-treated fetal bovine serum (DCC-FBS)
Vehicle	Dimethylsulfoxide (DMSO)
Vehicle control	0.1% of DMSO as final concentration (six-wells for pre-validations and three-wells for inter-laboratory validations)
Final concentration of vehicle	0.1%
Positive control for calculation of PC10 or PC50 values *	100 pM of 17β-Estradiol (E2) in six-wells for pre-validations 1 nM of E2 in three-wells for inter-laboratory validations
Positive control for dose response curve	17β-Estradiol (E2); 0.1 pM, 1 pM, 10 pM, 100 pM, 1 nM, 10 nM and 100 nM
Number of test chemicals within pre-validation	22 chemicals for preliminary assessment. 48 chemicals for comparison with receptor binding assays* 48 chemicals for comparison with uterotrophic assays* 46 chemicals for comparison with data listed as ICCVAM reference chemicals *: Not the same set of chemicals
Number of test chemicals within inter-laboratory validations	9 coded test chemicals and one positive reference chemical (E2)
Number of assays per chemical	Three-runs of each chemical (in triplicate) on separate days for the inter-laboratory validation study
Concentrations tested	Test Chemicals: 10 pM, 100 pM, 1 nM, 10 nM, 100 nM, 1 μM and 10 μM
Cell density	10^4 cells/well
Incubation time with test chemicals	20-24 hours

40 The original protocol used for inter-laboratory validation was designed for use with a Glo-type luciferase assay reagent (Steady Glo luciferase reagent, Promega). However, to avoid the variation of results originating from the sensitivity of the luminometer used for the assay, the original protocol was amended to allow use of two types of assay reagents, both Glo-type and Flash-type reagents. The combined use of a luciferase reagent and a luminometer at each participating laboratory is tabulated in Table 2.

Table 2 The Combined Use of a Luciferase Reagent and a Luminometer at Each Participating Laboratory in Inter-laboratory Validation Study

Name of Participating Lab.	Luminometer	Type of Luciferase Reagent
CERI	Lumister [BMG]	Flash [Promega] (Promega, E1500)
Sumitomo	Top-count [Packard]	Glo [Promega] (Promega, E2510)
Otsuka Pharm.	ARVO [PerkinElmer]	Flash [Promega] (Promega, E1500)
Kaneka	Top-count [Packard]	Glo [Promega] (Promega, E2510)

5.1.1 Cell line (stable clone: hERα-carrying HeLa cells)

41 hER-HeLa-9903 cell line is an estrogen responsive stable transformant derived from a HeLa cell line. This cell line was established by Sumitomo Chemical Co. as follows: human cervical tumor cells (HeLa; ATCC No. CCL-2) purchased from ATCC through Dai-Nippon Pharmaceutical Company (Osaka, Japan) were stably transfected with both plasmids human ERα expression vector and a firefly luciferase reporter vector bearing five tandem repeats of estrogen-responsive element (ERE) driven by a mouse metallothionein promoter TATA element. A vitellogenin ERE was selected because of its high reactivity to estrogen in the preliminary experiments.

Enhancer (ERE)	5'-TCGACAAAGTCAGGTCACAGTGACCTGATCAAG-3'
Promoter	5'-GATCTCGACTATAAAGAGGGCAGGCTGTCCTCTAAGCGTCACCACGACTTCA-3'

42 The human ERα expression vector was generated by insertion of a RT-PCR amplified full-length of human ERα cDNA (Genbank Accession No. 47621M), with an efficient Kozak's translation initiator sequence, from a commercial human ovary mRNA (Clontech, Palo Alto, CA), into the blunting site of a pRc/RSV vector (Invitrogen, San Diego, CA).

43 Functional ERα, ERβ, AR, TRα and TRβ could not be detected in the host cell (HeLa; ATCC No. CCL-2), when tested by mock transfection assays with each hormone responsive reporter construct. Further, the established cell line, hER-HeLa-9903, was confirmed to be free of any mycoplasma infection.

44 The hER-HeLa-9903 cell line was obtained from Sumitomo Chemical Co., Ltd. and then distributed to each participating laboratory.

45 This cell line is available from the Owner, Sumitomo Chemical Co., Ltd. under the independent contract.

5.1.2 Medium (support protocols Nos. 1-4, APPENDIX 2 and APPENDIX 3)

46 Eagle's Minimum Essential Medium without phenol red (EMEM, Nissui Pharmaceutical Co.), supplemented with a 10% dextran-coated-charcoal (DCC)-treated fetal bovine serum (DCC-FBS) was used for both the propagation and the assay. This DCC-FBS was prepared at CERI, and was subsequently provided to each participating laboratory for the inter-laboratory validation study.

5.1.3 Chemical exposure to cells

5.1.3-.1) For pre-validations (support protocol No. 5, APPENDIX 2)

47 Each test chemical used for this pre-validation study was dissolved in dimethysulfoxide (DMSO) at 10 mM as a stock solution. The final concentration of DMSO in the assay medium was 0.1%, which did not affect the cells.

48 The ranges of test concentrations of the test chemical were 10 µM, 1 µM, 100 nM, 10 nM, 1 nM, 100 pM, and 10 pM (10^{-11}-10^{-5}M). The ranges of test concentrations of 1 µM, 100 nM, 10 nM, 1 nM, 100 pM, 10 pM and 1 pM (10^{-12}-10^{-6}M) were only used for the initial test to demonstrate the stability of the test system.

49 In order to prepare the desired concentrations of test chemicals, the 10 mM of stock solution was first serially diluted in common ratios of 10 with DMSO to obtain 1 mM, 100 µM, 10 µM, 10 µM, 1 µM, 100 nM and 10 nM. Then, these diluted samples in DMSO were further diluted with serum-free EMEM to prepare three-fold concentrations of the desired test concentrations (1.5 µL of DMSO soln. in 500 µL of serum-free EMEM). Lastly, the desired test concentrations in quadruplicate were prepared by adding 50 µL of each sample solution to each well of the assay plates, containing $1x10^4$ cells/well/100 µL as illustrated in the assignment table (Table 3).

50 Positive control wells (n=6) treated with a natural ligand (100 pM of E2) and vehicle control wells (n=6) treated with DMSO (0.1%) alone were prepared on every assay plate.

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

52 Cytotoxicity evaluation was conducted by examining baseline induction. If a substance

induced decreased luciferase activity below baseline, the substance was considered to be cytotoxic.

Table 3 Plate Dose Assignment Table: Pre-validation Study

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

VC: Vehicle control (DMSO at 0.1%); PC: Positive control (100 pM of E2)

5.1.3-.2) For inter-laboratory validations (support protocol No. 5, APPENDIX 3)

53 The stock solutions of test chemicals were prepared at CERI, where they were coded and then provided to each participating laboratory.

54 The 10 mM of stock solutions at each participating laboratory were serially diluted in common ratios of 10 with DMSO to obtain 1 mM, 100 µM, 10 µM, 10 µM, 1 µM and 100 nM. Further diluted chemical solutions with serum-free EMEM were prepared to obtain final concentrations of 10 µM, 1 µM, 100 nM, 10 nM, 1 nM, 100 pM, and 10 pM (10^{-11}-10^{-5}M) for the test chemicals in the assay plate in triplicate in the same manner as shown for the pre-validation.

55 On the basis of sensitivity of the assay system, the concentration range to be tested was set at 10^{-11}-10^{-5}M. The assay system can detect estrogenic activity of well-known weak estrogenic chemicals in this concentration range, such as bisphenol A (BPA) and nonylphenol. This fixed-concentration strategy could allow the assay to achieve high-throughput assay performance as a screening test method for providing mechanistic information, which would be placed at level 2 in the OECD conceptual framework.

56 A full dose response range of E2 was assigned in all assay plates to monitor the accuracy of chemical dilution procedure in the inter-laboratory study.

57 In the inter-laboratory validation study, an analysis of each triplicate, for each concentration of a test chemical, was employed to achieve the high-throughput assay

format.

58 Positive control wells (n=3) treated with a natural ligand (1 nM of E2) and vehicle control wells (n=3) treated with DMSO alone, were prepared on every assay plate.

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

60 The test chemicals and the vehicle and positive control substances were all assigned to the assay wells in accordance with the assignment table for inter-laboratory validation study (Table 4).

61 In some assay systems using microtiter plates, the consideration of an edge effect would be necessary before starting assays because of differences between wells located on the edge and the center of the assay plate, with regard to the evaporative loss of medium and efficacy of gas exchange, etc. In cases that such edge effects would be expected, 36 wells on the edge of a 96-well plate should not be used for the assay. However, following an independent assessment, it was confirmed that the assay system using hER-HeLa-9903 cell line did not show any edge effects that would affect the assay results for practical use (APPENDIX 4).

Table 4 Plate Dose Assignment Table: Inter-laboratory Validation Study

	Chemical 1			Chemical 2			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	→	→	BL	→	→	→	→	→	PC	→	→

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

5.1.4 Reagent for stably transfected TA assays and detection instrument (support protocol No. 5, APPENDIX 3)

62 A standard luciferase assay system (Promega, E1500) was used in the pre-validation study conducted at CERI. One of two types of commercial luciferase assay reagents, the standard luciferase assay system (Promega, E1500) or Steady-Glo Luciferase Assay

System (Promega, E2520), was used for measuring luciferase activity in each laboratory as the preliminary test before the inter-laboratory validation study.

63 The type of luciferase assay reagent used for each inter-laboratory validation study was dependent upon the sensitivity of the luminometer used at that particular participating laboratory. Selection of the assay reagent was determined by the results of a preliminary test that confirmed the assay conditions at each participating laboratory.

5.1.5 Test chemical

5.1.5-.1) Dose selection

64 The test concentration range employed in this assay was determined based upon the sensitivity of the assay, whilst also ensuring that there were no problems with solubility and cytotoxicity.

65 As described above, on the basis of sensitivity of the assay system, the concentration range at 10^{-11}-10^{-5}M can detect estrogenic activity of well-known weak estrogenic chemicals in this concentration range, such as bisphenol A (BPA) and nonylphenol (NP), allowing the high-throughput assay performance as a screening test method for providing mechanistic information.

5.1.5-.2) Selection of test chemicals

For pre-validation

66 To examine the stability of the assay system as a part of the pre-validation study, three chemicals, an endogenous estrogen (17β-estradiol; E2), a weak estrogen, bisphenol A (BPA) and negative substance in the range 10^{-11}M-10^{-6}M, testosterone (TS), were tested a total of 13 times in repeated tests.

67 To demonstrate the performance of the assay system in detecting estrogenic activity, 22 chemicals (Table 5) were selected from a chemical list that provided median EC50 values as determined by using different assay systems, such as the mammalian reporter gene assay, the mammalian cell-proliferation assay, or the yeast reporter gene assay in the ICCVAM report (ICCVAM, 2003). Some chemicals in this list were excluded on the basis of unavailability, or due to regulatory restrictions, such as the substances under emission control by Japanese Law concerning the Evaluation of Chemical Substances and Regulation of their Manufacture, etc. (Law No. 117, 1973, as last amended by Law No.49,

2003).

Table 5 Chemicals Used for the Quantitative Comparison (selected from ICCVAM list (ICCVAM, 2003))

Chemical	Cas No.	Manufacturer	Lot. No	Purity
Ethynyl Estradiol	57-63-6	Wako	KSN3933	98%
Diethylstilbestrol	56-53-1	Wako	7488C	N.S.
17α-Estradiol	57-91-0	Wako	ELJ1532	97%
17β-Estradiol	50-28-2	Wako	ACL1188	>97%
Estrone	53-16-7	Wako	TPN4558	98%
Zearalenone	17924-92-4	Sigma	50K4014	99.7%
17α-Methyltestosterone	58-18-4	Wako	TPE6748	97%
β-Zearalenol	71030-11-0	Sigma	40K4092	>90%
Coumestrol	479-13-0	Fluka	400248	95%
Estriol	50-27-1	Wako	DLM3617	98%
4-tert-Octylphenol	140-66-9	Wako	09802JQ	99%
Genistein	446-72-0	Wako	NNP1712	98%
4-Nonylphenol	84852-15-3	TCI	FGE01	98%
Testosterone,19-Nor	434-22-0	Sigma	108H0648	>99%
Daidzein	486-66-8	Wako	HC-1408	97%
Phloretin	60-82-2	Sigma	99H7000	99.6%
Levonorogestrel	797-63-7	Sigma	30K0711	99%
Bisphenol A	80-05-7	TCI	GF01	>99%
Naringenin	480-41-1	Aldrich	14722PG	N.S.
Methoxychlor	72-43-5	Wako	YWL9207	>97%
Progesterone	57-83-0	Sigma	98H0893	99%
Atrazine	1912-24-9	Wako	MSF9593	>98%

Aldrich : Aldrich Chemical Co., Inc. (Sigma-Aldrich corp.)
Fluka : Fluka Chemie AG (Sigma-Aldrich corp.)
Sigma : Sigma Chemical Co. (Sigma-Aldrich corp.)
TCI : Tokyo Kasei Kogyo Co., Ltd.
Wako : Wako Pure Chemical Industries, Ltd.
N.S. : not specified

68 In order to evaluate the relevance and to provide the mechanism of action by the proposed stably transfected TA assay, 46 chemicals selected from the ICCVAM list, which provides both positive and negative estrogenic information (ICCVAM, 2003), were tested (Table 6).

69 The results obtained by applying the same protocols as the pre-validation study were compared as supplemental information to the results obtained from a receptor binding assay using recombinant hERα, and the uterotrophic assay. The 48 chemicals in Table 7 used for this comparison with the receptor binding were selected from the US EPA's core chemical list, proposed at the March 2002 Endocrine Disruptor Methods Validation Subcommittee meeting (EDMVS, 2002). The 48 chemicals for which uterotrophic assay data had already been tested were used for this comparison (Table 8). It should be noted that the range of chemicals used for the comparison with the binding assay and immature rat uterotrophic assay were not identical but differed according to data availability.

70 The receptor binding assay was performed as follows: a solution (10 μL, final conc. 0.2 nM) of approximately 10 nM of recombinant human estrogen receptor ligand binding domain fused with glutathione S-transferase, expressed in E. coli, was dissolved in Tris-HCl (pH 7.4, 70 μL) containing 1 mM EDTA, 1 mM EGTA, 1 mM NaVO3, 10% glycerol, 10 mg/ml γ-globulin, 0.5 mM phenylmethylsulfonyl fluoride, and 0.2 mM leupeptin. After adding the sample solution (10 μL) of each chemical and 5 nM [2,4,6,7,16,17-3H] of 17β-estradiol (10 μL), the solution was incubated for 1 h at 25°C. Free radioligand was removed by incubation with 0.2% activated charcoal and 0.02% dextran in PBS (pH 7.4) for 10 min at 4°C followed by filtration. Chemicals were tested in the concentration range of 10^{-11}-10^{-4}M. The data were fitted to Hill's equation by using the GraphPad Prism computer program, and IC50 values were calculated. Then relative binding affinity (RBA) to the 17β-estradiol was calculated. Any chemicals possessing RBA values were defined as positive chemicals in the receptor binding assays.

71 For the immature rat uterotrophic assays, chemicals were dissolved in olive oil and injected subcutaneously into the back of immature (19-day-old) female rats; each group consisted of six rats that were injected once a day for three consecutive days. A vehicle control group was injected solely with olive oil, and a positive control group was injected with ethynyl estradiol (EE). The dose levels were determined based on the results of a preliminary range finding study. The dosing volume was 2 mL/kg of body weight. Animals were sacrificed by exsanguinations under deep ether anesthesia approximately 24 hours after the final dosing, and their uteri were carefully dissected, free of adhering fat and mesentery, and weighed. The blotted weight changes in the uterus from the test group after giving chemicals to immature female rats for three days were compared with those of the vehicle control group. When there was a statistically significant difference from the control group determined by the two-tailed Student's t test, the change in the uterus was judged positive.

Table 6 Selected Chemicals Used to Examine the Concordance Between the Stably Transfected TA Assay and the Data Reported in the ICCVAM Report (2003)

Chemical	CAS No.	Manufacturer	Lot. No.	Purity
17α-Ethinyl estradiol	57-63-6	Wako	KSN3933	98%
Diethylstilbestrol	56-53-1	Wako	7488C	N.S.
17α-Estradiol	57-91-0	Wako	ELJ1532	97%
17β-Estradiol	50-28-2	Wako	ACL1188	>97%
Zearalenone	17924-92-4	Sigma	50K4014	N.S.
Estrone	53-16-7	Wako	TPN4558	98%
Methyl testosterone	58-18-4	Wako	TPE6748	97%
Coumestrol	479-13-0	Fluka	400248	<95%
Genistein	446-72-0	Wako	NNP1712	98%
p- n-Nonylphenol	104-40-5	Wako		N.S.
Bisphenol B	77-40-7	TCI	FIC01	N.S.
Daidzein	486-66-8	Wako	HC-1408	97%
4-Cumylphenol	599-64-4	Wako	PAK1144	98%
Bisphenol A	80-05-7	TCI	GF01	>99%
p,p' -Methoxychlor	72-43-5	Wako	YWL9207	>97%
Apigenin	520-36-5	Aldrich	00902BU	N.S.
Tamoxifen	10540-29-1	ICN	4636C	N.S.
Kepone (Chlordecone)	143-50-0	Wako		99%
Butylbenzyl phthalate	85-68-7	Wako	KSJ8408	99%
Kaempferol	520-18-3	Wako	ELK6128	95%
4-tert- Octylphenol	140-66-9	Wako	YWE9213	97%
Atrazine	1912-24-9	Wako	MSF9593	>98%
Progesterone	57-83-0	Sigma	98H0893	99%
Testosterone	58-22-0	Wako	ACG5233	>97%
Corticosterone	50-22-6	Sigma	128H0744	95%
Phenobarbital	57-30-7	Wako	ACE1373	98.00%
Vinclozolin	50471-44-8	Wako	HCQ9724	99%
Cyproterone acetate	427-51-0	Sigma	65H0687	N.S.
Flutamide	13311-84-7	Sigma	87H1511	98
Linuron	330-55-2	Dr.Ehrensofter	70226	100%
Mifepristone	84371-65-3	Sigma	19H0828	98%
Procymidone	32809-16-8	Wako	HCH9638	99%
Clomiphene citrate	50-41-9	Sigma	28 0308	N.S.
Ethyl paraben	120-47-8	Wako	ELH6061	99%
Norethynodrel	68-23-5	Sigma	88F0192	N.S.
4-Androstenedione	63-05-8	Sigma	116H0463	98%
2-*sec-* Butylphenol	89-72-5	Wako	KSR1873	98%
Diethylhexyl phthalate	117-81-7	Wako	ELE1799	97%
Morin	480-16-0	Fluka	404144	N.S.
Phenolphthalin	81-90-3	Wako	ELP7131	N.S.
Haloperidol	52-86-8	ICN	85689	90%
Ketoconazole	65277-42-1	Wako	78353	N.S.
Reserpine	50-55-5	RBI	SNV-494A	97.5%
Spironolactone	52-01-7	Sigma	41K1534	97%
L-Thyroxine	51-48-9	TCI	GF01	98%
17β-Trenbolone	10161-33-8	Sigma aldrich	024K0877	>98%

Aldrich : Aldrich Chemical Co., Inc. (Sigma-Aldrich corp.)
Fluka : Fluka Chemie AG (Sigma-Aldrich corp.)
ICN : ICN Biomedicals, Inc.
Kanto : Kanto Chemical Co,. Inc.
Sigma : Sigma Chemical Co. (Sigma-Aldrich corp.)
RBI : SIGMA-RBI
TCI : Tokyo Kasei Kogyo Co., Ltd.
Wako : Wako Pure Chemical Industries, Ltd.
N.S. : not specified

Table 7 Chemicals Used to Examine the Concordance between the Stably Transfected TA Assay and Receptor Binding Assays

Chemical	CAS No.	Manufacturer	Lot. No.	Purity
Ethynyl estradiol	57-63-6	Wako	KSN3933	98%
17β-Estradiol	50-28-2	Wako	ACL1188	>97%
Hexestrol	84-16-2	Wako	LDQ2218	N.S.
Estrone	53-16-7	Wako	TPN4558	98%
17α-Estradiol	57-91-0	Wako	ELJ1532	97%
Norethynodrel	68-23-5	Sigma	88F0192	N.S.
Coumestrol	479-13-0	Fluka	400248	<95%
Genistein	446-72-0	Wako	NNP1712	98%
4-*tert-* Octylphenol	140-66-9	Wako	YWE9213	97%
Daidzein	486-66-8	Wako	HC-1408	97%
Nonylphenol (mixture)	25154-52-3	Aldrich	00504CU	N.S.
Bisphenol B	77-40-7	TCI	FIC01	N.S.
Testosterone propionate	57-85-2	Sigma	98H0566	N.S.
Bisphenol A	80-05-7	TCI	GF01	>99%
5α-Dihydrotestosterone	521-18-6	Wako	TPJ4827	95%
Kaempferol	520-18-3	Wako	ELK6128	95%
4-alpha-Cumylphenol	599-64-4	Wako	PAK1144	98%
17α-Methyltestosterone	58-18-4	Wako	TPE6748	97%
Morin	480-16-0	Fluka	404144	N.S.
Vinclozolin	50471-44-8	Wako	HCQ9724	99%
Testosterone	58-22-0	Wako	ACG5233	>97%
Tamoxifen	10540-29-1	ICN	4636C	N.S.
Clomiphene citrate	50-41-9	Sigma	28 0308	N.S.
di(2-Ethylhexyl)phthalate	117-81-7	Wako	ELE1799	97%
RU-486	84371-65-3	Sigma	19H0828	98%
Methoxychlor	72-43-5	Wako	YWL9207	>97%
Fenarimol	60168-88-9	Kanto	707S7109	97%
para-sec -butylphenol	99-71-8	TCI	FHF01	>98%
Dibutyl phthalate	84-74-2	Wako		for Anal. of Phthalic Acid Esters
Phenolphthalin	81-90-3	Wako	ELP7131	N.S.
Cyproterone acetate	427-51-0	Sigma	65H0687	N.S.
Ethyl *p* -Hydroxybenzoate	120-47-8	Wako	ELH6061	99%
2,4,5-Trichlorophenoxyacetic acid	93-76-5	Wako	HCL9884	98.7%
p,p' -DDE	72-55-9	Wako	YWG9700	99%
Ketoconazol	65277-42-1	Wako	78353	N.S.
Androstenedione	63-05-8	Sigma	116H0463	98%
Progesterone	57-83-0	Sigma	98H0893	99%
Haloperidol	52-86-8	ICN	85689	90%
Medroxyprogesterone	520-85-4	Sigma	59H0579	N.S.
Spironolactone	52-01-7	Sigma	41K1534	97%
L-thyroxine	51-48-9	TCI	GF01	98%
Reserpine	50-55-5	RBI	SNV-494A	97.5%
Corticosterone	50-22-6	Sigma	128H0744	95%
Phenobarbital	50-06-6	Maruishi	8603	N.S.
Linuron = Lorox	330-55-2	Dr.Ehrensofter	70226	100%
Procymidon	32809-16-8	Wako	HCH9638	99%
Atrazine	1912-24-9	Wako	MSF9593	>98%
Flutamide	13311-84-7	Sigma	87H1511	98

Aldrich : Aldrich Chemical Co., Inc. (Sigma-Aldrich corp.)
Fluka : Fluka Chemie AG (Sigma-Aldrich corp.)
ICN : ICN Biomedicals, Inc.
Kanto : Kanto Chemical Co,. Inc.
N.S. : not specified
Maruishi : Maruishi Pharmaceutical. Co., Ltd.
Sigma : Sigma Chemical Co. (Sigma-Aldrich corp.)
RBI : SIGMA-RBI
TCI : Tokyo Kasei Kogyo Co., Ltd.
Wako : Wako Pure Chemical Industries, Ltd.

Table 8 Chemicals Used to Examine the Concordance between the Stably Transfected TA Assay and Immature Rat Uterotrophic Assays

Chemical Name	Cas No.	Manufacture	Lot No.	Purity
Ethynyl Estradiol	57-63-6	Wako	KSN3933	>97%
Equilin	474-86-2	Sigma	97H1529	100%
Estrone	53-16-7	Wako	TPN4558	98%
17α-Estradiol	57-91-0	Wako	ACL1188	>97%
Zearalenone	17924-92-4	Sigma	50K4014	N.S.
4-(1-Adamantyl)phenol	29799-07-3	Aldrich	11608MR	97%
2,2-bis(4-Hydroxyphenyl)-4-methyl-n-pentane	6807-17-6	Wako	PTM1337	100%
Genistein	446-72-0	Wako	NNP1712	98%
Norethrindrone	68-22-4	Wako	DWM4647	100%
4-*tert*-Octylphenol	140-66-9	Wako	09802JQ	99%
4,4'-(Hexafluoroisopropylidene)diphenol	1478-61-1	Aldrich	05328PI	97%
Daidzein	486-66-8	Wako	HC-1408	97%
Nonylphenol (mixture)	25154-52-3	Kanto	109281	97%
Bisphenol B	77-40-7	TCI	FIC01	100%
4,4'-Thiobisphenol	2664-63-3	TCI	JC01	100%
Testosterone enanthate	315-37-7	Wako	KSL4869	100%
Bisphenol A	80-05-7	TCI	GF01	>99%
2,2',4,4'-Tetrahydroxybenzophenone	131-55-5	Wako	ELN6605	98%
2,4,4'-Trihydroxybenzophenone	1470-79-7	Aldrich	04417JN	95%
p-Dodecyl-phenol	104-43-8	Kanto	209D2209	N.S.
5α-Dihydrotestosterone	521-18-6	Wako	TPJ4827	95%
4-Hydroxyazobenzene	1689-82-3	Wako	LDM7343	96%
4-Cyclohexylphenol	1131-60-8	TCI	FIJ01	100%
4-α-Cumylphenol	599-64-4	Wako	PAK1144	98%
4,4'-Dihydroxybenzophenone	611-99-4	Wako	LDR1808	99%
4-Hydroxybenzophenone	1137-42-4	Aldrich	04419CO	98%
3,3,3',3'-Tetramethyl-1,1'-spirobisindane-5,5',6,6'-tetrol	77-08-7	TCI	GG01	99%
p-(*tert*- Pentyl)phenol	80-46-6	Wako	ELF1567	100%
4-(Phenylmethyl)phenol	101-53-1	TCI	FHG01	100%
17α-Methyltestosterone	58-18-4	Wako	ELG7538	100%
4-*n*-Amylphenol	14938-35-3	TCI	FIF01	99%
4,4'-(Octahydro-4,7-methano-5H-inden-5-ylidene)bisphenol	1943-97-1	ACROS	A008394601	100%
Levonorogestrel	797-63-7	Sigma	30K0711	99%
Methoxychlor	72-43-5	Wako	YWL9207	>97%
4-*n*-Octylphenol	1806-26-4	Wako	JSL9944	99%
Diphenyl-*p*-Phenylenediamine	74-31-7	Wako	ELH7269	97%
4,4'-Dimethoxybenzophenone	90-96-0	TCI	FIH01	100%
Dicyclohexyl phthalate	84-61-7	Wako	RIG9061	100%
Diethyl phthalate	84-66-2	Wako	ELH6895	99%
di-*n*-Butyl phthalate	84-74-2	Wako	ACE7193	N.S.
di(2-Ethylhexyl)adipate	103-23-1	Wako	LDR4958	100%
p-n-Nonylphenol	104-40-5	TCI	10425	99%
di(2-Ethylhexyl)phthalate	117-81-7	Wako	ELH6895	99%
Benzophenone	119-61-9	Wako	HCM9879	100%
Tributyltin chloride	1461-22-9	Wako	LDN5508	98%
Octachlorostyrene	29082-74-4	Kanto	106121	100%
Hematoxylin	517-28-2	Sigma	99H3645	N.S.
4,4'-Dimethoxytriphenylmethane	7500-76-7	ERC	1040701	100%

Aldrich : Aldrich Chemical Co., Inc. (Sigma-Aldrich corp.)
Fluka : Fluka Chemie AG (Sigma-Aldrich corp.)
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N.S. : not specified
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RBI : SIGMA-RBI
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Wako : Wako Pure Chemical Industries, Ltd.

For inter-laboratory validations

72 For the inter-laboratory validation study in order to evaluate the protocol transferability among laboratories and to evaluate the relevance of the assay system, nine test chemicals including six positive chemicals that exhibit a wide range of strength of estrogenic activity and three presumed negatives within the test concentration range from 10^{-11}-10^{-5}M were selected (Table 9).

73 Moreover, the full dose response range of E2 was measured in all assay plates to monitor the accuracy of chemical dilution procedure, and to evaluate reproducibility of positive control responses at the participating laboratories.

Table 9 Chemicals Used for Inter-laboratory Validation Study

Chemical	CAS No.	Manufacturer	Lot. No.	Purity
17β-Estradiol	50-28-2	Wako	ACK5754	99%
17α-Estradiol	57-91-0	Wako	ELJ1532	97% ,HPLC ,for Biochem.
Genistein	446-72-0	Wako	VIR1711	98%
4-tert-Octylphenol	140-66-9	Wako	YWE9213	97% ,cGC ,for Environment Anal.
Bisphenol A	80-05-7	TCI	GF01	>99%
p-tert-Pentylphenol	80-46-6	Wako	KSQ2664	97% ,GC
17α-Methyltestosterone	58-18-4	Wako	TPE6748	97% ,HPLC ,for Biochem.
Hematoxylin	517-28-2	Wako	LDK7723	N.S.
Diethylhexyl phthalate	117-81-7	Wako	ELE1799	97% ,GC
Benzophenone	119-61-9	Wako	RLH9114	99% ,cGC ,for Environment Anal.

TCI : Tokyo Kasei Kogyo Co., Ltd.
Wako : Wako Pure Chemical Industries, Ltd.
N.S. : not specified

5.1.5-.3) Test chemical supply

74 All chemicals used in the studies were obtained from a domestic distributor. For the inter-laboratory validation study, 10 mM solutions of test chemicals in dimethylsulfoxide (DMSO) were prepared by CERI, and they were then coded and distributed to the participating laboratories.

5.2 Data Recording and Analyses

75 The luminescence signal data as read by a luminometer were processed, and the average for the vehicle control (V.C.) wells was calculated. Positive control data below a fold

induction threshold of 4, was not observed. The value for each test well was divided by the average value of the V.C. wells in order to obtain individual relative transcriptional activities. Then the average transcriptional activity was calculated for each concentration of the test chemical.

76 If Hill's logistic equation is applicable to dose response data, EC50 was calculated by following equation:

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

where X is the logarithmic concentration of the test chemical, Y is the response, and Y starts at the Bottom and goes to the Top with a sigmoid shape.

Data were analyzed using the commercial software Prism, version 3.00 (Graphpad Software Inc.), and the EC50 value (the concentration producing a 50% peak response) was calculated by applying a logistic equation.

77 Furthermore, the PC50 and PC10 values were also calculated. These PC50 and PC10 values were defined as the test chemical concentrations estimated to elicit either a 10% or a 50% transcription activity when compared with the positive control (PC) response of 100 pM or 1 nM of 17β-estradiol (E2) for pre-validation and inter-lab validation, respectively, in each assay plate. Each PC value was calculated by a simple linear regression using two variable data points in the transcription activity (Fig. 4).

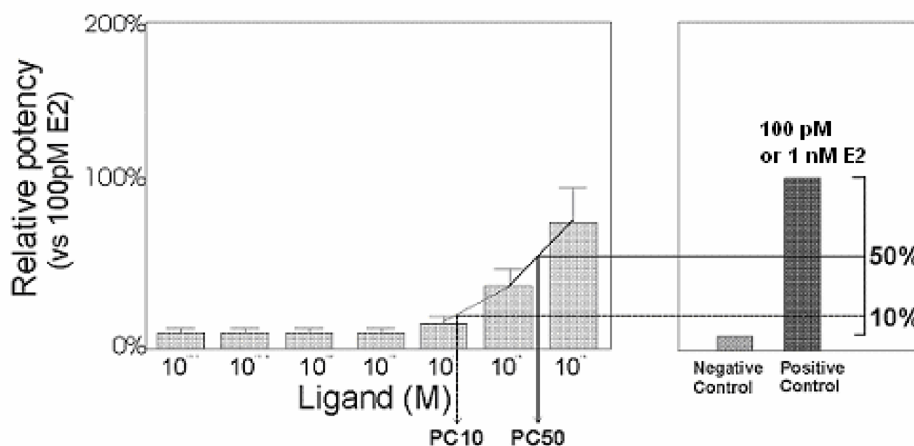

Fig. 4 Definition of PC50 and PC10 Values

78 A common spreadsheet prepared by CERI was provided to all participating laboratories and used throughout all the studies.

Example of the excel spread sheet for processing raw data (Prepared by CERI)

NC_Ave	967
NC_SD	117

Raw - NC_Ave

6285.67	6273.67	7181.67	7564.67	7732.67	7642.67	6801.67	6947.67	6994.67	5925.67	5535.67	6389.67
1447.67	2188.67	4319.67	7183.67	6853.67	6840.67	4901.67	3412.67	3548.67	7265.67	5936.67	6382.67
273.667	785.667	1257.67	3575.67	3472.67	3467.67	1623.67	1240.67	911.667	6786.67	6998.67	6374.67
205.667	600.667	457.667	548.667	479.667	505.667	418.667	269.667	284.667	5823.67	6396.67	6161.67
246.667	20.6667	179.667	19.6667	98.6667	128.667	-35.333	-104.33	5.66667	2870.67	2817.67	2810.67
-96.333	109.667	52.6667	69.6667	-100.33	-131.33	-21.333	-49.333	12.6667	408.667	391.667	459.667
-6.3333	50.6667	106.667	-1.3333	-57.333	-155.33	39.6667	-95.333	0.66667	-21.333	89.6667	169.667
9.66667	111.667	-121.33	10.6667	-135.33	-158.33	-258.33	-23.333	-159.33	6449.67	6191.67	6306.67

PC_Ave 6316

Transcriptional activity of PC = (PC_Ave + NC_Ave)/NC_Ave 7.53

Relative transcriptional activity = (Raw - NC_Ave)/PC_Ave

0.9952	0.9933	1.1371	1.1977	1.2243	1.2100	1.0769	1.1000	1.1075	0.9382	0.8765	1.0117
0.2292	0.3465	0.6839	1.1374	1.0851	1.0831	0.7761	0.5403	0.5619	1.1504	0.9399	1.0106
0.0433	0.1244	0.1991	0.5661	0.5498	0.5490	0.2571	0.1964	0.1443	1.0745	1.1081	1.0093
0.0326	0.0951	0.0725	0.0869	0.0759	0.0801	0.0663	0.0427	0.0451	0.9220	1.0128	0.9756
0.0391	0.0033	0.0284	0.0031	0.0156	0.0204	-0.0056	-0.0165	0.0009	0.4545	0.4461	0.4450
-0.0153	0.0174	0.0083	0.0110	-0.0159	-0.0208	-0.0034	-0.0078	0.0020	0.0647	0.0620	0.0728
-0.0010	0.0080	0.0169	-0.0002	-0.0091	-0.0246	0.0063	-0.0151	0.0001	-0.0034	0.0142	0.0269
0.0015	0.0177	-0.0192	0.0017	-0.0214	-0.0251	-0.0409	-0.0037	-0.0252	1.0212	0.9803	0.9985

	25	0		29	0		33	0			37	0	
Concentration (M)	Ave	SD	Rank	Ave	SD	Rank	Ave	SD	Rank	Concentration (M)	Ave	SD	Rank
1.E-05	1.0419	0.0825	1	1.2107	0.0133	1	1.0948	0.0159	1	1.E-07	0.9421	0.0677	4
1.E-06	0.4199	0.2361	2	1.1019	0.0308	2	0.6261	0.1303	2	1.E-08	1.0336	0.1071	2
1.E-07	0.1223	0.0779	3	0.5550	0.0097	3	0.1993	0.0564	3	1.E-09	1.0640	0.0502	1
1.E-08	0.0667	0.0317	4	0.0810	0.0055	4	0.0514	0.0130	4	1.E-10	0.9701	0.0456	3
1.E-09	0.0236	0.0184	5	0.0130	0.0089	5	-0.0071	0.0088	7	1.E-11	0.4485	0.0052	5
1.E-10	0.0035	0.0168	7	-0.0085	0.0171	6	-0.0031	0.0049	6	1.E-12	0.0665	0.0056	6
1.E-11	0.0080	0.0089	6	-0.0113	0.0123	7	-0.0029	0.0110	5	1.E-13	0.0126	0.0152	7
Max	1.0419			1.2107			1.0948				1.0640		
Max - Value(1.E-11 M)	1.0339			1.2220			1.0977				1.0514		
PC50 or PC10	PC50、PC10			PC50、PC10			PC50、PC10				PC50、PC10		
PC50	#######			#######			#######				#######		
PC10	#######			#######			#######				#######		

For PC10 calculation

log [Concentration (M)]	25 / 0			29 / 0			33 / 0			log [Concentration (M)]	37 / 0		
	((Raw - NC_Ave)/PC_Ave)- Value(1.E-11 M)												
-5	1.0339	≥10%PC	×	1.2220	≥10%PC	×	1.0977	≥10%PC	×	-7	0.9295	≥10%PC	×
-6	0.4119	≥10%PC	×	1.1132	≥10%PC	×	0.6290	≥10%PC	×	-8	1.0211	≥10%PC	×
-7	0.1143	≥10%PC	#######	0.5663	≥10%PC	#######	0.2022	≥10%PC	#######	-9	1.0514	≥10%PC	×
-8	0.0587	<10%PC	×	0.0923	<10%PC	×	0.0543	<10%PC	×	-10	0.9576	≥10%PC	×

79 Two-by-two table analyses were performed to evaluate accuracy (concordance) ([a+d]/[a+b+c+d]), sensitivity (a/[a+c]), and specificity (d/[b+d]) of the proposed stably transfected TA assay system, by comparing it with a reference assay method, because there is no "gold standard" test method that could be compared with this assay.

		New Test Outcome		
		Positive	Negative	Total
Reference Test Classification	Positive	a	c	a+c
	Negative	b	d	b+d
	Total	a+b	c+d	a+b+c+d

80 In order to examine the concordance of the outcomes of the proposed assay system with the results listed in the ICCVAM Recommended Substances for Validation of In Vitro ER TA Agonism Assays, two-by-two table analysis was conducted upon 46 selected chemicals.

81 The performance of the assay system using hER-HeLa-9903 cells was evaluated using a two-by-two table analysis with reference data obtained from either a receptor binding assay or an immature rat uterotrophic assay; both of these assay types were conducted at CERI.

6. RESULTS

6.1 Stability of response of hER-HeLa-9903 cell line

82 The reliability of the assay system using hER-HeLa-9903 cells line was evaluated by analyzing the reproducibility of the test systems' biological responses to E2 as a potent estrogen, to TS, and to BPA. The assays for these three chemicals were repeated 13 times over a four-month period. The duration of the test was decided upon pursuant to the longest subculture period from one stock tube of the cell line.

83 The individual response curves for transcriptional activity for the 13 assays (Fig. 5) and the changes in the positive control (100 pM of E2) response during the study period (Fig. 6) are shown.

84 E2 produced a typical sigmoidal response in all 13 experiments. The maximum transcriptional activities induced by E2 were from 4.77 to 15.4-fold (Fig. 5). No increase or decrease of time-dependent tendencies was observed with regard to the positive control responses (Fig. 6). The mean Log_{10}[EC50 (M)] ± Standard deviation (SD) for E2 was -11.17 ± 0.25 (n=13) and the 95% confidential interval ranged from -11.02 to -11.32. The 95% confidential interval for E2 was within acceptable and normal variation observed for such assays.

85 When the raw numbers were assessed in, a range of $\log_{10}$[PC50 (M)] of E2 as measured in 13 experiments, the narrow range was evident (from -10.87 to -11.58), despite the extensively wide range of assay concentrations (10^{-12}-10^{-6}M).

86 The precise EC50 values of the other two chemicals, BPA and TS, could not be calculated because these chemicals did not demonstrate a complete sigmoidal dose response curve over the concentration range tested (10^{-12}-10^{-6}M).

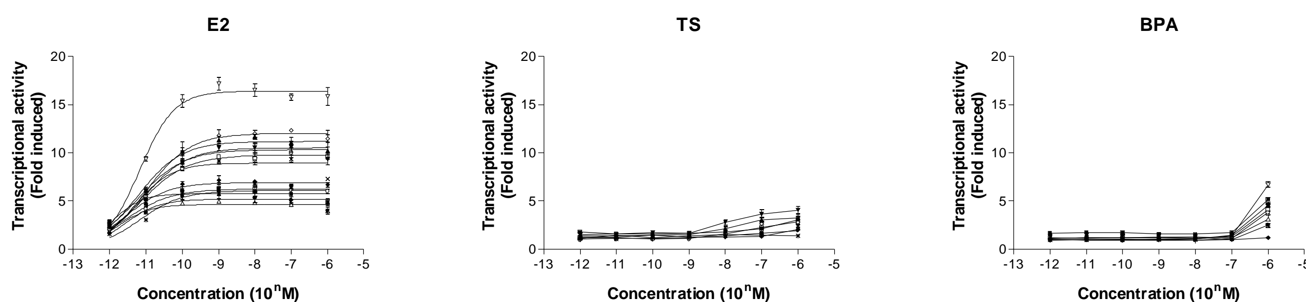

Fig. 5 Individual Dose-response Curves for 17β-estradiol (E2), Testosterone (TS) and Bisphenol A (BPA) in 13 Assays –Transcriptional Activity

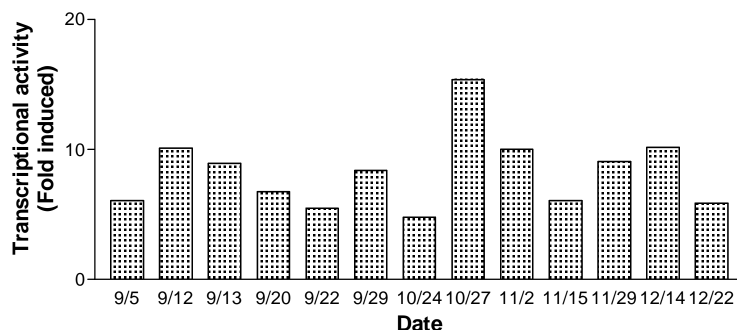

Fig. 6 Changes in the Positive control (100 pM of E2) Response during the Study Period

6.2 Relevance of the assay system

87 The fact that there is no "gold standard" data that can be used to evaluate the relevance of the proposed stably transfected TA assay should be taken into consideration; i.e., no validated assay to detect estrogenic activity is currently available. One possible approach to demonstrate the capacity of any transfected TA assay system for detecting estrogenic activity of chemicals is to compare the results with available data collected from other assays that are designed to detect estrogenic activity.

88 The EC50s for 22 selected chemicals (as shown in Table 10), the relationships between logEC50s obtained from the proposed assay, and the median logEC50s referred to the ICCVAM report (2003) which are derived from EC50 values from different assay systems (including the mammalian reporter-gene assay, the mammalian cell-proliferation assay, and the yeast reporter-gene assay), and of which any are expected to detect estrogenic activity, are shown in Fig. 8.

89 Note that for 17α-methyltestosterone, genistein, phloretin and naringenin, the PC50 values are shown in place of the EC50 values in Table 10 because the response curves of those chemicals did not exhibit sigmoidal responses, and the EC50 values of these chemicals could not be calculated using Hill's logistic equation.

90 With regard to levonorogestrel and methoxychlor, neither the EC50 nor the PC50 value could be calculated because the response curves were incomplete and did not show more than 50% of PC response (see the response curve shown below in Fig. 7). Thus, the EC50 values were considered to be over 10^{-5} M. However, the PC50 values for these two chemicals appeared to be around 10^{-5} M, judging from the appearance of the curves.

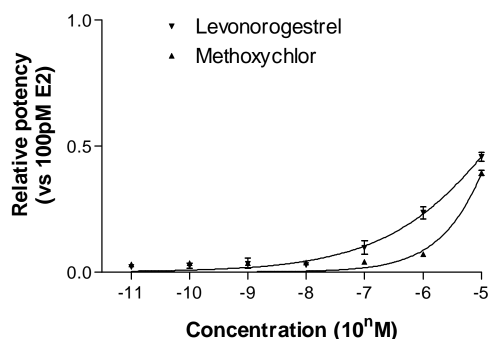

Fig. 7 Dose response curves of levonorogestrel and methoxychlor

91 For accurate calculations of EC50 values with Hill's equation, at least four data points containing the basal response and the saturated response are required. The dose response curve of the two chemicals discussed above, levonorogestrel and methoxychlor, did not appear to reach the saturated response. Moreover, most of the weak estrogenic compounds that elicit transcriptional activity over 10^{-6}M would show a similar dose response curve to these two compounds.

92 In this regard, the PC50 value is regarded as a relative E2 estrogenic activity value that is

normalized by E2. This parameter can be obtained with only two data points. The PC50 values can also be calculated in cases of weak estrogenic compounds as the relative estrogenic activity to the natural estrogen.

93 Log_{10}[EC50 (M)] values obtained in the proposed stably transfected TA assay for several known chemicals listed in ICCVAM report (2003) correlate well with the values reported by ICCVAM (2003). As shown in Fig. 8-1, the correlation coefficient between the Log_{10}[EC50 (M)]of proposed test outcomes and that of original data was successful (R^2=0.802, n=20).

94 Although available data are limited, Log_{10}[EC50 (M)] obtained from the stably transfected TA assay using the hER-HeLa-9903 cell line showed high consistency with the data obtained by the ER-CALUX and HELN-ERα cell systems. As shown in Fig. 8-2, the correlation coefficient between the Log_{10}[EC50 (M)] of the proposed test outcomes and that reported in other ER/TA assay systems were R^2= 0.987 (vs. ERα-CALUX , n=8), R^2= 0.938 (vs. HELN-ERα cell system , n=7) and R^2= 0.922 (vs. LUMI-CERL™ , n=7).

95 As for the regression formula for each individual assay system, the slopes of the formula against ERα CALUX and LUMI-CELL™ were nearly 1.0 (0.956 for ERα-CALUX, 1.01 for LUMI-CELL™), however that for HELN-ERα cell systems was 0.712.

Table 10 EC50 Values Obtained from the Stably Transfected TA Assay using HeLa-hER-9903 and the Median EC50 Values Reported in the Other Assays for Detection of Estrogenic Activity

Chemical Name	EC50(M)				
	HeLa-hER-9903§	Reference*	ERα-CALUX#	HELN-ERα¶	LUMI-CELL™ $
Ethynyl Estradiol	5.68E-12	1.10E-11	7.94E-12	8.00E-12	NA
Diethylstilbestrol	2.40E-11	1.89E-11	3.98E-11	N.A.	1.83E-11
17α-Estradiol	6.04E-10	4.60E-11	1.58E-09	N.A.	NA
17β-Estradiol	8.17E-12	1.00E-10	1.58E-11	1.70E-11	8.44E-12
Estriol	1.91E-11	7.10E-10	1.26E-10	1.60E-10	NA
Estrone	4.89E-10	3.20E-09	1.00E-09	6.60E-10	NA
Zearalenone	9.05E-10	3.43E-09	N.A.	N.A.	1.66E-09
17α-Methyltestosterone	(4.11E-06)	1.08E-08	N.A.	N.A.	NA
Beta-Zearalenol	4.79E-09	1.50E-08	N.A.	N.A.	NA
Coumestrol	6.05E-08	1.50E-08	N.A.	1.60E-08	1.94E-08
4-*tert*-Octylphenol	1.01E-07	5.00E-08	N.A.	N.A.	NA
Genistein	(2.45E-08)	6.20E-08	5.01E-08	3.80E-08	7.03E-07
4-Nonylphenol	4.91E-07	9.45E-08	N.A.	N.A.	NA
Testosterone,19-Nor	5.91E-08	2.12E-07	2.00E-07	N.A.	NA
Daidzein	4.99E-06	2.90E-07	N.A.	1.50E-07	2.05E-06
Phloretin	(4.95E-06)	3.00E-07	N.A.	N.A.	NA
Levonorogestrel	(ca. 1.00E-05)	3.30E-07	N.A.	N.A.	NA
Bisphenol A	4.55E-07	3.99E-07	N.A.	N.A.	NA
Naringenin	(1.48E-06)	1.00E-06	N.A.	N.A.	4.48E-06
Methoxychlor	(ca. 1.00E-05)	8.85E-06	7.94E-06	N.A.	NA
Progesterone	-	-	-	N.A.	NA
Atrazine	-	-	N.A.	N.A.	NA

§: The EC values in the parenthesis indicates PC50 value instead of EC50, because the response curve was a non-sigmoidal one.
*: quoted from ICCVAM (2003).
#: quoted from Sonneveld et al. (2006).
¶: quoted from Escande et al. (2006).
$: calculated from the values as ug/mL units published in Jefferson et al. (2002)
-: Negative response
N.A.: Not available

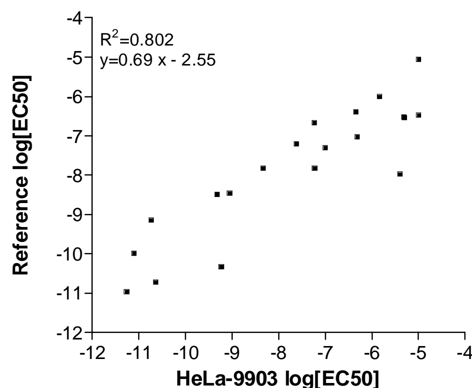

Fig. 8-1 The Relationship between LogEC50s and Median Log EC50s in the ICCVAM Report (2003)

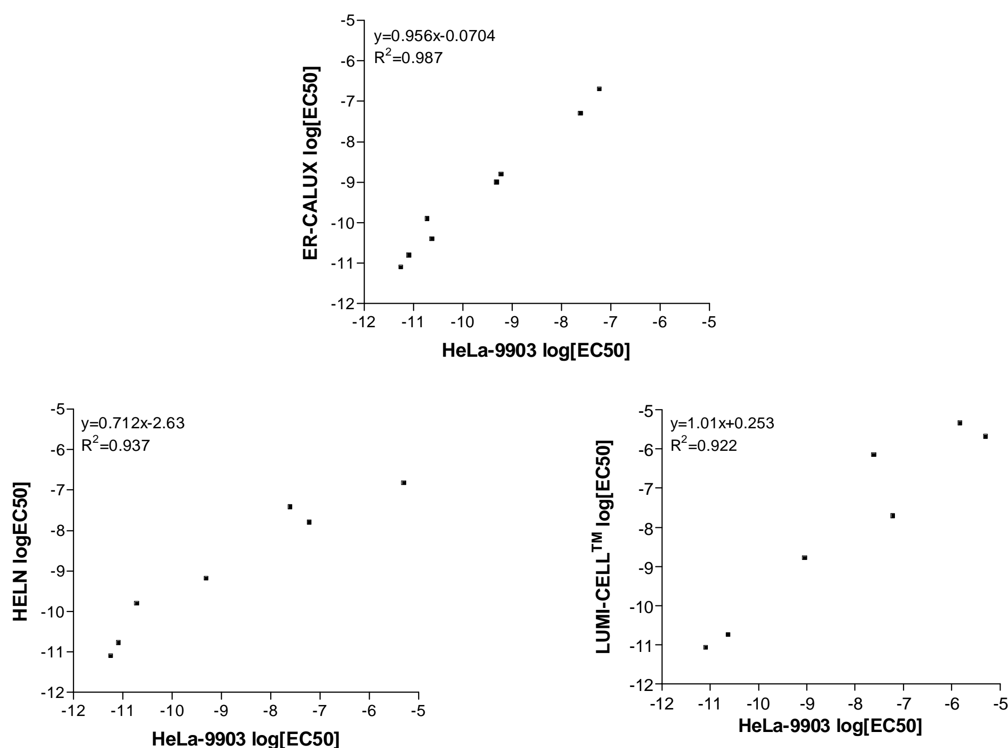

Fig. 8-2 The Relationship of LogEC50s between the Data Obtained in Proposed TA Assay System and the Other ER/TA Assay System using ERα-CALUX, HELN-ERα or LUMI-CELL™ Assay Systems

6.3 Overview assessment of the stably transfected TA assay using hER-HeLa-9903

96 The positive/negative result outcomes reported as the PC50 of the stably transfected TA assay using hER-HeLa-9903 cell line were compared with 46 chemicals recommended by ICCVAM for appraising the performance of new assay results (ICCVAM, 2003). The

results of two-by-two table analyses are shown in Table 11 and the positive/negative outcomes of this proposed assay system and the data reported in the ICCVAM report are represented in Table 12.

97 The concordance between the results obtained from the stably transfected TA assay using hER-HeLa-9903 cell line and the reference data in the ICCVAM report was 80%. Further, sensitivity and specificity rates were 79% and 82%, respectively.

98 The consistency between the proposed assay system (using PC50s as comparative parameters) and the ICCVAM reference data was found to be satisfactory.

Table 11 Two-by-two Table Analysis of 46 Selected Chemicals Listed in the ICCVAM Report (2003) as Recommended Chemicals for ER/TA assay

		Stably transfected TA assay (PC50 based)		
		Positive	Negative	Total
Listed in ICCVAM report (2003)	Positive	19	5	24
	Negative	4	18	22
	Total	23	23	46

Concordance	80%
Sensitivity	79%
Specificity	82%

Table 12 The Positive/negative Outcomes from the hERα Mediated Proposed Stably Transfected TA Assay (PC50 based) and the Data Reported in ICCVAM Report (2003)

Chemical name	CAS	ICCVAM	PC50
17α-Ethinyl estradiol	57-63-6	P (2/2)	P
Diethylstilbestrol	56-53-1	P (8/8)	P
17α-Estradiol	57-91-0	P (2/2)	P
17β-Estradiol	50-28-2	P (77/77)	P
Zearalenone	17924-92-4	P (8/8)	P
Estrone	53-16-7	P (3/3)	P
Methyl testosterone	58-18-4	P (2/2)	P
Coumestrol	479-13-0	P (8/8)	P
Genistein	446-72-0	P (11/11)	P
p- n-Nonylphenol	104-40-5	P (4/4)	N
Bisphenol B	77-40-7	P (2/2)	P
Daidzein	486-66-8	P (5/5)	P
4-Cumylphenol	599-64-4	P (2/2)	P
Bisphenol A	80-05-7	P (15/15)	P
p,p' -Methoxychlor	72-43-5	P (12/13)	-
Apigenin	520-36-5	P (6/6)	P
Tamoxifen	10540-29-1	P (5/7)	-
Kepone (Chlordecone)	143-50-0	P (4/6)	P
Butylbenzyl phthalate	85-68-7	P (3/4)	P
Kaempferol	520-18-3	P (2/2)	P
4-tert- Octylphenol	140-66-9	P (2/3)	P
Atrazine	1912-24-9	N (3/3)	N
Progesterone	57-83-0	N (2/2)	N
Testosterone	58-22-0	N (2/2)	P
Corticosterone	50-22-6	N (1/1)	N
Phenobarbital	57-30-7	N (1/1)	N
Vinclozolin	50471-44-8	N (1/1)	P
Cyproterone acetate	427-51-0	N (1/1)	N
Flutamide	13311-84-7	N (1/1)	N
Linuron	330-55-2	N (1/1)	N
Mifepristone	84371-65-3	N (1/1)	N
Procymidone	32809-16-8	N (1/1)	N
Clomiphene citrate	50-41-9	P	-
Ethyl paraben	120-47-8	P	-
Norethynodrel	68-23-5	P	P
4-Androstenedione	63-05-8	N	-
2-*sec*- Butylphenol	89-72-5	N	N
Diethylhexyl phthalate	117-81-7	N	N
Morin	480-16-0	N	P
Phenolphthalin	81-90-3	N	N
Haloperidol	52-86-8	N	N
Ketoconazole	65277-42-1	N	N
Reserpine	50-55-5	N	N
Spironolactone	52-01-7	N	N
L-Thyroxine	51-48-9	N	-
17β-Trenbolone	10161-33-8	N	P

P: Positive, N: Negative, -: the response was not reached to PC50 value but responded enough to calculate PC10 value. These chemicals are regarded as negatives in two-by-two analysis.

6.4 Supplemental information that supports the performance of the assay test system for detection of estrogenic activity

99 In order to provide information supporting the performance of the proposed assay system for the detection of estrogenic activity, a different set of 48 chemicals that had been tested in both *in vitro* ERα binding assays and immature rat uterotrophic assays (the latter for the detection of *in vivo* endpoints for estrogenic activity), were compared with the data generated from the proposed assay system using a two-by-two table analysis. All data were obtained by CERI.

100 The data for 48 chemicals were subjected to examine the performance of the assay system using hER-HeLa-9903 cell line by a two-by-two table analysis. As shown in Table 13 and Table 14, the assay performance parameters, such as concordance, sensitivity and specificity, were 77%, 71 and 83%, respectively.

Table 13. Two-by-two Table Analysis of the Stably Transfected TA Assay and Receptor Binding Assay with 48 Selected Chemicals

		Stably transfected TA assay (PC50 based)		
		Positive	Negative	Total
Binding assay	Positive	17	7	24
	Negative	4	20	24
	Total	21	27	48

Concordance	77%
Sensitivity	71%
Specificity	83%

Table 14. The Comparison between the Results Obtained

in the ER Binding Assay and the Stably Transfected TA Assay

Chemical name	CAS No.	Relative potency in Reporter gene assay (E2=100)	Relative binding affinity in binding assay (E2=100)
Ethynyl estradiol	57-63-6	>81.7	142
17β-Estradiol	50-28-2	>81.7	126
Hexestrol	84-16-2	29.2	37.6
Estrone	53-16-7	1.39	44.2
17α-Estradiol	57-91-0	1.27	80.1
Norethynodrel	68-23-5	0.543	0.282
Coumestrol	479-13-0	0.0408	0.264
Genistein	446-72-0	0.0334	0.12
4-*tert*- Octylphenol	140-66-9	0.0111	0.124
Daidzein	486-66-8	0.0054	0.18
Nonylphenol (mixture)	25154-52-3	0.00518	0.143
Bisphenol B	77-40-7	0.00388	0.593
Testosterone propionate	57-85-2	0.0028	N.B.
Bisphenol A	80-05-7	0.00278	0.195
5α-Dihydrotestosterone	521-18-6	0.00155	0.0218
Kaempferol	520-18-3	0.000673	0.029
4-alpha-Cumylphenol	599-64-4	0.00051	0.107
17α-Methyltestosterone	58-18-4	0.000199	N.D.
Morin	480-16-0	0.000196	0.0011
Vinclozolin	50471-44-8	0.000107	N.B.
Testosterone	58-22-0	8.35E-05	N.D.
Tamoxifen	10540-29-1	-	47
Clomiphene citrate	50-41-9	-	37
di(2-Ethylhexyl)phthalate	117-81-7	-	0.071
RU-486	84371-65-3	-	0.0594
Methoxychlor	72-43-5	-	0.00238
Fenarimol	60168-88-9	-	0.00179
para-sec -butylphenol	99-71-8	-	0.00177
Dibutyl phthalate	84-74-2	-	N.D.
Phenolphthalin	81-90-3	-	N.D.
Cyproterone acetate	427-51-0	-	N.D.
Ethyl *p* -Hydroxybenzoate	120-47-8	-	N.D.
2,4,5-Trichlorophenoxyacetic acid	93-76-5	-	N.B.
p,p' -DDE	72-55-9	-	N.B.
Ketoconazol	65277-42-1	-	N.B.
Androstenedione	63-05-8	-	N.B.
Progesterone	57-83-0	-	N.B.
Haloperidol	52-86-8	-	N.B.
Medroxyprogesterone	520-85-4	-	N.B.
Spironolactone	52-01-7	-	N.B.
L-thyroxine	51-48-9	-	N.B.
Reserpine	50-55-5	-	N.B.
Corticosterone	50-22-6	-	N.B.
Phenobarbital	50-06-6	-	N.B.
Linuron = Lorox	330-55-2	-	N.B.
Procymidon	32809-16-8	-	N.B.
Atrazine	1912-24-9	-	N.B.
Flutamide	13311-84-7	-	N.B.

-: PC50 was not derived, N.D.: Not Determined, N.B.: Not Bound.
Footnote: Results of the stably transfected TA assay are represented as the relative estrogenic potency of E2

101 The PC50 based positive/negative outcomes of 48 chemicals from the stably transfected TA assay using hER-HeLa-9903 cell line and from an immature rat uterotrophic assay were compared and the results of two-by-two table analysis are shown in Table 15. The original data is shown in Table 16.

102 The concordance between the results obtained from the stably transfected TA assay using hER-HeLa-9903 cell line and the immature rat uterotrophic assay was 90%. Further, sensitivity and specificity were 91% and 88%, respectively.

103 Although the proposed stably transfected TA assay system shows good concordance with other *in vitro* and *in vivo* ER screening tests, it is important to caution that the TA assay is not a one to one alternative replacement method for any other existing *in vivo* test methods, but is a stand-alone screening test method for prioritizing or grouping substances in general categories of potential modes of action, and can be used in the OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals (adopted by OECD/EDTA 6).

Table 15 Two-by-two Table Analysis of the Stably Transfected TA Assay and the Immature Rat Uterotrophic Assay with 48 Selected Chemicals

		Stably transfected TA assay		
		Positive	Negative	Total
Uterotrophic assay	Positive	29	3	32
	Negative	2	14	16
	Total	31	17	48

Concordance	90%
Sensitivity	91%
Specificity	88%

Table 16 A Comparison of the hERα Mediated Proposed Stably Transfected TA Assay and the Immature Rat Uterotrophic Assay

Chemical Name	CAS	PC10(pM)	PC50 (pM)	Reporter gene		Uterotrophic assay
				PC10	PC50	
Ethynyl Estradiol	57-63-6	>10	>10	P	P	P
Equilin	474-86-2	>10	75	P	P	P
Estrone	53-16-7	30	588	P	P	P
17α-Estradiol	57-91-0	72	644	P	P	P
Zearalenone	17924-92-4	24	644	P	P	P
4-(1-Adamantyl)phenol	29799-07-3	1248	18594	P	P	P
2,2-bis(4-Hydroxyphenyl)-4-methyl-n-pentane	6807-17-6	1892	19903	P	P	P
Genistein	446-72-0	2242	24459	P	P	P
Norethrindrone	68-22-4	1006	49474	P	P	P
4-*tert*-Octylphenol	140-66-9	1846	73676	P	P	P
4,4'-(Hexafluoroisopropylidene)diphenol	1478-61-1	6906	80249	P	P	P
Daidzein	486-66-8	17606	151271	P	P	N
Nonylphenol (mixture)	25154-52-3	11530	157618	P	P	P
Bisphenol B	77-40-7	23576	210679	P	P	P
4,4'-Thiobisphenol	2664-63-3	20087	213679	P	P	P
Testosterone enanthate	315-37-7	17140	270712	P	P	P
Bisphenol A	80-05-7	20157	294271	P	P	P
2,2',4,4'-Tetrahydroxybenzophenone	131-55-5	106427	328223	P	P	P
2,4,4'-Trihydroxybenzophenone	1470-79-7	43765	374950	P	P	P
p-Dodecyl-phenol	104-43-8	23645	410096	P	P	P
5α-Dihydrotestosterone	521-18-6	104122	527786	P	P	P
4-Hydroxyazobenzene	1689-82-3	164424	1082903	P	P	P
4-Cyclohexylphenol	1131-60-8	64256	1507661	P	P	P
4-α-Cumylphenol	599-64-4	149373	1600708	P	P	P
4,4'-Dihydroxybenzophenone	611-99-4	124213	1648224	P	P	P
4-Hydroxybenzophenone	1137-42-4	1096217	2596825	P	P	P
3,3,3',3'-Tetramethyl-1,1'-spirobisindane-5,5',6,6'-tetrol	77-08-7	143472	3156712	P	P	N
p-(*tert*- Pentyl)phenol	80-46-6	401969	3456682	P	P	P
4-(Phenylmethyl)phenol	101-53-1	1198024	4073138	P	P	P
17α-Methyltestosterone	58-18-4	173235	4109650	P	P	P
4-*n*-Amylphenol	14938-35-3	177639	4615960	P	P	P
4,4'-(Octahydro-4,7-methano-5H-inden-5-ylidene)bisphenol	1943-97-1	37162	-	P	N	P
Levonorogestrel	797-63-7	104707	-	P	N	P
Methoxychlor	72-43-5	1228849	-	P	N	N
4-*n*-Octylphenol	1806-26-4	1255876	-	P	N	N
Diphenyl-*p*-Phenylenediamine	74-31-7	2300407	-	P	N	P
4,4'-Dimethoxybenzophenone	90-96-0	2497084	-	P	N	N
Dicyclohexyl phthalate	84-61-7	2527731	-	P	N	N
Diethyl phthalate	84-66-2	4461009	-	P	N	N
di-*n*-Butyl phthalate	84-74-2	8505555	-	P	N	N
di(2-Ethylhexyl)adipate	103-23-1	-	-	N	N	N
p-n-Nonylphenol	104-40-5	-	-	N	N	N
di(2-Ethylhexyl)phthalate	117-81-7	-	-	N	N	N
Benzophenone	119-61-9	-	-	N	N	N
Tributyltin chloride	1461-22-9	-	-	N	N	N
Octachlorostyrene	29082-74-4	-	-	N	N	N
Hematoxylin	517-28-2	-	-	N	N	N
4,4'-Dimethoxytriphenylmethane	7500-76-7	-	-	N	N	N

*: All data concerning the stably transfected TA assay and immature rat uterotrophic assay were determined in the Hita laboratory, CERI-Japan.
-: Could not be determined.
P: Positive, N: Negative
Positive/Negative based decision of stably transfected TA assay was made based on the PC50 values.
Positive/Negative based decision for the uterotrophic assay was made when positive response was observed in agonist tests.

6.5 Inter-laboratory reproducibility (reliability) and protocol transferability.

104 For the inter-laboratory validation study, assays were performed three times on separate days with nine coded test chemicals and one positive control substance, E2. The reproducibility of E2 responses are shown in Table 17 using four different parameters, $\log_{10}$[PC10 (M)], $\log_{10}$[PC50 (M)] and $\log_{10}$[EC50 (M)].

105 The mean Log_{10}[PC50 (M)], Log_{10}[PC10 (M)], and Log_{10}[EC50 (M)] measured in a same day at each participating laboratory ranged from -10.46 to -11.28, from -11.71 to -12.33 and from -10.36 to -11.19, respectively. These data demonstrated the high reproducibility of the assay system with regard to the positive control (E2) responses.

106 Log_{10}[PC50 (M)], Log_{10}[PC10 (M)] and Log_{10}[EC50 (M)] values obtained for nine test chemicals and positive control substance, E2, in three separate experiments are shown in Table 18, Table 19 and Table 20. Except for the EC50 values of 17α-Methyltestosterone, all the assay results showed high reproducibility within all parameters at any of the participating laboratories. Although there were differences in the luciferase detection system used, i.e., reagents and luminometer, the outputs obtained from each laboratory were consistent.

107 The EC50 value for 17α-Methyltestosterone could not always. The reason for this was due to the incomplete dose response curve given by 17α-Methyltestosterone, similar to the cases were mentioned in section 6.2. However it was possible to calculate PC50 values for 17α-Methyltestosterone for all experiments conducted in each participating laboratory with one exception at one laboratory, with high reproducibility (See Table 18 and Table 19). Consequently it can be concluded that there is a great advantage to using PC values as an assay parameter.

108 As for the parameters used to evaluate the assay results, the PC50 value is capable of making a sharp distinction between estrogenic compounds and non-estrogenic compounds. PC10 can also distinguish positive compounds; however, positive responses were noted in some experiments with regard to presumed negative chemicals, Hematoxylin, Diethylhexyl phthalate and Benzophenone. Similar positive results have also been reported in the literature, but have been negative in other reports, the latter in some cases may depend on the cut off of 10^{-5}M (Blair et al., 2000; ICCVAM, 2003; Suzuki et al., 2005; Yamasaki et al., 2002).

Table 17 The Reproducibility of the Assay System with a Positive Control Substance, 17β-Estradiol

Test Substance	Test vial No.	Laboratory	Trial	Log_{10} [PC10 (M)]				Log_{10} [PC50(M)]				Log_{10} [EC50(M)]			
				Data	Mean	SE	SD	Data	Mean	SE	SD	Data	Mean	SE	SD
17β-Estradiol	37	ceri	1-1	-11.91				-10.69				-10.79			
			1-2	-11.89	-11.84	0.06	0.10	-10.69	-10.63	0.06	0.10	-10.76	-10.71	0.07	0.11
			1-3	-11.73				-10.52				-10.58			
			2-1	-12.42				-10.98				-11.01			
			2-2	-12.65	-12.33	0.22	0.38	-11.22	-11.03	0.10	0.17	-11.13	-11.02	0.06	0.10
			2-3	-11.91				-10.90				-10.93			
			3-1	-11.98				-11.11				-11.17			
			3-2	-12.41	-12.27	0.14	0.25	-11.36	-11.28	0.08	0.14	-11.15	-11.19	0.03	0.05
			3-3	-12.41				-11.37				-11.25			
	38	sumitomo	1-1	-11.74				-11.06				-11.12			
			1-2	>-13.00	-11.74	-	-	-10.83	-10.63	0.01	0.01	-10.48	-10.73	0.01	0.01
			1-3	>-13.00				-11.59				-11.49			
			2-1	-12.01				-10.51				-10.51			
			2-2	-11.62	-11.71	0.15	0.27	-10.54	-10.89	0.01	0.01	-10.46	-10.87	0.01	0.01
			2-3	-11.50				-10.50				-10.46			
			3-1	-12.12				-11.05				-10.93			
			3-2	-11.82	-11.94	0.09	0.16	-10.77	-11.00	0.01	0.01	-10.71	-11.02	0.00	0.01
			3-3	-11.87				-10.63				-10.55			
	39	otsuka	1-1	-11.58				-10.44				-10.41			
			1-2	-11.56	-11.57	0.01	0.01	-10.48	-10.46	0.02	0.03	-10.37	-10.39	0.02	0.03
				-											
			2-1	-11.09				-10.38				-10.31			
			2-2	-11.58	-11.34	0.24	0.34	-10.43	-10.40	0.02	0.03	-10.29	-10.30	0.01	0.02
				-											
			3-1	-11.84				-10.55				-10.69			
			3-2	-11.64	-11.81	0.09	0.16	-10.75	-10.73	0.10	0.17	-10.70	-10.73	0.04	0.07
			3-3	-11.95				-10.90				-10.81			
	40	kaneka	1-1	-12.61				-11.08				-11.04			
			1-2	>-13.00	-12.20	0.40	0.57	-11.04	-10.94	0.12	0.20	-11.06	-10.91	0.14	0.24
			1-3	-11.80				-10.71				-10.63			
			2-1	-11.88				-10.65				-10.63			
			2-2	-12.23	-11.99	0.12	0.21	-10.51	-10.57	0.04	0.07	-10.47	-10.55	0.05	0.08
			2-3	-11.85				-10.56				-10.57			
			3-1	-11.66				-10.48				-10.38			
			3-2	-11.72	-11.71	0.02	0.04	-10.37	-10.46	0.04	0.08	-10.26	-10.36	0.05	0.09
			3-3	-11.75				-10.52				-10.43			
Total	MAX			-11.09 *	-11.71 *	0.22 *	0.38 *	-10.37	-10.46 *	0.12 *	0.20 *	-10.26	-10.36 *	0.14 *	0.24 *
	MIN			-12.65 *	-12.33 *	0.02 *	0.04 *	-11.59	-11.28 *	0.01 *	0.01 *	-11.49	-11.19 *	0.00 *	0.01 *
	Ave.			-11.89 *	-11.95 *	0.11 *	0.20 *	-10.77	-10.82 *	0.06 *	0.10 *	-10.72	-10.81 *	0.05 *	0.08 *

*excepted shaded region

-: Data were excluded because the assay was conducted on separate days (one of three runs conducted by Otsuka pharm.)

Table 18 The Reproducibility of Log_{10}[PC50 (M)] in the Assay System

Test Substance	Test vial No.	Laboratory	Trial	Log_{10} [PC50 (M)] Data	within-Lab			between-Lab		
					Mean	SE	SD	Mean	SE	SD
Hematoxylin	1	ceri	1	-				-	-	-
			2	-	-	-	-			
			3	-						
	2	sumitomo	1	-						
			2	-	-	-	-			
			3	-						
	3	otsuka	1	-						
			2	-	-	-	-			
			3	-						
	4	kaneka	1	-						
			2	-	-	-	-			
			3	-						
17α-Estradiol	5	ceri	1	-9.04				-8.85	0.16	0.32
			2	-9.46	-9.30	0.13	0.23			
			3	-9.39						
	6	sumitomo	1	-9.22						
			2	-8.36	-8.79	0.25	0.43			
			3	-8.79						
	7	otsuka	1	-8.33						
			2	-8.46	-8.52	0.13	0.23			
			3	-8.77						
	8	kaneka	1	-9.15						
			2	-8.66	-8.79	0.18	0.32			
			3	-8.56						
Benzophenone	9	ceri	1	-				-	-	-
			2	-	-	-	-			
			3	-						
	10	sumitomo	1	-						
			2	-	-	-	-			
			3	-						
	11	otsuka	1	-						
			2	-	-	-	-			
			3	-						
	12	kaneka	1	-						
			2	-	-	-	-			
			3	-						
Bisphenol A	13	ceri	1	-6.50				-6.40	0.07	0.15
			2	-6.57	-6.61	0.08	0.13			
			3	-6.76						
	14	sumitomo	1	-6.52						
			2	-6.48	-6.41	0.09	0.16			
			3	-6.22						
	15	otsuka	1	-6.14						
			2	-6.46	-6.30	0.09	0.16			
			3	-6.31						
	16	kaneka	1	-6.53						
			2	-6.18	-6.28	0.12	0.21			
			3	-6.14						
Diethylhexyl phthalate	17	ceri	1	-				-	-	-
			2	-	-	-	-			
			3	-						
	18	sumitomo	1	-						
			2	-	-	-	-			
			3	-						
	19	otsuka	1	-						
			2	-	-	-	-			
			3	-						
	20	kaneka	1	-						
			2	-	-	-	-			
			3	-						

Table 18 – continued

Genistein	21	ceri	1	-6.77	-7.40	0.32	0.55	-7.26	0.07	0.14
			2	-7.66						
			3	-7.78						
	22	sumitomo	1	-7.42	-7.10	0.16	0.28			
			2	-6.89						
			3	-6.99						
	23	otsuka	1	-7.23	-7.35	0.10	0.17			
			2	-7.27						
			3	-7.54						
	24	kaneka	1	-7.52	-7.18	0.17	0.29			
			2	-7.07						
			3	-6.97						
17α-Methyltestosterone	25	ceri	1	-5.75	-5.84	0.05	0.08	-5.56	0.11	0.22
			2	-5.87						
			3	-5.90						
	26	sumitomo	1	-5.49	-5.31	0.11	0.20			
			2	-5.10						
			3	-5.35						
	27	otsuka	1	-5.73	-5.62	0.11	0.16			
			2	-						
			3	-5.50						
	28	kaneka	1	-5.60	-5.47	0.08	0.15			
			2	-5.51						
			3	-5.31						
4-tert-Octylphenol	29	ceri	1	-6.85	-7.16	0.19	0.33	-6.83	0.12	0.23
			2	-7.12						
			3	-7.51						
	30	sumitomo	1	-6.67	-6.61	0.07	0.11			
			2	-6.68						
			3	-6.48						
	31	otsuka	1	-6.89	-6.80	0.06	0.10			
			2	-6.82						
			3	-6.70						
	32	kaneka	1	-6.72	-6.76	0.02	0.04			
			2	-6.80						
			3	-6.75						
p-tert-pentylphenol	33	ceri	1	-6.08	-6.39	0.21	0.36	-6.03	0.12	0.24
			2	-6.30						
			3	-6.79						
	34	sumitomo	1	-5.91	-5.83	0.04	0.07			
			2	-5.82						
			3	-5.77						
	35	otsuka	1	-5.89	-5.94	0.06	0.10			
			2	-5.87						
			3	-6.06						
	36	kaneka	1	-6.00	-5.96	0.03	0.06			
			2	-5.89						
			3	-5.99						
17β-Estradiol	37	ceri	1	-10.63	-10.98	0.19	0.33	-10.75	0.10	0.20
			2	-11.03						
			3	-11.28						
	38	sumitomo	1	-10.63	-10.84	0.11	0.19			
			2	-10.89						
			3	-11.00						
	39	otsuka	1	-10.46	-10.53	0.10	0.18			
			2	-10.40						
			3	-10.73						
	40	kaneka	1	-10.94	-10.66	0.15	0.25			
			2	-10.57						
			3	-10.46						
MAX						0.32	0.55		0.16	0.32
MIN						0.02	0.04		0.07	0.14
Ave.						0.12	0.21		0.11	0.22

Table 19 The Reproducibility of Log_{10}[PC10 (M)] in the Assay System

Test Substance	Test vial No.	Laboratory	Trial	Log_{10} [PC10 (M)] Data	within-Lab Mean	within-Lab SE	within-Lab SD	between-Lab Mean	between-Lab SE	between-Lab SD
Hematoxylin			1	-				-8.12	-	-
	1	ceri	2	-	-8.12	-	-			
			3	-8.12						
			1	-						
	2	sumitomo	2	-	-	-	-			
			3	-						
			1	-						
	3	otsuka	2	-	-	-	-			
			3	-						
			1	-						
	4	kaneka	2	-	-	-	-			
			3	-						
17α-Estradiol			1	-10.49				-9.97	0.17	0.33
	5	ceri	2	-10.69	-10.39	0.21	0.37			
			3	-9.98						
			1	-10.47						
	6	sumitomo	2	-9.84	-10.05	0.21	0.36			
			3	-9.84						
			1	-9.65						
	7	otsuka	2	-9.33	-9.61	0.15	0.26			
			3	-9.85						
			1	-10.06						
	8	kaneka	2	-9.83	-9.84	0.12	0.21			
			3	-9.64						
Benzophenone			1	-				-7.61	-	-
	9	ceri	2	-	-	-	-			
			3	-						
			1	-						
	10	sumitomo	2	-	-	-	-			
			3	-						
			1	-						
	11	otsuka	2	-	-	-	-			
			3	-						
			1	-7.61						
	12	kaneka	2	-	-7.61	-	-			
			3	-						
Bisphenol A			1	-7.77				-7.82	0.20	0.41
	13	ceri	2	-7.51	-7.73	0.12	0.21			
			3	-7.92						
			1	-7.73						
	14	sumitomo	2	-7.28	-7.30	0.25	0.43			
			3	-6.88						
			1	-10.20						
	15	otsuka	2	-7.08	-8.04	1.09	1.88			
			3	-6.82						
			1	-10.81						
	16	kaneka	2	-6.97	-8.23	1.29	2.23			
			3	-6.92						
Diethylhexyl phthalate			1	-				-5.49	-	-
	17	ceri	2	-	-	-	-			
			3	-						
			1	-						
	18	sumitomo	2	-5.49	-5.49	-	-			
			3	-						
			1	-						
	19	otsuka	2	-	-	-	-			
			3	-						
			1	-						
	20	kaneka	2	-	-	-	-			
			3	-						

Table 19- continued.

Genistein	21	ceri	1	-7.18				-8.12	0.07	0.14
			2	-8.40	-8.09	0.46	0.80			
			3	-8.70						
	22	sumitomo	1	-8.70						
			2	-7.84	-8.13	0.29	0.50			
			3	-7.84						
	23	otsuka	1	-7.94						
			2	-7.94	-7.96	0.01	0.03			
			3	-7.99						
	24	kaneka	1	-8.92						
			2	-8.05	-8.31	0.31	0.54			
			3	-7.95						
17α-Methyltestosterone	25	ceri	1	-7.66				-7.24	0.20	0.40
			2	-7.40	-7.71	0.19	0.33			
			3	-8.06						
	26	sumitomo	1	-7.44						
			2	-6.32	-6.78	0.34	0.59			
			3	-6.58						
	27	otsuka	1	-7.06						
			2	-6.74	-7.06	0.19	0.33			
			3	-7.40						
	28	kaneka	1	-7.59						
			2	-7.45	-7.41	0.12	0.22			
			3	-7.17						
4-tert-Octylphenol	29	ceri	1	-7.90				-8.09	0.13	0.25
			2	-7.96	-8.12	0.19	0.34			
			3	-8.51						
	30	sumitomo	1	-9.67						
			2	-7.65	-8.44	0.62	1.08			
			3	-7.99						
	31	otsuka	1	-7.96						
			2	-7.84	-7.88	0.04	0.07			
			3	-7.84						
	32	kaneka	1	-7.85						
			2	-8.07	-7.93	0.07	0.12			
			3	-7.89						
p-tert-pentylphenol	33	ceri	1	-7.54				-7.55	0.12	0.24
			2	-7.67	-7.72	0.12	0.21			
			3	-7.96						
	34	sumitomo	1	-9.79						
			2	-6.70	-7.78	1.00	1.74			
			3	-6.86						
	35	otsuka	1	-7.42						
			2	-7.34	-7.43	0.05	0.09			
			3	-7.51						
	36	kaneka	1	-7.46						
			2	-6.97	-7.28	0.16	0.27			
			3	-7.40						
17β-Estradiol	37	ceri	1	-11.84				-11.87	0.12	0.24
			2	-12.33	-12.14	0.15	0.26			
			3	-12.27						
	38	sumitomo	1	-11.74						
			2	-11.71	-11.80	0.07	0.13			
			3	-11.94						
	39	otsuka	1	-11.57						
			2	-11.35	-11.58	0.13	0.23			
			3	-11.81						
	40	kaneka	1	-12.20						
			2	-11.99	-11.97	0.14	0.25			
			3	-11.71						
MAX						1.29	2.23		0.20	0.41
MIN						0.01	0.03		0.07	0.14
Ave.						0.29	0.50		0.14	0.29

Table 20 The Reproducibility of Log_{10}[EC50 (M)] in the Assay System

Test Substance	Test vial No.	Laboratory	Trial	Log_{10} [EC50(M)]						
				Data	intra-Lab			inter-Lab		
					Mean	SE	SD	Mean	SE	SD
alpha-Estradiol	5	ceri	1	-9.02	-9.25	0.12	0.20	-8.89	0.13	0.26
			2	-9.37						
			3	-9.37						
	6	sumitomo	1	-	-8.82	0.01	0.01			
			2	-8.83						
			3	-8.82						
	7	otsuka	1	-8.48	-8.64	0.12	0.21			
			2	-8.57						
			3	-8.88						
	8	kaneka	1	-9.04	-8.85	0.10	0.17			
			2	-8.69						
			3	-8.84						
Bisphenol A	13	ceri	1	-6.25	-6.31	0.08	0.13	-6.10	0.07	0.14
			2	-6.22						
			3	-6.46						
	14	sumitomo	1	-	-5.99	0.00	0.00			
			2	-5.99						
			3	-5.99						
	15	otsuka	1	-6.00	-6.07	0.06	0.10			
			2	-6.18						
			3	-6.03						
	16	kaneka	1	-6.15	-6.03	0.06	0.10			
			2	-5.97						
			3	-5.98						
Genistein	21	ceri	1	-5.95	-5.72	0.20	0.34	-4.77	0.74	1.49
			2	-5.33						
			3	-5.88						
	22	sumitomo	1	-	-5.62	0.34	0.49			
			2	-5.27						
			3	-5.96						
	23	otsuka	1	-5.00	-2.57	2.55	4.41			
			2	-5.24						
			3	2.52						
	24	kaneka	1	-5.21	-5.18	0.20	0.35			
			2	-4.82						
			3	-5.51						
17α-Methyltestosterone	25	ceri	1	-	-6.06	-	-	-5.13	0.92	1.30
			2	-						
			3	-6.06						
	26	sumitomo	1	-5.73	-3.65	2.03	3.51			
			2	-5.63						
			3	0.41						
	27	otsuka	1	-5.53	-5.69	0.13	0.23			
			2	-5.95						
			3	-5.59						
	28	kaneka	1	-	-	-	-			
			2	-						
			3	-						

Table 20-continued

Test Substance	Test vial No.	Laboratory	Trial	Log10 [EC50(M)] Data	intra-Lab Mean	intra-Lab SE	intra-Lab SD	inter-Lab Mean	inter-Lab SE	inter-Lab SD
			1	-6.93						
	29	ceri	2	-6.93	-7.08	0.15	0.27			
			3	-7.39						
			1	-6.25						
	30	sumitomo	2	-6.36	-6.27	0.05	0.08			
			3	-6.20						
4-tert-Octylphenol			1	-6.80				-6.72	0.17	0.34
	31	otsuka	2	-7.06	-6.85	0.11	0.19			
			3	-6.70						
			1	-6.58						
	32	kaneka	2	-6.69	-6.67	0.05	0.08			
			3	-6.75						
			1	-5.92						
	33	ceri	2	-5.90	-6.21	0.30	0.52			
			3	-6.80						
			1	0.81						
	34	sumitomo	2	-5.61	-3.56	2.19	3.78			
			3	-5.88						
p-tert-pentylphenol			1	-5.71				-5.36	0.61	1.22
	35	otsuka	2	-6.18	-5.95	0.14	0.23			
			3	-5.98						
			1	-5.55						
	36	kaneka	2	-5.84	-5.73	0.09	0.16			
			3	-5.80						
			1	-10.69						
	37	ceri	2	-10.97	-10.92	0.13	0.22			
			3	-11.12						
			1	-11.03						
	38	sumitomo	2	-10.48	-10.75	0.16	0.28			
			3	-10.73						
17β-Estradiol			1	-10.39				-10.67	0.10	0.20
	39	otsuka	2	-10.34	-10.48	0.12	0.21			
			3	-10.72						
			1	-10.82						
	40	kaneka	2	-10.47	-10.53	0.15	0.26			
			3	-10.32						
MAX *				-10.43	-	-	-	-	-	-
MIN *						0.00	0.00		0.07	0.14
Ave. *						0.37	0.64		0.39	0.71

* Excepting for Hematoxylin, Benzophenone and Diethylhexyl phthalate

109 An independent statistical assessment of the inter-laboratory reproducibility (reliability) and protocol transferability components of the validation of the assay was conducted [1)].

110 In addition the results from individual runs as well as overall variability (within-laboratory or between-laboratory) were similarly independently assessed. A summary of overall between-laboratory standard deviation (SD) estimates for positives are presented in Table 21. The full report is available in Appendix 6.

[1]) These analyses were conducted by Dr Yutaka Aoki, ASPH Fellow at the US EPA.

Table 21 Summary of Overall Between-laboratory SD of LogPC10 and LogPC50

Chemical	Log_{10}[PC10 (M)]	Log_{10}[PC50 (M)]
17α-Estradiol	0.31	0.29
Bisphenol A	0.29	0.27
Genistein	0.31	0.15
17α-Methyltestosterone	0.21	0.21
4-*tert*-Octylphenol	0.15	0.18
p-*tert*-Pentylphenol	0.30	0.08
17β-Estradiol	0.21	0.24
Arithmetic mean	0.25	0.20
	0.26*	0.20*

* For all chemicals other than 17β-Estradiol

111 Overall between-laboratory SD of 0.25 means that a future parameter estimate from a laboratory drawn from a universe of laboratories like the four laboratories in the inter-laboratory study is expected to fall in the range between 0.33 times the true value and 3.1 times the true value ($0.33 = 1/3.1 = 10^{-1.95*0.25}$) with a probability of 95%. The SDs of Log_{10}[PC10 (M)] and Log_{10}[PC50 (M)] were in the range of the minimum and maximum ratios to the true value of 0.25 and 4.0, respectively. The additional independent statistical analysis (Appendix 6) concluded that the level of variability of this assay seemed satisfactorily low for the intended use of the assay.

112 These results clearly demonstrate the high reproducibility, technical transferability and strength of the stably transfected TA assay system using hER-HeLa-9903 cell line.

7. DISCUSSION

113 Numerous chemicals found in the environment, as well as some synthetic chemicals may disrupt the endocrine functions of wildlife and humans. At the present time, there is global concern regarding endocrine disruption effects resulting from chemical exposure, particularly those mediated by the ER. To ensure the safety of chemicals, an effective procedure for screening chemicals for endocrine modulating activity has been pursued by regulatory agencies in several countries, including the United States Environment Protection Agency (US-EPA), Japan and Europe.

114 The endocrine disrupter testing and assessment task force (EDTA) was established in 1997 and the OECD conceptual framework for testing and assessment of potential endocrine disrupting chemicals from both new and existing substances was agreed upon at the 6th EDTA meeting (OECD, 2002). This framework is not a testing scheme but rather a

toolbox that contains various tests, each of which can contribute information about detecting the hazards of endocrine disruption. Within this toolbox framework, there are five levels, each level corresponding to a different level of biological complexity. Some *in vitro* assays, such as the transcriptional activation (TA) assays and receptor binding assays, have been proposed and incorporated as "Level 2" *in vitro* assays to provide mechanistic information for prioritization purposes.

115 In the US, the US-EPA developed a chemical screening and testing program consisting of a tiered system to evaluate the endocrine disrupting effects of chemicals (Earl-Gray L. Jr., 1998). In this program, the hormone receptor mediated reporter gene assay system is proposed for pre-screening and the Tier 1 screening battery. Within the European Union (EU), the development and validation of internationally agreed test methods to assess endocrine disruption in people and wildlife is part of the European Community Strategy on Endocrine Disrupting Substances (COM (99) 706), both within the OECD and as part of the development of an appropriate EU testing strategy. The EC Registration, Evaluation and Authorisation of CHemicals 'REACH' programme is expected to enter into force in 2007 (EDSTAC, 1998; ECB, 2006). In Europe, several *in vitro* TA assays are currently being validated within the EU integrated project ReProTect, and receptor binding assays internationally, with the US, Japan and Europe, under the OECD umbrella.

116 In order to develop and validate a test protocol to support the development of test guidelines for the detection of chemicals possessing the potential estrogenic activity through human estrogen receptor α (hERα), we conducted a series of validation tests for the hERα mediated stably transfected TA assay established in Japan under the agreement of the 1st OECD VMG-NA meeting that Japan would take lead in this assay.

117 Validation work on the hERα mediated stably transfected TA assay using a stable clone consisted of both pre-validation and inter-laboratory validation. The pre-validation work was conducted in the Chemicals Evaluation and Research Institute (CERI), Japan and the inter-laboratory validation study was conducted within four Japanese domestic laboratories upon the initiative of CERI.

118 Under the pre-validation study, the stability of the responses to E2, BPA and TS were measured in the range from 10^{-12} to 10^{-6}M. E2 produced a typical sigmoidal response in all 13 experiments and the mean Log_{10}[EC50 (M)] ± SD for E2 was -11.17 ± 0.25 and the 95% confidential interval ranged from -11.02 to -11.32. The 95% confidential interval for E2 was within acceptable and normal variation observed for such assays. The precise EC50 values of the other two chemicals, BPA and TS, could not be calculated because

these chemicals could not show complete sigmoidal dose response over the concentration range tested.

119 As for the results of the inter-laboratory validation study, statistical analysis revealed that the reproducibility within four participating laboratories of this assay system appeared to have acceptably low between-laboratory variation (in-house analysis and Appendix 6). The results showed that the test system has highly reliable and that the test protocol used in this study is adequately transferable for practical use.

120 The additional independent statistical analysis (Appendix 6), recommends that with respect to the endpoint parameter to be used for this assay system, Hill equation-based nonlinear regression be used for estimating PC10 values, because it has an advantage over linear interpolation, in terms of accuracy and precision. However, for practical purposes, the authors do not agree for the following reasons:

i) As the linear regression based PC values can achieve high-throughput performance and as this type of assay will require high-throughput performance to screen a vast number of chemicals for prioritization, before the implementation of higher level tests, and the linear regression based PC10 is easy to apply for batch-processing in spread sheet of Microsoft Excel.
ii) The calculation of Hill equation-based PC values requires at least 4 data points although linear regression-based PC values requires only 2 data points. Accordingly it would not be possible to calculate the Hill equation-based PC value for putative weak estrogens that can induce transactivation only at the highest concentration.
iii) The application of Hill equation-based PC values would be employed after consideration of the purpose of this assay, the applicability to batch-processing and the applicability to weak estrogens. With regards to the estimation of PC50, both the linear regression-based and the Hill equation-based can provide similar results.

121 Log_{10}[EC50 (M)] obtained with the proposed assay system showed high consistency with the data obtained by the ERα-CALUX, HELN-ERα and LUMI-CELL™ assay systems at. R^2=0.987 (n=8), R^2=0.937 (n=7) and R^2=0.922 (n=7) respectively. (Sonneveld et al, 2006; Escade et al, 2006; Jefferson et al., 2002). Moreover, the correlation coefficient between log_{10}[EC50 (M)] obtained from the stably transfected TA assay using hER-HeLa-9903 cell line and logEC50s in the ICCVAM report (2003) was successful (R^2=0.802, n=20). As for the regression formula for each individual assay system, the slopes of the formula against ERα-CALUX and LUMI-CELL™ were nearly 1.0 (0.956 for ERα-CALUX, 1.01 for LUMI-CELL™), however the slope for HELN-ERα cell systems was 0.712. These results

suggest that the assay system using hER-HeLa-9903 gives much the same EC50 values with the assay systems using ERα-CALUX and LUMI-CELL™. On the same time, the assay system using hER-HeLa-9903 tend to give slightly higher EC50 values for weak estrogenic compounds compared with HELN-ERα cell systems. However the Speaman's correlation coefficient between both assay systems is 0.9643. Accordingly this trend gives no problem to the assay system for the main purposes of this assay system; detection and prioritizing of the estrogenic activity of chemicals.

122 The results obtained by the stably transfected TA assay and the information given in the ICCVAM report (2003) were compared with regard to 46 chemicals. The information in ICCVAM report (2003) was collected based on several different *in vitro* assay systems to detect estrogenic activities, and the assay performance parameters for the stably transfected TA assay, concordance, sensitivity and specificity, were 80%, 79% and 82%, respectively.

123 So as to provide supplemental information, the results obtained from the receptor binding assay using hERα and the stably transfected TA assay were compared with regard to 48 chemicals. The concordance, sensitivity and specificity, were 77%, 71% and 83%, respectively.

124 Furthermore, as a part of supplemental information, the results obtained by the uterotrophic assay and the stably transfected TA assay were also compared with regard to 48 chemicals, and the concordance, sensitivity and specificity, were 90%, 91% and 88%, respectively.

125 The high concordance observed when comparing the endpoints of the stably transfected TA ER assay and the other ER endpoints, including those provided in the ICCVAM report (2003), the ER binding assay and the immature rat uterotrophic assay suggest that the outcomes of the stably transfected TA assay can provide reliable information about the biological effect of chemicals mediated by receptor-ligand interaction.

126 Accordingly, the overall assay performance of the stably transfected TA assay system using the hER-HeLa-9903 cell line was deemed satisfactory for practical use, and in accordance with GD 34 (See Table 22).

127 This validation report was completed with the kind assistance of the preliminary validation assessment panel of the **'Japanese multi-laboratories validation study of a stably transfected ER alpha mediated reporter gene assay in Japan' (PVAP).** The

final report from PVAP can be found in Appendix 7. The summary of queries from PVAP and corresponding answers are provided in Appendix 8.

Table 22 Checklist to Assess Whether the Validation Principles in OECD GD34 were Met, Partially Met, or Not Met by the Japanese Multi-laboratories Validation Study of the Stably Transfected TA Assay.

Principles	Met /Not met	Explanation and Justification
a) The rationale for the test method should be available.	MET	The proposed test method is used to provide mechanistic information and used for the purposes of prioritizing or grouping substances that has a potential estrogenic activity mediated estrogen receptor alpha.
b) The relationship between the test method's endpoint(s) and the (biological) phenomenon of interest should be described.	MET	The endpoint is a luciferase activity that is produced as a result of transcriptional activation of the reporter gene. Stimulation of reporter gene expression in response to ER agonists, is thought to be mediated by direct binding where E2-liganded ER binds directly to estrogen responsive element (ERE) and interacts directly with coactivator proteins and components of the RNA polymerase II transcription initiation complex resulting in enhanced transcription.
c) A detailed protocol for the test method should be available.	MET	This is provided in the draft report appendices. Further statistical discussions on data analysis and decision criteria are provided in paragraphs 3.11 and 4.10 and appendices 2 and 3.
d) The intra-, and inter-laboratory reproducibility of the test method should be demonstrated.	MET	Demonstrated.
e) Demonstration of the test method's performance should be based on the testing of reference chemicals representative of the types of substances for which the test method will be used. A sufficient number of the reference chemicals should have been tested under code to exclude bias.	NOT FULLY MET	Reference chemicals are necessary to establish the relevance and reliability of the proposed test and should include a minimum number of chemicals possessing expected range of response (strong, moderate, weak and negative). Nine coded chemicals and one positive chemical, E2, (Table 9) possessing expected ranges of response were tested under the inter-laboratory validation, and relevance and reliability were demonstrated. Data were collected at the lead laboratory for further comparison with 46 chemicals selected from the ICCVAM list, and these data give a strong indication of relevance of the proposed test method (paper in preparation).
f) The performance of the test method should have been evaluated in relation to relevant information from the species of concern, and existing relevant toxicity testing data.	MET	Relevant information obtained from the ICCVAM ED list, and results for selected chemicals were compared with this list. All data used for this comparison were produced at the lead laboratory. Additionally a data comparison was conducted with the proposed test method and the hERα Binding assay (and data from the immature rat uterotrophic assay) with good concordance.
g) Ideally, all data supporting the validity of a test method should have been obtained in accordance with the principles of GLP.	NOT FULLY MET	The pre-validation and data collection for comparison with ICCVAM list or hERα binding assay were not conducted to GLP, but in the spirit of GLP. The inter laboratory validation however was conducted to GLP. While GLP is ideal, for practical purposes, the fact that components of this validation and data comparison was not always to GLP is considered acceptable.
h) All data supporting the assessment of the validity of the test method should be available for expert review.	MET	A detailed test protocol is available, and data is available for independent review. Benchmark: The responses of positive control (E2) and vehicle control (DMSO) wells in each assay plate act as a benchmark such that reproducible results can be obtained when generating PC_{10} and PC_{50} values normalized by the positive control response.

7.1 Limitations of the assay, and further validation considerations

7.1.1 Function of this test method and application of a prediction model.

128 The "Solna Principles"(1996) and GD34 specify that a series of reference chemicals must be utilized to demonstrate the test method's performance, but with flexibility appropriate to the test method undergoing validation. Where an *in vitro* test method is intended as an alternative method for *in vivo* testing, a prediction model can be defined to clarify the limitations of the *in vitro* assay to predict the *in vivo* results representing current scientific knowledge. The test method validated in this report addresses the generally accepted nuclear receptor mediated mechanism of ERα activation only. It has not been directly extrapolated to the complex *in vivo* estrogenic situation in the format of a prediction model algorithm. However as part of the EDTA Conceptual Framework toolbox, users might wish to develop this test method as an alternative for specified *in vivo* ERα screening assays, by utilizing the test method to produce data for different purposes, including the development of a prediction model.

7.1.2 Detection of anti-estrogenic activity

129 This validation effort only considered agonists. For screening and prioritization purposes, ideally chemicals would also be assessed for antagonistic activity. The test method described in this report can also address this need and preliminary data are available. Although antagonists were not included in this validation effort, the antagonist protocol is included in APPENDIX 5, together with data for three strong antagonists which so far have been tested nine times by the CERI laboratory. Additional data for 250 chemicals can be provided on request.

130 In the near future, the currently validated protocol could be updated and extended with the optimization of the antagonist ERα TA assay, as and when such a protocol might be supported and made available in a catch-up validation manner.

7.1.3 Non-receptor mediated luminescence signals

131 Non-receptor mediated luminescence signals have been reported at concentrations higher than 1 µM of the phytoestrogens genistein, daidzein and biochanin A (Escade et al., 2006). Escade et al. observed an over activation of the luciferase reporter gene in a stably transfected ER HeLa cell line (HELN-ERα, HELN-ERβ and the parental HELN cell line).

This effect has also been previously reported for genistein (Kuiper et al., 1998), and indicates that luciferase expression obtained at high concentrations of phytoestrogens needs to be examined carefully in such stably transfected TA assay systems. However, this effect has not been reported in the literature with respect to the ERα screening of industrial chemicals, which is the intended regulatory use of this proposed test method.

7.1.4 Metabolic capability and TA assays

132 This ER TA assay method does not include metabolism considerations, beyond the capacity to screen substances that are also metabolic products of parent compounds.

133 Metabolism is known to be a bottleneck in the development of *in vitro* tests for regulatory purposes (Coecke et al 2006). For instance, we have conducted a study on 64 chemicals using S9 mix and performed in a stably transfected TA assay and two potential problems were observed to be associated with the use of S9. *Trans*-Stilbene was used as a reference agonist because it needs to be hydroxylated to *trans*-4-hydroxystilbene and *trans*-4,4'-dihydroxystilbene in order to be active. With the amounts of S9 required, E2 at normally active concentrations was inactivated, while higher concentrations of E2 were again more active with S9, than without. This is explained by an inversion of the concentration response curve that has a maximum at about 100 pM. The second problem that was encountered was the low reproducibility (unpublished data: Takeyoshi et al, extract from the OECD draft Detailed Review Paper, "The use of metabolising systems for in vitro testing of endocrine disruptors", June 2006).

8. CONCLUSIONS

134 Results of the inter-laboratory validation study within four Japanese domestic laboratories showed the high reproducibility of the assay system and good technical transferability of the assay protocols.

135 One of the primary purposes of prescreening procedures, such as the stably transfected TA assay and the receptor binding assay, is to prioritize chemicals for subsequent testing at the higher screening stages. Accordingly, a high concordance and a low false negative rate are required for prescreening procedures. Two-by-two table analytical comparison of the results of the stably transfected TA assay with those of *in vivo* screening tests, such as the uterotrophic assay, revealed that the stably transfected TA assay demonstrated a high concordance and low false negative rate. These results suggested that the stably

transfected TA assay is a promising method to be utilized in the prescreening process of an endocrine disruptor testing strategy.

136 The stably transfected TA assay system can be conducted with approximately 100 chemicals within a week at a relatively low cost (approximately $1,290, €1,700, ¥200,000 per chemical).

137 Moreover, the system employs an established cell line, so the system is compliant with the 3R policies, and it can furthermore contribute to the reduction of animals being tested for regulatory purposes, with respect to ER mediated endocrine disruption, particularly with respect to *in vivo* assays such as the uterotrophic assay.

138 A Japanese human ER mediated stably transfected TA assay system using hER-HeLa-9903 is well-established and has been shown to be a well-validated assay for development of an OECD test guideline for the detection of chemicals possessing potential estrogenic activity through hERα. The assay is a therefore a promising method to use in the prescreening process of an endocrine disruptor screening strategy.

9. RECOMMENDATIONS

139 Currently, there are many types of luciferase reagents and luminometers. To produce reproducible results, a wide dynamic range of raw signal counts between positive and negative (vehicle) control responses would be required. In our experience, the dynamic range between positive and vehicle control responses depends upon the combination of the luciferase reagent and the sensitivity of the luminometer used for the study. Accordingly, any suitable combination of a luciferase assay reagent and luminometer should be determined in the individual laboratory by preliminary testing with several control compounds, such as E2, BPA, etc.

140 With regards to the parameters used for the study, historically the EC50 value has been used for indicating the relative biological activity of chemicals. Calculation of EC50, using Hill's logistic equation, requires at least four data points and complete sigmoidal dose response to estimate accurate and reproducible values. Some weak estrogens cannot give complete sigmoidal dose responses in the stably transfected TA assay, and it is difficult to obtain accurate EC50 values. In the case of these weak estrogens, PC10 and PC50 values calculated using linear regression can be obtained with accuracy and reproducibility. PC50 values can also provide the relative estrogenic potency and this

parameter reflects ER mediated biological effects from the results of comparative studies with ER binding and/or immature rat uterotrophic assays. Moreover a high-throughput assay design can be achieved by using PC values and fixed-dose format. Taking these factors together, PC values are promising parameters for TA assays.

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APPENDIX 1 List of Participating Laboratories

Testing facility 1 (Coordination and enforcement of the study)
Hita laboratory
Chemicals Evaluation and Research Institute (CERI)
3-822, Ishii-machi, Hita-shi, Oita 8770061, Japan

Testing facility 2 (Enforcement of the study)
Environmental Health Science Laboratory,
Sumitomo Chemical Co., Ltd.
1-98, Kasugade-naka 3-chome, Konohana-ku,
Osaka 554-8558, Japan

Testing facility 3 (Enforcement of the study)
EDC Analysis Center, Otsuka Life Science Initiative,
Otsuka Pharmaceutical Co., Ltd.
224-18, Ebisuno Hiraishi, Kawauchi-cho, Tokushima
7710195, Japan

Testing facility 4 (Enforcement of the study)
KANEKA Techno-Research Co., Ltd.
1-8, Miyamae-cho, Takasago-cho, Takasago-shi,
Hyogo 6768688, Japan

APPENDIX 2 Standard operating procedure (SOP) for detection of estrogenic activity using the reporter gene assay

STANDARD OPERATING PROCEDURE (SOP)

for detection of estrogenic activity using the reporter gene assay

Description: This document provides a methodology for detecting the estrogenic activity of chemicals by the reporter gene assay technique using the hER-HeLa-9903 cell line.

Chemicals Evaluation and Research Institute

CERI-Japan

Materials and Methods

1. Test chemicals

Test chemicals should be dissolved in dimethylsulfoxide (DMSO) at a concentration of 10 mM.

2. Competitive substance

17β-Estradiol (E2)

3. Vehicle for chemical stock solutions

Dimethylsulfoxide (DMSO) should be used for the vehicle.

4. Materials

4.1 Cell lines

The hERα-*HeLa*-9903 stable cell line (Sumitomo Chemicals Co.) will be used for the assay.

4.2 Cell cultures (See support protocols No.1 – No. 4)

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.

4.3 Preparation of chemicals

All chemicals are dissolved in DMSO at a concentration of 10 mM, and those solutions are 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. In the case of positive control substance (E2), stock solutions are prepared at concentrations of 100 µM, 10 µM, 1 µM, 100 nM, 10 nM, 1 nM and 100 pM.

4.4 Preparation of cells

Assay plates are prepared according to the support protocol No. 5.

4.5 Reagents for the luciferase assay

A commercial luciferase assay reagent, Steady-Glo Luciferase Assay System (Promega, E2510 and its equivalents) or a standard luciferase assay system (Promega, E1500 and its equivalents) are used in this study. A bottle of Luciferase Assay Substrate is dissolved with the Luciferase Assay Buffer. The dissolved substrate should either be used immediately or stored below -20 C.

When using the standard luciferase assay system, Cell Culture Lysis Reagent (Promega,

E1531) should be used before adding the substrate.

4.7 Chemical exposure

Each test chemical diluted in DMSO are 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 quadruplicate.

To achieve the above-described test conditions, each chemical stock solution should be serially diluted in a common ratio of 1:10 with DMSO in order to obtain 10 µM, 10 µM, 1 µM, 100 nM, 10 nM and 1 nM working solutions. Exactly 1.5 µL of 10 mM chemical stock and 6 working solutions will dilute in serum-free EMEM (500 µL).

Then 50µL of the diluted test samples will be added to each well of the assay plate according to the assignment table shown in Fig.1.

Positive control wells (n=6) treated with 100 pM of E2 and vehicle control wells (n=6) treated with DMSO alone will be prepared on every assay plate. After adding the chemicals, the assay plates will be incubated in a CO_2 incubator for 20-24 hours to induce the reporter gene products.

Fig.1 Typical assignment of the assay plate for the agonist assay

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

VC: Vehicle control (DMSO at 0.1%); PC: Positive control (100 pM of E2)

4.8 Luciferase assay (See support protocol No. 6)

Luciferase activity will be measured with the luciferase assay reagent and a luminometer according to the manufacturer's instructions.

5. Analysis of data

The luminescence signal data are processed, and the average for the negative control wells were calculated. The integrated value for each test well is divided by the average integrated value of the negative control wells to obtain individual relative transcriptional activity. Then the average transcriptional activity is calculated for each concentration of the test chemical. The PC50 and PC10 values are 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 spread sheet. If Hill's logistic equation is applicable to dose response data, EC50 should be calculated by the 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 Fig 2.

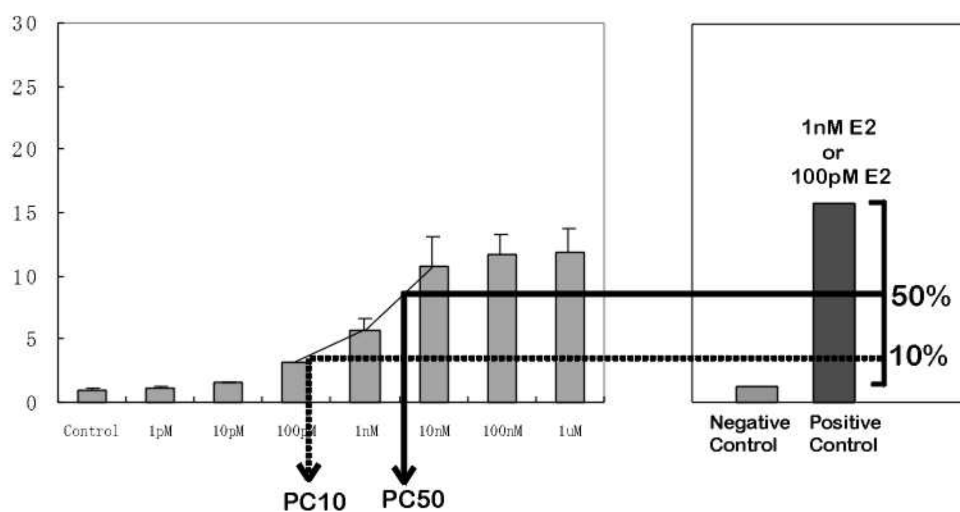

Fig. 2 Description of PC10 and PC50

SUPPORT PROTOCOLS

No.1 Preparation of the medium

Reagents

Eagle's Minimal Essential medium without neutral red (Nissui Pharmaceutical Co.)

10% Sodium bicarbonate ($NaHCO_3$)

Dissolve 10 grams of $NaHCO_3$ to a final volume of 100 mL with water. Next, the solution should be sterilized using a vacuum-driven bottle-top sterilization filter unit and stored at room temperature.

3% Glutamine

Dissolve 3 grams of glutamine to a final volume of 100 mL with water. Next, the solution should be sterilized using vacuum-driven bottle-top sterilization filter unit. Prepared 3% Glutamine should be stored in aliquots under -20°C.

Dextran-coated charcoal (DCC)-treated Fetal bovine serum (DCC-FBS)

Prepared and provided by CERI-Japan.

Preparation of EMEM*

Add the following reagents into a 1L conical glass flask and then add Milli-Q water to bring the total volume to one liter:

- 9.4 grams of pre-made powder medium
- 18 mL 10% Sodium Bicarbonate
- 3% Glutamine 12 mL

Preparation of 10%FBS-EMEM*

Add 56 mL of dextran-coated charcoal (DCC)-treated fetal bovine serum (DCC-FBS) to 500 mL EMEM.

*EMEM and 10%FBS-EMEM should be stored in a refrigerator after being sterilized with a vacuum-driven bottle-top sterilization filter unit.

SUPPORT PROTOCOLS

No. 2. Reconstitution of cells from the frozen stock

1. Remove the vial from the liquid nitrogen or freezer and immediately transfer it to a 37°C water bath.
2. While holding the tip of the vial, gently agitate the vial.
3. When completely thawed, transfer the cell stock into 5mL pre-warmed 10%FBS-EMEM in 15 a mL conical tube.
4. Centrifuge the tube at 1100 rpm (200-300 x g) for 5-min, then remove the supernatant carefully.
5. Resuspend the cell with 10 mL of 10%FBS-EMEM and place it in a 90 mm culture dish.
6. Incubate the cells in a 5% CO_2 incubator at 37°C.

SUPPORT PROTOCOLS

No. 3. Propagation

1. Remove the medium from the culture dish with a sterile pipette or sucker.
2. Rinse the cell with 5 mL of PBS.
3. Remove the PBS with a sterile pipette or sucker.
4. Add 2 mL of Trypsin-EDTA solution (0.25% Trypsin + 0.02%EDTA/PBS), enough to coat the bottom of the culture dish, and then remove the excess.
5. Allow the Trypsin treated cells to stand for about 3-min. in a 5% CO_2 incubator at 37°C. (Monitor the cells under a microscope. The cells are beginning to detach when they appear rounded.)
6. Tap the dish gently.
7. Wash with 5 mL of 10%FBS-EMEM to remove the adherent cells.
8. Count the number of cells.
9. Dilute the cell suspension with 10%FBS-EMEM to 0.4-1.0 x 10^5 cells/mL.
10. Place 10 mL of cell suspension in a 90 mm culture dish.
11. Incubate the cells in a 5% CO_2 incubator at 37°C.

SUPPORT PROTOCOLS

No. 4. Preparation of frozen stock

1. Remove the medium from the culture dish with a sterile pipette or sucker.
2. Rinse the cell with 5 mL of PBS.
3. Remove the PBS with a sterile pipette or sucker.
4. Add 2 mL of Trypsin-EDTA solution, enough to coat the bottom of the culture dish, and then remove the excess.
5. Allow the Trypsin-treated cell to stand for about 3-min. in a 5% CO_2 incubator at 37°C. (Monitor the cells under a microscope. The cells are beginning to detach when they appear rounded.)
6. Tap the dish gently.
7. Wash with 5 mL of 10%FBS-EMEM to remove the adherent cells.
8. Count the number of cells.
9. Centrifuge the tube at 1100 rpm (200-300 x g) for 5-min., and remove the supernatant carefully.
10. Add Cell-Banker* (Juji Field Inc.) and resuspend the cell at a density of ca. 1 x 10^4 cells/mL.
11. Make 1 mL aliquots of cell stock.
12. Freeze and store the cell stock below -80°C.**

*A conventional freeze medium (90% FBS/10% DMSO) can be used in place of Cell-Banker.
**Storage in liquid nitrogen would be preferable for long-term storage (more than 3 months).

SUPPORT PROTOCOLS

No. 5 Preparation of the assay plate

Prepare a dish of cultured hERα-HeLa-9903 cells

1. Remove the medium from the culture dish with a sterile pipette or sucker.
2. Rinse the cells with 5 mL of PBS.
3. Remove the PBS with a sterile pipette or sucker.
4. Add 2 mL of Trypsin-EDTA solution, enough to coat the bottom of the culture dish, and then remove the excess.
5. Allow the Trypsin-treated cells to stand for about 3-min. in a 5% CO_2 incubator at 37°C. (Monitor the cells under a microscope. The cells are beginning to detach when they appear rounded)
6. Tap the dish gently.
7. Wash with 5 mL of 10%FBS-EMEM to remove the adherent cells.
8. Count the number of cells.
9. Centrifuge the tube at 1100 rpm (200-300 x g) for 5min, and remove the supernatant carefully.
10. Resuspend the cell with 10%FBS-EMEM to obtain a final cell density of 1 x 10^5 cells/mL.
11. Add 100μL of cell suspension into each well of a 96 well assay plate (Nunc #136102 or an equivalents).
12. Incubate the cell in a 5% CO_2 incubator at 37°C for 3-h.
13. Proceed to test, positive and vehicle chemical exposure of assay plate.

SUPPORT PROTOCOLS

No. 6-1. Chemiluminescence detection with a standard luciferase reagent

Reagents

Cell lysis reagent (4.5x): Dilute 10 mL of 5×Cell Culture Lysis Reagent (CCLR, #E1531) with 45 mL of distilled water.

Luciferase Assay Reagent: Add 1 vial 105 mL of Luciferase Assay buffer (Promega, #E4550) into a vial containing Luciferase Assay Substrate (Promega, #E4550), and dissolve the substrate thoroughly. Store the substrate below -20°C if necessary.

Chemiluminescence Detection

1. Flick and drain off the contents of the assay plate.
2. Add 100µL of PBS to the well to wash the plate.
3. Flick and drain off the contents of the assay plate.
4. Add 100µL of PBS to the well to wash the plate again.
5. Flick and drain off the contents of the assay plate.
6. Add 15 µL of Cell lysis reagent (4.5x) to the wells.
7. Incubate for 10-min. at room temperature.
8. Add 50 µL of Luciferase Assay Reagent to the wells.
9. Read the plates on a chemiluminescence plate reader.

SUPPORT PROTOCOLS

No. 6-2. Chemiluminescence detection with a luciferase reagent using the Steady-Glo Luciferase Assay System

Reagents

Luciferase Assay Reagent: Add 1 vial (100 mL) of Luciferase Assay buffer into a vial containing Luciferase Assay Substrate (Promega, #E2520), and dissolve the substrate thoroughly. Store the substrate below -20°C if necessary.

Chemiluminescence Detection

1. Remove 50μL of the assay medium from all wells of the assay plate.
2. Add 100μL of the Luciferase Assay Reagent to wells.
3. Allow to stand for 5-min.
4. Read the plates on a chemiluminescence plate reader.

APPENDIX 3 Protocol used for the inter-laboratory validation study

Study Code: R10-xxxx

Multi-lab validation study for the ERα mediated reporter gene assay

xx, 2005

Chemicals Evaluation and Research Institute
CERI-Japan

Multi-lab validation study for the ERα mediated reporter gene assay

Participating laboratories

Testing facility 1 (coordination and enforcement of the study)

Testing facility 2 (enforcement of the study)

Testing facility 3 (enforcement of the study)

Testing facility 4 (enforcement of the study)

Aim of the study: To appraise the reliability and reproducibility of the ERα mediated reporter gene assay method by conducting the assays within multiple laboratories utilizing the same test chemicals. The technical transferability will be also evaluated in this study.

GLP: This study will be conducted in compliance with the "OECD principle of Good Laboratory Practice," November 26, 1997.

Proposed study dates

Start	Date
Completion	Date

Persons concerned in the study

Study director

Person in charge of individual studies conducted by each laboratory

Testing facility 1:

Testing facility 2:

Testing facility 3:

Testing facility 4:

Quality Assurance Supervisor

Peer reviewers

(To be announced)

Materials and Methods

1. Test chemicals

The test chemicals to be used in this study are listed in Table 1. All chemicals will be coded and provided by CERI as 10 mM solutions in dimethylsulfoxide (DMSO).

2. Positive control substance

2.1 Chemical name

17β-Estradiol (E2)

2.2 Lot No.

ACK5754

2.3 Manufacturer

Wako Pure Chemicals, Japan

2.4 Storage

To be stored at room temperature in a shading bottle.

3. Vehicle control (vehicle for chemical stock solutions)

3.1 Chemical name

Dimethylsulfoxide (DMSO)

3.2 Lot No.

PKF5322

3.3 Manufacturer

Wako Pure Chemicals, Japan

3.4 Storage

To be stored at room temperature

4. Materials

4.1 Test systems

The hERα-*HeLa*-9903 stable cell line system (Sumitomo Chemicals Co.) will be used for the assay.

Each laboratory should conduct three series of assays with the nine test chemicals listed in Table 1 on independent days.

4.2 Cell lines

hERα-*HeLa*-9903 will be provided by the Sumitomo Chemical Co. Ltd.

4.3 Cell culture (See support protocols No.1 – No. 4)

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.

4.4 Preparation of chemicals

All test chemicals dissolved in DMSO at 10 mM will be serially diluted with the same solvent at a common ratio of 1:10 in order to prepare concentrations of 1 mM, 100 μM, 10 μM, 1 μM, 100 nM and 10 nM in DMSO. In the case of positive control substance (E2), DMSO solutions will be prepared at concentrations of 100 μM, 10 μM, 1 μM, 100 nM, 10 nM, 1 nM and 100 pM.

4.5 Preparation of cells

Assay plates will be prepared according to support protocol No. 5.

4.6 Reagents for luciferase assay

A commercial luciferase assay reagent, Steady-Glo Luciferase Assay System (Promega, E2510 and its equivalents) or a standard luciferase assay system (Promega, E1500 and its equivalents) will be employed in this study. A bottle of Luciferase Assay Substrate is dissolved with the Luciferase Assay Buffer. Dissolved substrate should either be used immediately or stored below -20°C.

In cases where the standard luciferase assay system is used, Cell Culture Lysis Reagent (Promega, E1531) should be used before adding the substrate.

4.7 Chemical exposure

Each test chemical diluted in DMSO will be added to the wells for final concentrations of 10 μM, 1 μM, 100 nM, 10 nM, 1 nM, 100 pM, and 10 pM (10^{-11}-10^{-5}M) for the test chemicals and 100 nM, 10 nM, 1 nM, 100 pM, 10 pM, 1 pM, 0.1 pM (10^{-13}-10^{-7}M) for the positive control substance, in triplicate.

To achieve the above-described test conditions, each chemical stock solution provided by CERI should be serially diluted in a common ratio of 1:10 with DMSO in order to obtain 10 μM, 10 μM, 1 μM, 100 nM, 10 nM and 1 nM working solutions. Exactly 1.5 μl of 10 mM chemical stock and 6 working solutions will dilute in serum-free EMEM (500 μl).

Then 50μl of the diluted test samples will be added to each well of the assay plate according to the assignment table shown below.

Positive control wells (n=3) treated with a natural ligand (1 nM of E2 for stable cell assay) and vehicle control wells (n=3) treated with DMSO alone will be prepared on every assay plate. Serial dilution of E2 will be tested in all assay plates to examine the reproducibility of dose responses to E2. After adding the chemicals, the assay plates will be incubated in a

CO_2 incubator for 20-24 hours to induce the reporter gene products.

Test chemicals, as well as negative and positive control substances, should all be assigned as below.

Plate dose assignment of test chemicals in the assay plate

	Test Chemical 1			Test Chemical 2			Test Chemical 3			PC (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	→	→	BL	→	→	→	→	→	PC	→	→

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

4.8 Luciferase assay (See support protocol No. 6)

Luciferase activity will be measured with the luciferase assay reagent and a luminometer according to the manufacturer's instructions.

4.9. While edge effects are not considered to affect the final data for practical purposes, this needs to be monitored by individual laboratories.

5. Analysis of data

The luminescence signal data will be processed, and the average for the vehicle control wells will be calculated. The integrated value for each test well will be divided by the average integrated value of the vehicle control wells to obtain individual relative transcriptional activity. Then the average transcriptional activity will be calculated for each concentration of the test chemical. The PC50 and PC10 values will 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 spread sheet provided by CERI-Japan. 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 shown in Fig. 2.

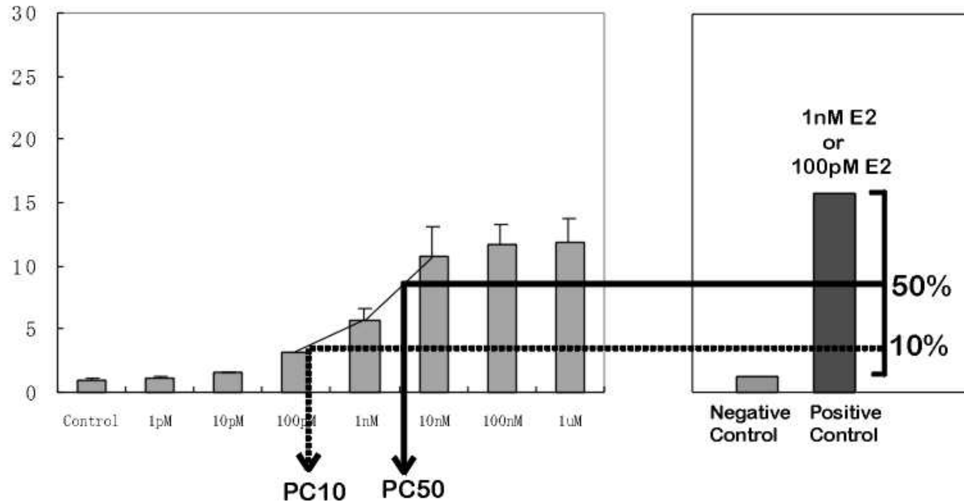

Fig. 2 Description of PC10 and PC50

6. Records

The records listed below should be retained at each laboratory.

Study protocol and its amendments

Amendments should be prepared if some modification is to be made regarding the original protocol. In this case, approval of the amendment by CERI would be required.

Standard Operating Procedures (SOPs)

SOPs or instruction manuals should be prepared by each laboratory.

Chemicals

With regard to the test chemicals and the positive control substance supplied by CERI, records of usage, storage, return and other related records should be retained at each laboratory.

Cell

With regard to the cells, records of acquisition, propagation, storage, usage, passage number of cells used for assay, and other related records should be retained at each laboratory.

Reagents

With regard to the reagents used in the assay, records of the manufacturer's name, the lot

number, usage, and related records should be retained at each laboratory. In the case of reagents made at the laboratory, the recipes and the records of preparation, storage, usage and other related records should be retained at each laboratory.

Equipments

All equipment used in the study should have corresponding records of the manufacturer's name, usage, maintenance and periodical inspection at each laboratory.

Main study

All records with regard to the cells, reagents, equipment, dates of the assays performed, researchers participating in the study, and other relevant records should be retained at each laboratory.

Data

All raw data derived from the study and the records of processing of data should be retained at each laboratory.

7. Inspection of the study

To assure GLP compliance, the lead quality assurance personnel would inspect the operations in the study, including records and data, as the occasion demands. If inappropriate cases are found, remedial actions would be required. All records related to the inspection should be retained by CERI.

8. Evaluation of the results of multi-lab validation studies

All data obtained by each laboratory should be filled in the common spread sheets provided by CERI, and will be collected at CERI. Then the reliability, reproducibility and technical transferability of this assay method will be evaluated by CERI.

9. Reporting

The report will contain details of the test substances, methodology, results and interpretation of data. A GLP statement and a Quality Assurance statement will be included in the report.

10. Peer review

The final report will be prepared after completing peer review of this study and related data by the external specialists.

Table 1 Candidate chemical list for multi-lab validation studies

Chemical	CAS No.	Manufacturer	Lot. No.	Purity
17β-Estradiol	50-28-2	Wako	ACK5754	99%
17α-Estradiol	57-91-0	Wako	ELJ1532	97% ,HPLC ,for Biochem.
Genistein	446-72-0	Wako	VIR1711	98%
4-tert-Octylphenol	140-66-9	Wako	YWE9213	97% ,cGC ,for Environment Anal.
Bisphenol A	80-05-7	TCI	GF01	>99%
p-tert-Pentylphenol	80-46-6	Wako	KSQ2664	97% ,GC
17α-Methyltestosterone	58-18-4	Wako	TPE6748	97% ,HPLC ,for Biochem.
Hematoxylin	517-28-2	Wako	LDK7723	N.S.
Diethylhexyl phthalate	117-81-7	Wako	ELE1799	97% ,GC
Benzophenone	119-61-9	Wako	RLH9114	99% ,cGC ,for Environment Anal.

TCI : Tokyo Kasei Kogyo Co., Ltd.
Wako : Wako Pure Chemical Industries, Ltd.
N.S. : not specified

SUPPORT PROTOCOLS

No. 1. Preparation of the medium

Reagents

Eagle's Minimal Essential medium without neutral red (Nissui Pharmaceutical Co.).

10% Sodium bicarbonate ($NaHCO_3$)

Dissolve 10 grams of $NaHCO_3$ to a final volume of 100 mL with water. Next, the solution should be sterilized using a vacuum-driven bottle-top sterilization filter unit and stored at room temperature.

3% Glutamine

Dissolve 3 grams of glutamine to a final volume of 100 mL with water. Next, the solution should be sterilized using vacuum-driven bottle-top sterilization filter unit. Prepared 3% Glutamine should be stored in aliquots under -20°C.

Dextran-coated charcoal (DCC)-treated Fetal bovine serum (DCC-FBS)

Prepared and provided by CERI-Japan.

Preparation of EMEM *

Add the following reagents into a 1L conical glass flask and then add sufficient Milli-Q water to bring the total volume to one liter:

- 9.4 grams of pre-made powder medium
- 18 mL 10% Sodium Bicarbonate
- 3% Glutamine 12 mL

Preparation of 10%FBS-EMEM *

Add 56 mL of dextran-coated charcoal (DCC)-treated fetal bovine serum (DCC-FBS) to 500 mL EMEM.

* EMEM and 10%FBS-EMEM should be stored in a refrigerator after being sterilized with a vacuum-driven bottle-top sterilization filter unit.

6/8/2005

SUPPORT PROTOCOLS

No. 2. Reconstitution of cells from the frozen stock

1. Remove the vial from the liquid nitrogen or freezer and immediately transfer it to a 37°C water bath.
2. While holding the tip of the vial, gently agitate the vial.
3. When completely thawed, transfer the cell stock into 5mL pre-warmed 10%FBS-EMEM in a 15 mL conical tube.
4. Centrifuge the tube at 1100 rpm (200-300 x g) for five minutes; then remove the supernatant carefully.
5. Resuspend the cell with 10 mL of 10%FBS-EMEM and place it in a 90 mm culture dish.
6. Incubate the cells in a 5% CO_2 incubator at 37°C.

6/8/2005

SUPPORT PROTOCOLS

No. 3. Propagation

1. Remove the medium from the culture dish with a sterile pipette or sucker.
2. Rinse the cell with 5 mL of PBS.
3. Remove the PBS with a sterile pipette or sucker.
4. Add 2 mL of Trypsin-EDTA solution (0.25% Trypsin + 0.02%EDTA/PBS), enough to coat the bottom of the culture dish, and then remove the excess.
5. Allow the Trypsin-treated cell to stand for about three minutes in a 5% CO_2 incubator at 37°C.

   (Monitor the cells under a microscope. The cells are beginning to detach when they appear rounded.)
6. Tap the dish gently.
7. Wash with 5 mL of 10%FBS-EMEM to remove the adherent cells.
8. Count the number of cells.
9. Dilute the cell suspension with 10%FBS-EMEM to 0.4-1.0 x 10^5 cells/mL.
10. Place 10 mL of cell suspension in a 90 mm culture dish.
11. Incubate the cells in a 5% CO_2 incubator at 37°C.

6/8/2005

SUPPORT PROTOCOLS

No. 4. Preparation of frozen stock

1. Remove the medium from the culture dish with a sterile pipette or sucker.
2. Rinse the cell with 5 mL of PBS.
3. Remove the PBS with a sterile pipette or sucker.
4. Add 2 mL of Trypsin-EDTA solution, enough to coat the bottom of the culture dish, and then remove the excess.
5. Allow the Trypsin-treated cell to stand for about three minutes in a 5% CO_2 incubator at 37°C.

   (Monitor the cells under a microscope. The cells are beginning to detach when they appear rounded.)
6. Tap the dish gently.
7. Wash with 5 mL of 10%FBS-EMEM to remove the adherent cells.
8. Count the number of cells.
9. Centrifuge the tube at 1100 rpm (200-300 x g) for five minutes, and remove the supernatant carefully.
10. Add Cell-Banker* (Juji Field Inc.) and resuspend the cell at a density of ca. 1×10^4 cells/mL.
11. Make 1 mL aliquots of cell stock.
12. Freeze and store the cell stock below -80°C.**

* A conventional freeze medium (90% FBS/10% DMSO) can be used in place of Cell-Banker.

** Storage in liquid nitrogen would be preferable for long-term storage (more than three months).

SUPPORT PROTOCOLS

No. 5. Preparation of the assay plate

Prepare a dish of cultured hERα-HeLa-9903 cells

1. Remove the medium from the culture dish with a sterile pipette or sucker.
2. Rinse the cells with 5 mL of PBS.
3. Remove the PBS with a sterile pipette or sucker.
4. Add 2 mL of Trypsin-EDTA solution, enough to coat the bottom of the culture dish, and then remove the excess.
5. Allow the Trypsin-treated cells to stand for about three minutes in a 5% CO_2 incubator at 37°C.
   (Monitor the cells under a microscope. The cells are beginning to detach when they appear rounded.)
6. Tap the dish gently.
7. Wash with 5 mL of 10%FBS-EMEM to remove the adherent cells.
8. Count the number of cells.
9. Dilute the cell suspension with 10%FBS-EMEM to obtain a final cell density of 1×10^5 cells/mL.
10. Add 100μL of cell suspension into each well of a 96-well assay plate (Nunc #136102 or an equivalent).
11. Incubate the cell in a 5% CO_2 incubator at 37°C for three hours.
12. Proceed to chemical exposure.

SUPPORT PROTOCOLS

No. 6-1. Chemiluminescence detection with a standard luciferase reagent

Reagents

Cell lysis reagent (4.5x): Dilute 10 mL of 5×Cell Culture Lysis Reagent (CCLR, #E1531) with 45 mL of distilled water.

Luciferase Assay Reagent: Add 1 vial 105 mL of Luciferase Assay buffer (Promega, #E4550) into a vial containing Luciferase Assay Substrate (Promega, #E4550), and dissolve the substrate thoroughly. Store the substrate below -20°C if necessary.

Chemiluminescence Detection

1. Flick and drain off the contents of the assay plate.
2. Add 100µL of PBS to the well to wash the plate.
3. Flick and drain off the contents of the assay plate.
4. Add 100µL of PBS to the well to wash the plate again.
5. Flick and drain off the contents of the assay plate.
6. Add 15µL of Cell lysis reagent (4.5x) to the wells.
7. Incubate for ten minutes at room temperature.
8. Add 50µL of Luciferase Assay Reagent to the wells.
9. Read the plates on a chemiluminescence plate reader.

SUPPORT PROTOCOLS

No. 6-2. Chemiluminescence detection with a luciferase reagent using the Steady-Glo Luciferase Assay System

Reagents

Luciferase Assay Reagent: Add 1 vial 100 mL of Luciferase Assay buffer into a vial containing Luciferase Assay Substrate (Promega, #E2520), and dissolve the substrate thoroughly. Store the substrate below -20°C if necessary.

Chemiluminescence Detection

1. Remove 50μL of the assay medium from all wells of the assay plate.
2. Add 100μL of the Luciferase Assay Reagent to wells.
3. Allow to stand for five minutes.
4. Read the plates on a chemiluminescence plate reader.

APPENDIX 4 Consideration of the edge effects on assay system

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In some assay systems using microtiter plates, an edge effect could not be ignored because of differences among wells located on the edge and center of the assay plate with regard to the evaporative loss of medium and efficacy of gas exchange, etc. In the case that clear edge effects would be expected, wells on edge should not be used for the assay. We have conducted some experiments to ensure the edge effects on the assay system.

1) Experiment 1

-The distribution of luminescent intensity in a assay plate were examined by measuring the chemiluminescence signal of all the wells on a assay plate stimulated with the positive control substance, 100 pM of E2 or the vehicle (dimethylsulphoxide, DMSO, final concentration at 0.1%). This experiment was conducted according to the SOP attached in APPENDIX 3. For this experiment, two assay plates were prepared and each plate was treated with 100pM of E2 or vehicle (DMSO) only.

Data obtained in this experiment were shown in Table 1-1 and 1-2 for the vehicle treated and 100 pM of E2 treated plates, respectively, and distributions of the signals summarized by A-H rows were shown in Figure 1.

To ensure the edge effect, we analyzed the results by Tukey's multiple comparison test, and no significant differences were noted between any combination of two rows within the assay plate treated with 100pM of E2 (Table 2). There was no edge effect like tendency with regard to the signals assessed by rows.

Table 1-1 The raw data of the luminescence intensity of each well in vehicle treated plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	86365	79024	76496	83248	85988	81890	93131	88834	87276	78168	76700	80736
B	80704	76268	70380	73748	81874	86122	84618	84650	76936	74563	68252	73993
C	76433	72350	70490	74196	82125	81011	85254	82163	82506	76684	75183	81209
D	79746	78694	76991	82612	81120	77509	81919	83770	85702	85205	77328	89445
E	82808	84465	84151	88399	85713	78082	78306	84129	84610	87496	89380	93677
F	84387	84591	87252	87566	81882	73293	77703	82392	82335	86526	87529	89918
G	87802	83955	88155	86098	79307	80539	88630	82832	82424	86037	89282	98113
H	91484	85855	90950	96438	83853	84340	94427	86673	88703	87121	94248	100445

Table 1-2 The raw data of the luminescence intensity of each well in 100 pM of E2 treated plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	346042	343608	332467	341833	345735	330433	368585	340607	354527	353288	350776	365038
B	339203	343584	320572	330794	350077	363582	375117	333872	273424	348778	336318	364027
C	344746	334767	326343	330480	341527	337382	348118	352423	296933	335503	327194	360773
D	340868	334398	323838	328494	325864	342328	336620	357601	306261	343029	331450	356468
E	358698	351616	330936	331312	327300	328431	331719	332233	339180	336033	319145	346731
F	350525	341323	332451	332616	331305	327803	330822	346087	324437	343160	335356	338104
G	325856	322668	321498	340247	341825	326217	336702	326778	336938	313453	321078	339857
H	315021	322362	331422	348797	353862	343254	349129	335674	332641	342695	350474	348436

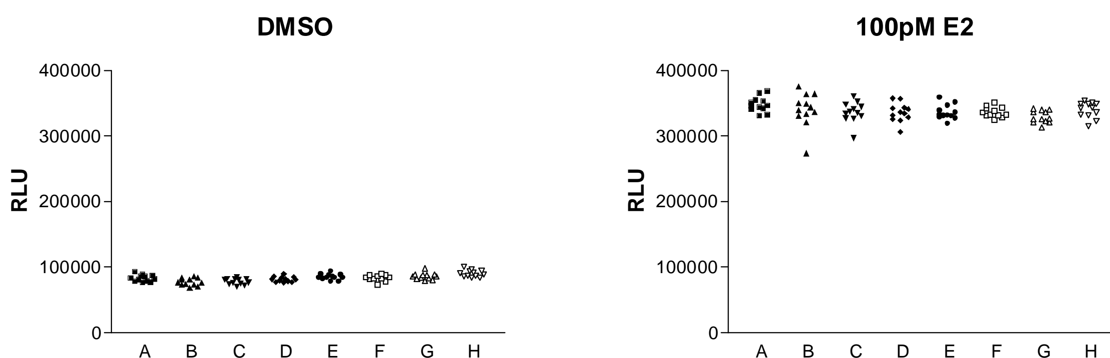


Figure 1 Distribution of signals summarized by A-H rows

To ensure existence or nonexistence of the edge effect, the results were analyzed by Tukey's multiple comparison tests. Significant differences were noted between some combinations of rows in the vehicle control plate treated with DMSO, however there was no tendency specific to edge effect (Table 2-1). As for the positive control plate treated with E2, no significant differences were noted between any combination of two rows with in the assay plate treated with 100pM of E2 (Table 2-2).

Therefore, the edge effects were unlikely with regard to the signals assessed by rows.

Table 2-1 Summary of the statistical analysis of chemiluminescent signals in the assay plate treated with DMSO

Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff.
A vs B	5479	3.875	P > 0.05	-749.1 to 11710
A vs C	4854	3.433	P > 0.05	-1374 to 11080
A vs D	1485	1.05	P > 0.05	-4744 to 7713
A vs E	-1947	1.377	P > 0.05	-8175 to 4281
A vs F	-626.5	0.4431	P > 0.05	-6855 to 5602
A vs G	-2943	2.081	P > 0.05	-9171 to 3285
A vs H	-7223	5.109	P < 0.05	-13450 to -995.3
B vs C	-624.7	0.4418	P > 0.05	-6853 to 5603
B vs D	-3994	2.825	P > 0.05	-10220 to 2234
B vs E	-7426	5.252	P < 0.01	-13650 to -1198

B vs F	-6106	4.318	P > 0.05	-12330 to 122.6
B vs G	-8422	5.956	P < 0.01	-14650 to -2194
B vs H	-12700	8.983	P < 0.001	-18930 to -6474
C vs D	-3370	2.383	P > 0.05	-9598 to 2858
C vs E	-6801	4.81	P < 0.05	-13030 to -572.9
C vs F	-5481	3.876	P > 0.05	-11710 to 747.2
C vs G	-7798	5.515	P < 0.01	-14030 to -1569
C vs H	-12080	8.542	P < 0.001	-18310 to -5850
D vs E	-3431	2.427	P > 0.05	-9659 to 2797
D vs F	-2111	1.493	P > 0.05	-8339 to 4117
D vs G	-4428	3.131	P > 0.05	-10660 to 1800
D vs H	-8708	6.158	P < 0.01	-14940 to -2480
E vs F	1320	0.9336	P > 0.05	-4908 to 7548
E vs G	-996.5	0.7047	P > 0.05	-7225 to 5232
E vs H	-5277	3.732	P > 0.05	-11500 to 951.3
F vs G	-2317	1.638	P > 0.05	-8545 to 3911
F vs H	-6597	4.665	P < 0.05	-12830 to -368.8
G vs H	-4280	3.027	P > 0.05	-10510 to 1948

Table 2-2 Summary of the statistical analysis of chemiluminescent signals in the assay plate treated with E2

Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff.
A vs B	7799	1.854	P > 0.05	-10730 to 26320
A vs C	11400	2.71	P > 0.05	-7129 to 29920
A vs D	12140	2.887	P > 0.05	-6382 to 30670
A vs E	11630	2.766	P > 0.05	-6891 to 30160
A vs F	11580	2.753	P > 0.05	-6946 to 30100
A vs G	18320	4.355	P > 0.05	-206.6 to 36840
A vs H	8264	1.965	P > 0.05	-10260 to 26790
B vs C	3597	0.8551	P > 0.05	-14930 to 22120
B vs D	4344	1.033	P > 0.05	-14180 to 22870
B vs E	3835	0.9117	P > 0.05	-14690 to 22360
B vs F	3780	0.8987	P > 0.05	-14750 to 22310
B vs G	10520	2.501	P > 0.05	-8006 to 29040
B vs H	465.1	0.1106	P > 0.05	-18060 to 18990
C vs D	747.5	0.1777	P > 0.05	-17780 to 19270
C vs E	237.9	0.05657	P > 0.05	-18290 to 18760
C vs F	183.3	0.04359	P > 0.05	-18340 to 18710
C vs G	6923	1.646	P > 0.05	-11600 to 25450
C vs H	-3132	0.7446	P > 0.05	-21660 to 15390
D vs E	-509.6	0.1212	P > 0.05	-19030 to 18020
D vs F	-564.2	0.1341	P > 0.05	-19090 to 17960
D vs G	6175	1.468	P > 0.05	-12350 to 24700
D vs H	-3879	0.9223	P > 0.05	-22400 to 14650
E vs F	-54.59	0.01298	P > 0.05	-18580 to 18470
E vs G	6685	1.589	P > 0.05	-11840 to 25210
E vs H	-3369	0.8011	P > 0.05	-21890 to 15160
F vs G	6739	1.602	P > 0.05	-11790 to 25260

F vs H		-3315	0.7881	P > 0.05	-21840 to 15210
G vs H		-10050	2.391	P > 0.05	-28580 to 8471

2) Experiment 2

The differences in dose responsiveness of positive control substance (E2) in concentration range of 10^{-13}-10^{-7} M were tested twice. This experiment was also conducted according to the SOP attached in APPENDIX 3. The plate format used for this experiment is as shown below. PC50 values were calculated according to the SOP in APPENDIX 3. Data obtained in this experiment were shown in Figure 2.

	Edge			Center			Center			Edge		
	Series-1			Series-2			Series-3			Seris-4		
	1	2	3	4	5	6	7	8	9	10	11	12
A	100 nM	→	→	→	→	→	→	→	→	→	→	→
B	10 nM	→	→	→	→	→	→	→	→	→	→	→
C	1 nM	→	→	→	→	→	→	→	→	→	→	→
D	100 pM	→	→	→	→	→	→	→	→	→	→	→
E	10 pM	→	→	→	→	→	→	→	→	→	→	→
F	1 pM	→	→	→	→	→	→	→	→	→	→	→
G	0.1 pM	→	→	→	→	→	→	→	→	→	→	→
H	VC	→	→	→	→	→	PC	→	→	→	→	→

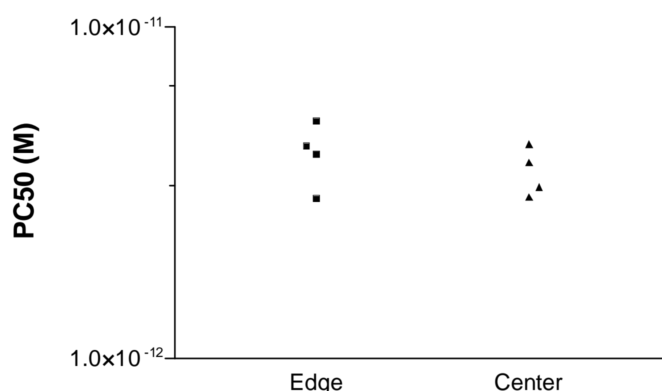


Figure 2 Distribution of PC50 values assessed by their location on the assay plate

PC50 values of E2 located in edge and center of the plate were 3.04-5.21 pM and 3.07-4.44 pM, respectively. Moreover, statistical analysis revealed that there was no significant difference between the assay area located in the edge and center of the assay plate (Unpaired *t*-test).

Raw data of Experiment 2-1

	17beta-Estradiol			17beta-Estradiol			17beta-Estradiol			17beta-Estradiol		
RawData	1	2	3	4	5	6	7	8	9	10	11	12
A	440627	399635	409292	402650	437981	405759	447283	420333	430729	416231	401129	395176
B	430915	415613	423252	526718	427225	437981	430795	420316	406845	408232	428071	399017
C	421972	423872	420802	431810	404825	431072	426726	430061	423651	422061	397427	399898
D	397146	360873	396754	382299	369957	382095	396253	370404	372197	363847	355563	320295
E	259166	251362	266256	255123	258664	262322	261584	249140	235775	233745	243913	237871
F	115148	117072	116043	129022	125771	119153	125634	124240	115262	119037	116966	114813
G	44148	41911	44109	46959	39296	43033	43153	42933	39834	41277	44629	54145
H	47768	36917	37891	36642	36336	34609	378313	387430	370126	371309	367892	343315

VC_Ave 38361
VC_SD 4731

Raw - VC_Ave											
402267	361275	370932	364290	399621	367399	408923	381973	392369	377871	362769	356816
392555	377253	384892	488358	388865	399621	392435	381956	368485	369872	389711	360657
383612	385512	382442	393450	366465	392712	388366	391701	385291	383701	359067	361538
358786	322513	358394	343939	331597	343735	357893	332044	333837	325487	317203	281935
220806	213002	227896	216763	220304	223962	223224	210780	197415	195385	205553	199511
76787.5	78711.5	77682.5	90661.5	87410.5	80792.5	87273.5	85879.5	76901.5	80676.5	78605.5	76452.5
5787.5	3550.5	5748.5	8598.5	935.5	4672.5	4792.5	4572.5	1473.5	2916.5	6268.5	15784.5
9407.5	-1443.5	-469.5	-1718.5	-2024.5	-3751.5	339953	349070	331766	332949	329532	304955

PC_Ave 331370

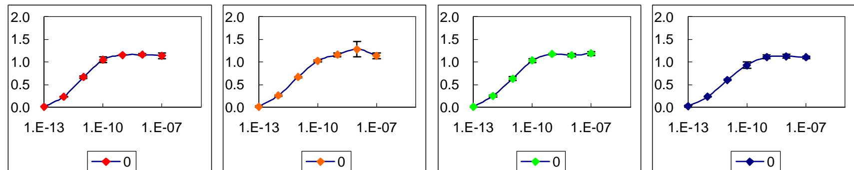
nscriptional activity of PC = (PC_Ave + VC_Ave)/VC_Ave 9.64

Relative transcriptional activity
= (Raw - VC_Ave)/PC_Ave

1.2139	1.0902	1.1194	1.0993	1.2060	1.1087	1.2340	1.1527	1.1841	1.1403	1.0948	1.0768
1.1846	1.1385	1.1615	1.4738	1.1735	1.2060	1.1843	1.1527	1.1120	1.1162	1.1761	1.0884
1.1577	1.1634	1.1541	1.1873	1.1059	1.1851	1.1720	1.1821	1.1627	1.1579	1.0836	1.0910
1.0827	0.9733	1.0815	1.0379	1.0007	1.0373	1.0800	1.0020	1.0074	0.9822	0.9572	0.8508
0.6663	0.6428	0.6877	0.6541	0.6648	0.6759	0.6736	0.6361	0.5958	0.5896	0.6203	0.6021
0.2317	0.2375	0.2344	0.2736	0.2638	0.2438	0.2634	0.2592	0.2321	0.2435	0.2372	0.2307
0.0175	0.0107	0.0173	0.0259	0.0028	0.0141	0.0145	0.0138	0.0044	0.0088	0.0189	0.0476
0.0284	-0.0044	-0.0014	-0.0052	-0.0061	-0.0113	1.0259	1.0534	1.0012	1.0048	0.9945	0.9203

	0 17beta-Estradiol			0 17beta-Estradiol			0 17beta-Estradiol			0 17beta-Estradiol		
Concentration (M)	Ave	SD	Rank	Ave	SD	Rank	Ave	SD	Rank	Ave	SD	Rank
1.E-07	1.1412	0.0647	3	1.1380	0.0590	3	1.1903	0.0410	1	1.1040	0.0328	3
1.E-08	1.1615	0.0231	1	1.2844	0.1648	1	1.1496	0.0362	3	1.1269	0.0448	1
1.E-09	1.1584	0.0047	2	1.1595	0.0464	2	1.1723	0.0097	2	1.1108	0.0409	2
1.E-10	1.0459	0.0629	4	1.0253	0.0213	4	1.0298	0.0436	4	0.9301	0.0698	4
1.E-11	0.6656	0.0225	5	0.6649	0.0109	5	0.6352	0.0390	5	0.6040	0.0154	5
1.E-12	0.2346	0.0029	6	0.2604	0.0152	6	0.2515	0.0170	6	0.2371	0.0064	6
1.E-13	0.0152	0.0039	7	0.0143	0.0116	7	0.0109	0.0056	7	0.0251	0.0201	7

Max	1.1615	1.2844	1.1903	1.1269
Max - Value(1.E-13 M)	1.1464	1.2701	1.1794	1.1018
PC50 or PC10	PC50、PC10	PC50、PC10	PC50、PC10	PC50、PC10
PC50	4.13E-12	3.91E-12	4.44E-12	5.21E-12
PC10	2.86E-13	2.55E-13	2.60E-13	2.96E-13



Raw data of Experiment 2-2

RawData

	1	2	3	4	5	6	7	8	9	10	11	12
	17beta-Estradiol			17beta-Estradiol			17beta-Estradiol			17beta-Estradiol		
A	417623	407102	427390	401056	415118	408177	415155	412447	399360	361752	338430	323728
B	432525	399290	398332	405751	410344	408138	404195	401749	391344	383711	361148	325995
C	435791	411232	419499	396024	416500	430491	417821	404350	398169	391703	364141	321396
D	381765	386704	371174	368269	364571	383540	371594	375149	368177	355489	329860	305095
E	272537	275576	276690	266649	271155	270315	276066	264992	259749	240602	243147	248553
F	128551	123589	118179	136025	125818	120409	126409	116077	121630	117219	116420	100396
G	26930	25690	27637	27880	23460	25588	28304	27643	24903	24602	27847	31158
H	18160	16127	16017	16417	16017	17273	403967	389615	367452	375027	357732	364997

VC_Ave 16669
VC_SD 871

Raw - VC_Ave											
400955	390434	410722	384388	398450	391509	398487	395779	382692	345084	321762	307060
415857	382622	381664	389083	393676	391470	387527	385081	374676	367043	344480	309327
419123	394564	402831	379356	399832	413823	401153	387682	381501	375035	347473	304728
365097	370036	354506	351601	347903	366872	354926	358481	351509	338821	313192	288427
255869	258908	260022	249981	254487	253647	259398	248324	243081	223934	226479	231885
111883	106921	101511	119357	109150	103741	109741	99408.5	104962	100551	99751.5	83727.5
10261.5	9021.5	10968.5	11211.5	6791.5	8919.5	11635.5	10974.5	8234.5	7933.5	11178.5	14489.5
1491.5	-541.5	-651.5	-251.5	-651.5	604.5	387299	372947	350784	358359	341064	348329

PC_Ave 359797

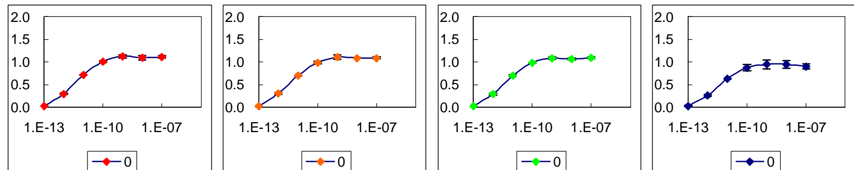
Relative transcriptional activity = (Raw - VC_Ave)/PC_Ave

nscriptional activity of PC = (PC_Ave + VC_Ave)/VC_Ave 22.59

1.1144	1.0852	1.1415	1.0683	1.1074	1.0881	1.1075	1.1000	1.0636	0.9591	0.8943	0.8534
1.1558	1.0634	1.0608	1.0814	1.0942	1.0880	1.0771	1.0703	1.0414	1.0201	0.9574	0.8597
1.1649	1.0966	1.1196	1.0544	1.1113	1.1502	1.1149	1.0775	1.0603	1.0424	0.9657	0.8469
1.0147	1.0285	0.9853	0.9772	0.9669	1.0197	0.9865	0.9963	0.9770	0.9417	0.8705	0.8016
0.7111	0.7196	0.7227	0.6948	0.7073	0.7050	0.7210	0.6902	0.6756	0.6224	0.6295	0.6445
0.3110	0.2972	0.2821	0.3317	0.3034	0.2883	0.3050	0.2763	0.2917	0.2795	0.2772	0.2327
0.0285	0.0251	0.0305	0.0312	0.0189	0.0248	0.0323	0.0305	0.0229	0.0220	0.0311	0.0403
0.0041	-0.0015	-0.0018	-0.0007	-0.0018	0.0017	1.0764	1.0365	0.9749	0.9960	0.9479	0.9681

	0 17beta-Estradiol			0 17beta-Estradiol			0 17beta-Estradiol			0 17beta-Estradiol		
Concentration (M)	Ave	SD	Rank	Ave	SD	Rank	Ave	SD	Rank	Ave	SD	Rank
1.E-07	1.1137	0.0282	2	1.0880	0.0195	2	1.0904	0.0235	1	0.9023	0.0533	3
1.E-08	1.0933	0.0541	3	1.0879	0.0064	3	1.0629	0.0190	3	0.9458	0.0808	2
1.E-09	1.1270	0.0347	1	1.1053	0.0482	1	1.0843	0.0279	2	0.9517	0.0985	1
1.E-10	1.0095	0.0221	4	0.9879	0.0279	4	0.9866	0.0097	4	0.8713	0.0700	4
1.E-11	0.7178	0.0060	5	0.7024	0.0067	5	0.6956	0.0232	5	0.6321	0.0113	5
1.E-12	0.2968	0.0144	6	0.3078	0.0220	6	0.2910	0.0144	6	0.2631	0.0264	6
1.E-13	0.0280	0.0027	7	0.0249	0.0061	7	0.0286	0.0050	7	0.0311	0.0091	7

Max	1.1270	1.1053	1.0904	0.9517
Max - Value(1.E-13 M)	1.0990	1.0803	1.0618	0.9206
PC50 or PC10	PC50, PC10	PC50, PC10	PC50, PC10	PC50, PC10
PC50	3.04E-12	3.07E-12	3.29E-12	4.38E-12
PC10	2.36E-13	2.26E-13	2.40E-13	2.70E-13



3) Experiment 3

The edge effects at all participating laboratories of inter-lab validation study were examined by analyzing the dose responsiveness of positive control substance (E2) prepared in all assay plates, i.e. 9 plates. In this trial, three PC50 values were calculated using the data in each single column (10, 11, or 12) for each assay plate (Table 3). Then PC50 obtained for each column were statistically compared by Tukey's multiple comparison tests for each participating laboratories.

Consequently, the distributions of the PC50 values obtained for each column in all participating laboratories showed much the same pattern, and the statistical analysis revealed that no significant differences between any combinations of PC50 values for each columns (Figure 3).

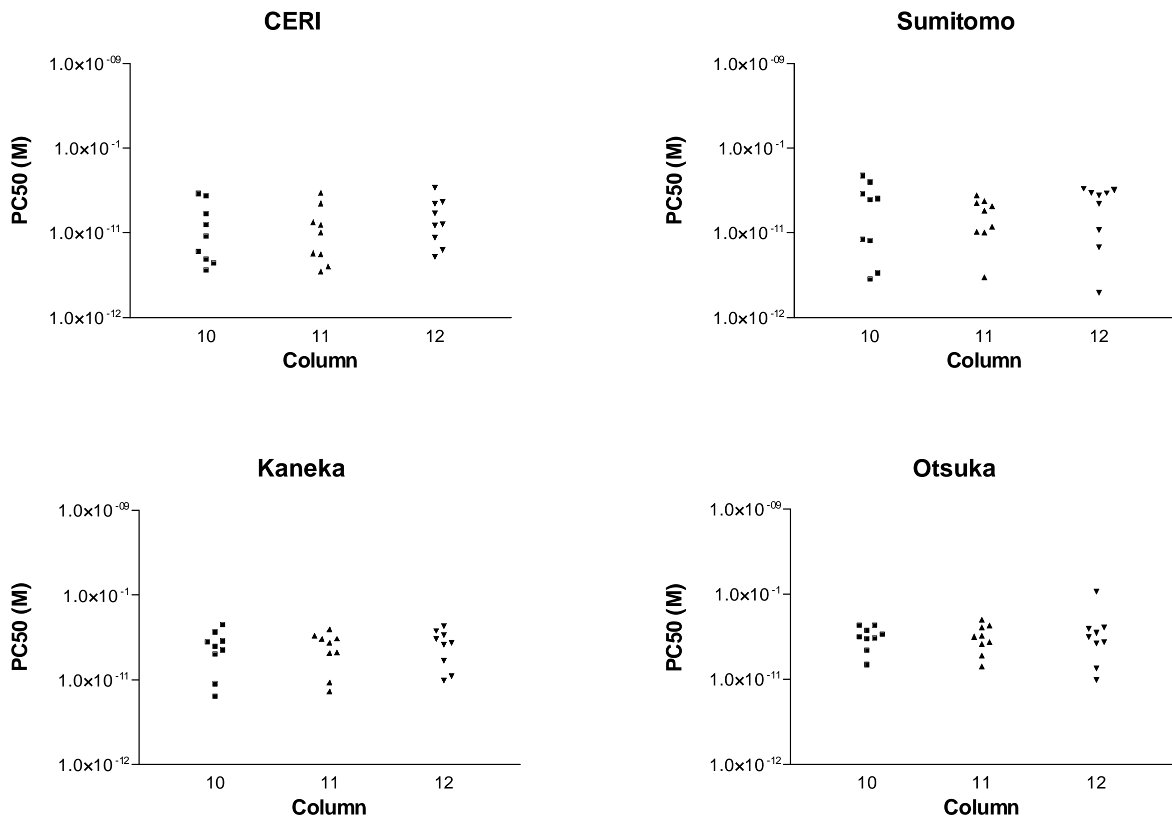


Figure 3 Distribution of PC values obtained for each column (10, 11 or 12)

Table 3 PC50 values calculated for each column of the assay plates of inter-lab validation study

		CERI			Sumitomo			Kaneka			Otsuka		
	Plate	10	11	12	10	11	12	10	11	12	10	11	12
Trial 1	1	1.67E-11	2.24E-11	2.20E-11	8.39E-12	1.18E-11	6.74E-12	6.41E-12	9.30E-12	9.84E-12	3.76E-11	4.04E-11	3.16E-11
	2	2.89E-11	1.34E-11	2.34E-11	3.96E-11	2.38E-11	3.20E-11	8.94E-12	7.27E-12	1.12E-11	4.30E-11	3.18E-11	2.73E-11
	3	2.74E-11	2.96E-11	3.40E-11	3.35E-12	1.00E-11	2.97E-11	2.25E-11	2.09E-11	1.68E-11	3.01E-11	5.03E-11	3.92E-11
Trial 2	1	9.19E-12	1.01E-11	1.22E-11	2.52E-11	1.03E-11	1.08E-11	2.00E-11	2.12E-11	2.61E-11	4.32E-11	4.28E-11	4.05E-11
	2	4.89E-12	5.56E-12	8.86E-12	2.88E-11	2.77E-11	2.95E-11	2.88E-11	3.06E-11	3.35E-11	3.07E-11	2.74E-11	3.52E-11
	3	1.25E-11	1.24E-11	1.27E-11	8.11E-12	2.05E-11	2.21E-11	2.78E-11	2.75E-11	2.75E-11	3.15E-11	3.22E-11	1.07E-10
Trial 3	1	5.96E-12	5.75E-12	1.70E-11	2.86E-12	3.01E-12	1.96E-12	3.65E-11	3.31E-11	3.03E-11	3.40E-11	2.59E-11	2.65E-11
	2	3.61E-12	4.02E-12	6.32E-12	4.73E-11	2.24E-11	3.32E-11	4.49E-11	3.93E-11	4.33E-11	2.18E-11	1.89E-11	1.34E-11
	3	4.40E-12	3.49E-12	5.20E-12	2.48E-11	1.84E-11	2.76E-11	2.48E-11	3.01E-11	3.78E-11	1.48E-11	1.43E-11	9.90E-12
Mean		1.26E-11	1.19E-11	1.58E-11	2.09E-11	1.64E-11	2.15E-11	2.45E-11	2.44E-11	2.63E-11	3.18E-11	3.15E-11	3.68E-11
SD		9.79E-12	8.95E-12	9.37E-12	1.62E-11	8.05E-12	1.19E-11	1.21E-11	1.07E-11	1.16E-11	9.26E-12	1.15E-11	2.84E-11

Conclusions:

Based on the results of experiment 1, 2 and 3, there are no significant edge effects with regard to the chemilumiscent signals induced by positive control substance (E2) within laboratory and inter-laboratories. As for the PC50 values calculated from four independent assay area of the assay plate, statistical analysis showed no significant difference between two test areas located in the both side (column 1-3 and column 10-12) and other two test areas located in the center (column 4-6 and column 7-9) of the assay plate. Moreover the distributions of the PC50 values for E2 obtained for each column (10, 11 or 12) in all participating laboratories showed much the same pattern, and the statistical analysis revealed that no significant differences between any combinations of PC50 values for each row.

These results suggest that there is no edge effect that would affect the results for practical purposes.

APPENDIX 5 Standard protocols for detecting of anti-estrogenic activity using the reporter gene assay

STANDARD PROTOCOLS

for detection of anti-estrogenic activity using the reporter gene assay

Description: This document provides a methodology for detecting the anti-estrogenic activity of chemicals by the reporter gene assay technique using the hER-HeLa-9903 cell line.

Chemicals Evaluation and Research Institute
CERI-Japan

Materials and Methods

1. Test chemicals

Test chemicals should be dissolved in dimethylsulfoxide (DMSO) at a concentration of 10 mM.

2. Competitive substance

17β-Estradiol (E2)

3. Vehicle for chemical stock solutions

Dimethylsulfoxide (DMSO) should be used for the vehicle.

4. Test system and operating procedures

4.1 Cell lines

The hERα-*HeLa*-9903 stable cell line (Sumitomo Chemicals Co.) will be used for the assay and the 9903-control cell, which consistently expresses firefly luciferase by the RSV promoter without stimulation, will be used for evaluating the cell-toxic effect of chemicals when an anti-estrogenic like effect is observed.

4.2 Cell cultures (See support protocols No.1 – No. 4)

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.

4.3 Preparation of chemicals

All chemicals will be dissolved in DMSO at a concentration of 10 mM, and those solutions will 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.

4.4 Preparation of cells

Assay plates will be prepared according to support protocol No. 5.

4.5 Reagents for the luciferase assay

A commercial luciferase assay reagent, Steady-Glo Luciferase Assay System (Promega, E2510 and its equivalents) or a standard luciferase assay system (Promega, E1500 and its equivalents) will be used in this study. A bottle of Luciferase Assay Substrate is dissolved with the Luciferase Assay Buffer. The dissolved substrate should either be used immediately or

stored below -20 C.

When using the standard luciferase assay system, Cell Culture Lysis Reagent (Promega, E1531) should be used before adding the substrate.

4.7 Chemical exposure

Each test chemical diluted in DMSO will 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.

Exactly 1.5 µL of 10 mM chemical stock and six working solutions will be diluted in serum-free EMEM (500 µL) containing 75 pM of E2.

Then 50 µL of the diluted test samples will be added to each well of the assay plate according to the assignment table in Fig. 1.

Reference control wells (n=6) treated with 25 pM of E2 without any other chemicals and vehicle control wells (n=6) treated with DMSO alone at concentration of 0.2% will be prepared on every assay plate. After adding the chemicals, the assay plates will be incubated in a CO_2 incubator for 20-24 hours to induce the reporter gene products.

Fig. 1 Typical assignment of the assay plate for the antagonist assay

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

VC: Vehicle control (DMSO only); RC: Reference control (25 pM E2 only)

In any case where an anti-estrogenic-like effect or decreasing trend in transcriptional activity is noted, the cytotoxicity of chemicals should be examined by using a HeLa-9903 control cell. Other luminescent cell viability assays are easily available, but use of the HeLa-9903 control cell is more specific to this test method. The cytotoxicity of chemicals will be evaluated by luciferase activity under the effect of test chemicals. The cytotoxicity assay will be performed in the same manner as the above-mentioned antagonist assay procedure, except HeLa-9903 control cells will be used. The plate format should be as shown in Fig. 2.

Fig. 2 Typical assignment of the assay plate for the cytotoxicity

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

VC: Vehicle control (DMSO only)

4.8 Luciferase assay (See support protocol No. 6)

Luciferase activity will be measured with the luciferase assay reagent and a luminometer in accordance with the manufacturer's instructions.

5. Analysis of data

The luminescence signal data will be processed, and the average for the vehicle control wells will be calculated. The integrated value for each test well will be divided by the average integrated value of the vehicle control wells in order to obtain individual relative transcriptional activities. Then the average transcriptional activity will be calculated for each concentration of the test chemical. Next the 50% inhibitory concentration against mean transcriptional activity induced by the reference wells (25 pM of E2) will be calculated and used for evaluating the anti-estrogenic activities of chemicals.

The above-described calculations will be made using commercial software with Hill's logistic equation showing below:

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.

In the cytotoxicity test, the luminescence signal data will be also processed, and the average of the vehicle control wells will be calculated. The integrated value for each test well will be divided by the average integrated value of the vehicle control wells in order to obtain individual relative transcriptional activities. When the transcriptional activities are reduced less than 80% of the mean transcriptional activities of the vehicle control wells, the concentration should be regarded as a cytotoxic concentration and thereby excluded for evaluation of any anti-estrogenic effects.

SUPPORT PROTOCOLS

No. 1. Preparation of the medium

Reagents

Eagle's Minimal Essential medium without neutral red (Nissui Pharmaceutical Co.).

10% Sodium Bicarbonate ($NaHCO_3$)

Dissolve ten grams of $NaHCO_3$ to a final volume of 100 mL with water. Next, the solution should be sterilized using a vacuum-driven bottle-top sterilization filter unit and stored at room temperature.

3% Glutamine

Dissolve three grams of glutamine to a final volume of 100 mL with water. Next, the solution should be sterilized using a vacuum-driven bottle-top sterilization filter unit. Prepared 3% Glutamine should be stored in aliquots lower than -20°C.

Dextran-coated charcoal (DCC)-treated fetal bovine serum (DCC-FBS)

Prepared and provided by CERI-Japan.

Preparation of EMEM *

Add the following reagents into a 1L conical glass flask and then add Milli-Q water to bring the total volume to one liter:

- 9.4 grams of pre-made powder medium
- 18 mL of 10% Sodium Bicarbonate
- 12 mL of 3% Glutamine

Preparation of EMEM containing 75pM of E2

Add 75nM of E2 to EMEM at a proportion of 1:1000 just prior to use.

Preparation of 10%FBS-EMEM *

Add 56 mL of dextran-coated charcoal (DCC)-treated fetal bovine serum (DCC-FBS) to 500 mL EMEM.

* Both EMEM and 10%FBS-EMEM should be stored in a refrigerator after being sterilized with a vacuum-driven bottle-top sterilization filter unit.

SUPPORT PROTOCOLS

No. 2. Reconstitution of cells from frozen stock

1. Remove the vial from the liquid nitrogen or the freezer and immediately transfer it to a 37°C water bath.
2. While holding the tip of the vial, gently agitate the vial.
3. When completely thawed, transfer the cell stock into 5 mL pre-warmed 10%FBS-EMEM in a 15 mL conical tube.
4. Centrifuge the tube at 1100 rpm (200-300 x g) for five minutes, and remove the supernatant carefully.
5. Resuspend the cell with 10 mL of 10%FBS-EMEM and place in a 90 mm culture dish.
6. Incubate the cells in a 5% CO_2 incubator at 37°C.

SUPPORT PROTOCOLS

No. 3. Propagation

1. Remove the medium from the culture dish with a sterile pipette or sucker.
2. Rinse the cells with 5 mL of PBS.
3. Remove the PBS with a sterile pipette or sucker.
4. Add 2 mL of Trypsin-EDTA solution (0.25% Trypsin + 0.02%EDTA/PBS), enough to coat the bottom of the culture dish, and then remove the excess.
5. Allow the Trypsin-treated cells to stand for about three minutes in a 5% CO_2 incubator at 37°C.
6. (Monitor the cells under a microscope. The cells are beginning to detach when they appear rounded.)
7. Tap the dish gently.
8. Wash with 5 mL of 10%FBS-EMEM to remove the adherent cells.
9. Count the number of cells.
10. Dilute the cell suspension with 10%FBS-EMEM to 0.4-1.0 x 10^5 cells/mL.
11. Place 10 mL of cell suspension in a 90 mm culture dish.
12. Incubate the cells in a 5% CO_2 incubator at 37°C.

SUPPORT PROTOCOLS

No. 4. Preparation of frozen stock

1. Remove the medium from the culture dish with a sterile pipette or sucker.
2. Rinse the cells with 5 mL of PBS.
3. Remove the PBS with a sterile pipette or sucker.
4. Add 2 mL of Trypsin-EDTA solution, enough to coat the bottom of the culture dish, and then remove the excess.
5. Allow the Trypsin-treated cell to stand for about three minutes in a 5% CO_2 incubator at 37°C.
6. (Monitor the cells under a microscope. The cells are beginning to detach when they appear rounded.)
7. Tap the dish gently.
8. Wash with 5 mL of 10%FBS-EMEM to remove the adherent cells.
9. Count the number of cells.
10. Centrifuge the tube at 1100 rpm (200-300 x g) for five minutes, and remove the supernatant carefully.
11. Add Cell-Banker* (Juji Field Inc.) and resuspend the cell at density of ca. 1 x 10^4 cells/mL.
12. Make 1 mL aliquots of cell stock.
13. Freeze and store the cell stock below -80°C.**

* A conventional freeze medium (90% FBS/10% DMSO) can be used in place of Cell-Banker.

** Storage in liquid nitrogen would be preferable for long-term storage (longer than three months).

SUPPORT PROTOCOLS

No. 5 Preparation of the assay plate

Prepare a dish of cultured hERα-HeLa-9903 cells

1. Remove the medium from the culture dish with a sterile pipette or sucker.
2. Rinse the cells with 5 mL of PBS.
3. Remove the PBS with a sterile pipette or sucker.
4. Add 2 mL of Trypsin-EDTA solution, enough to coat the bottom of the culture dish, and then remove the excess.
5. Allow the Trypsin-treated cell to stand for about three minutes in a 5% CO_2 incubator at 37°C.
6. (Monitor the cells under microscope. The cells are beginning to detach when they appear rounded.)
7. Tap the dish gently.
8. Wash with 5 mL of 10%FBS-EMEM to remove the adherent cells and transfer the cell suspension to a centrifuge tube.
9. Count the number of cells.
10. Centrifuge the tube at 1100 rpm (200-300 x g) for five minutes, and remove the supernatant carefully.
11. Resuspend the cell with 10%FBS-EMEM to obtain a final cell density of 1 x 10^5 cells/mL.
12. Add 100 μL of cell suspension into each well of a 96-well assay plate (Nunc #136102 or its equivalents).
13. Incubate the cells in a 5% CO_2 incubator at 37°C for three hours.
14. Proceed to chemical exposure.

SUPPORT PROTOCOLS

No. 6-1. Chemiluminescence detection with a standard luciferase reagent

Reagents

Cell lysis reagent (4.5x): Dilute 10 mL of 5×Cell Culture Lysis Reagent (CCLR, #E1531) with 45 mL of distilled water.

Luciferase Assay Reagent: Add 1 vial 105 mL of Luciferase Assay buffer (Promega, #E4550) into a vial containing Luciferase Assay Substrate (Promega, #E4550), and dissolve the substrate thoroughly. Store the substrate below -20°C if necessary.

Chemiluminescence detection

1. Flick and drain off the contents of the assay plate.
2. Add 100 µL of PBS to the well to wash the plate.
3. Again flick and drain off the contents of the assay plate.
4. Add 100µL of PBS to the well to wash the plate again.
5. Again flick and drain off the contents of the assay plate.
6. Add 15 µL of cell lysis reagent (4.5x) to wells.
7. Incubate for ten minutes at room temperature.
8. Add 50µL of Luciferase Assay Reagent to wells.
9. Read the plates on a chemiluminescence plate reader.

SUPPORT PROTOCOLS

No. 6-2. Chemiluminescence detection with luciferase reagent using Steady-Glo Luciferase Assay System

Reagents

Luciferase Assay Reagent: Add 1 vial (100 mL) of Luciferase Assay buffer into a vial containing Luciferase Assay Substrate (Promega, #E2520), and dissolve the substrate thoroughly. Store the substrate below -20°C if necessary.

Chemiluminescence Detection

1. Remove 50 µL of assay medium from all wells of assay plate.
2. Add 100 µL of Luciferase Assay Reagent to the wells.
3. Allow to stand for five minutes.
4. Read plates on a Chemiluminescence plate reader.

Appendix 6. Independent statistical analyses for inter-laboratory validation study

SUMMARY

As part of the preliminary peer review, further independent statistical analyses were conducted to examine inter-laboratory variability, and are provided in this appendix.

The statistical data analyses compare very favourably. In both cases, the assay demonstrated acceptable overall within-lab variability as well as between-lab variability. However, while the independent analyses are more complex and may yield greater precision, the precision does make a sufficient difference to the more practical statistical method used by CERI. The CERI PC50 measure also has the added benefit in being able to be obtained with only two data points. The PC50 values can also be calculated in cases of weak estrogenic compounds as the relative estrogenic activity to the natural estrogen. For this reason the CERI method is the method of choice as it is more accessible and user friendly for regulatory purposes.

INTRODUCTION

This appendix includes preliminary results of the additional analyses proposed and performed by a member of the preliminary peer review panel. The analyses will be finalized in the future and so the content of this appendix should be taken as provisional, interim results. Analytical strategies employed in this appendix have been developed and used for the data from certain *in vitro* assays other than the transfected ER gene reporter assay. As the attempt to adapt the strategies to the present data set was made, it was realized some additional considerations specific to the transfected ER gene reporter assay was necessary. Some tentative decisions were made based on these considerations, but they are subject to further changes in the future.

A version of assay variability assessment is already included in the body of the report. Specifically, overall within-lab and between-lab variability were estimated and interpreted. The additional analyses herein were performed with similar underling goals in mind although employing alternative methods at two levels: in generating run-specific estimates; and in further summarizing these run-specific estimates for each lab (and further summarizing lab-specific estimates obtained thereby across labs). As such, there are up to a total of four different combinations of methods applied to each parameter of interest as summarized below (detailed explanation for each of these procedures will be given later).

Method for run-specific estimates	Method for summarization	
	Traditional	DL
Linear interpolation	Original CERI analyses for logPC10 and logPC50	Additional analyses
Hill equation-based nonlinear regression	Original CERI analyses for logEC50 & additional analyses for logPC10 and logPC50	Additional analyses

The use of the additional procedures was proposed since these may have a potential for better performance than the methods used in the draft report and/or generate certain useful information unavailable from the procedures originally employed. These procedures were undertaken to explore the extent of any possible differences and evaluate whether the original procedures were sufficient for the intended regulatory use of this assay. Detailed explanations on these points were included in the summary minutes of three conference calls. Some rationale for the proposed improvements is briefly given below.

The method originally employed by CERI for generating run-specific estimates of logPC10 and logPC50 was linear interpolation implemented in a spreadsheet. As standard error (SE) was not reported originally, this appendix supplies the SEs, calculated using an add-on procedure implemented following the linear interpolation. CERI used Hill equation-based nonlinear regression available in the GraphPad Prism software to estimate logEC50. Similarly as no SEs for logEC50 was reported originally they are provided here.

SEs for linear interpolation-based logPC10 and logPC50 could be obtained using the delta method for nonlinear combination of regression coefficient from a linear regression. Although the linear interpolation is quite simple to perform (as demonstrated by the spreadsheet calculation shown by CERI) it may have some drawbacks: It may not be efficient because it uses only 6 data points (triplicate at two concentration levels) rather than all the data points available, i.e., 21 data points (triplicate at seven concentration levels); and it is expected to have some downward bias for logPC10, i.e., underestimation of logPC10 (and upward bias for logPC50, i.e., overestimation of logPC50, when the top plateau level of the underlying response is close to 50%) because an underlying concave (convex) curve is approximated by a line.

Intuitively, we may be able to improve our estimates of logPC10 and logPC50 by using all the data points available rather than linear approximation based on a portion of the whole data. A promising alternative that does just that is Hill equation-based nonlinear regression, which may be more efficient since it uses all the data, not only those with the average response levels “sandwiching”

the specific levels of interest (i.e., 10% or 50%). It reflects the underlying biological model more properly, thereby providing potentially more accurate logPC10 or logPC50. In CERI's original analysis logEC50 values already were estimated using a version of Hill equation-based nonlinear regression. logPC50 and logEC50 differ from each other in that the former corresponds to log10(concentration) that yields 50% of the response given by a standard compound at a pre-specified concentration while the latter corresponds to log10(concentration) that yields 50% of the response the maximum response level the test chemical produces. In this document log10 may be expressed simply as "log".

It was proposed that the DerSimonian-Laird (DL) random effects model be used as an alternative procedure for summarizing the run-specific estimates for each lab (and further summarizing the lab-specific estimates across labs). This procedure takes SE of individual run estimates into account and provides not only estimates of the overall between-run (lab) variability but also estimates of intrinsic between-run (lab) variability. The original analyses by CERI used what we call "traditional" in this appendix.

To sum, several combinations of parameters of interest and analytical procedures are performed, which are summarized below.

Estimation method for individual run	Method for summary					
	Traditional			DL		
	Parameter of interest					
	logPC10	logPC50	logEC50	logPC10	logPC50	logEC50
Linear interpolation	Tables 17, 19	Tables 17, 18	N.A.	Tables 6.1, 6.2	Tables 6.1, 6.3	N.A.
Hill equation-based nonlinear regression	Tables 6.4, 6.5	Tables 6.4, 6.6	Tables 17, 20, 6.4, 6.7	Tables 6.8, 6.9	Tables 6.8, 6.10	Tables 6.8, 6.11

Methods

As an alternative method for obtaining run-specific summary for logPC10 and logPC50, the use of Hill equation-based nonlinear regression was proposed. A version of the equation with four parameters, i.e., bottom, top, slope, and logPC10 (or logPC50), with a constraint of bottom = 0, was

initially proposed. The constraint of bottom = 0 seems justified since an appropriate blank value was subtracted from all response values.

After some exploratory analyses of the CERI data, it was decided to include another constraint of slope >= 0. This additional constraint keeps nonsensical logPC10 or logPC50 accompanied by a negative slope from being reported. Other constraints may also be used to keep nonsensical fit results from being generated, but in this preliminary analysis the constraint of slope >= 0 only was imposed. For logEC50 estimation, no constraint for the two remaining parameters (top levels and Hill slope) was imposed. It also was noticed that using a standard set of initial values for the three parameters resulted in failure to converge. Changing the initial value for logPC10 to log(the minimum concentration) achieved convergence in certain runs.

For estimation of logEC50, 4-parameter Hill equation-based nonlinear regression was used. Again, a constraint of slope >= 0 was imposed while no constraint for the other parameters were used.

Hill equation-based nonlinear regression occasionally generated rather imprecise logPC10 or logEC50. Estimates of these with SE (estimate) > 1 were excluded from further analyses. This cutoff is arbitrary. It represents a high degree of uncertainty and corresponds approximately to 180-fold difference between the upper and lower limits of PC10 (or EC50), which were obtained by exponentiating corresponding 95% confidence limits for logPC10 (or logEC50).

The proposed alternative method for summarizing run(lab)-specific estimates was DerSimonian-Laird random effects model. This generates, in addition to the estimate of overall between-variability, an estimate of intrinsic between-variability. In general, the overall (total) variability consists of two components: intrinsic between variability and overall within variability. That is, the following relationships hold:[2)]

Overall (total) within-lab variability =
Overall (total) between-run variability = intrinsic between-run variability
+ overall within-run variability

and

Overall (total) between-lab variability = intrinsic between-lab variability
+ overall within-lab variability

The complementary estimates of overall within-variability and intrinsic between-variability serve

[2)] The relationships hold in terms of variance under the assumption of independence between the underlying components for the two right-hand side terms.

certain practical purposes for a user of the assay.

In the traditional method, overall between-variability is estimated by obtaining SD of the point estimates for which a mean is calculated. SE of the mean is SD/sqrt(the number of point estimates summarized). In the DerSimonian-Laird (DL) random effects model, the overall between-variability is estimated by combining the estimate of intrinsic between-variability and estimates of within-variability. In the DL random effects model both SE of the mean and SD were calculated such that they can be compared to the counterparts from the traditional method. In addition, *p*-value for testing the null hypothesis of intrinsic between-variability = zero also was obtained for the DL model. This *p*-value is labeled as "homogeneity *p*-value" in the tables. Since the Q statistic-based test for these homogeneity *p*-value is known to be underpowered, a *p*-value below 0.1~0.15 (as opposed to usual 0.05) may be taken as some evidence for existence of non-zero intrinsic between-variability. In estimating overall between-variability, the traditional method ignores SE of the estimates being summarized, thereby taking within-variability into account only through apparent overall between-variability, which sometimes can be misleadingly small. As such, the traditional method underestimates overall between-variability when the intrinsic between-variability is small relative to within-variability. Other than this difference, the traditional method and DL method are expected to yield comparable results in terms of overall between-variability estimates, which is of our primary interest in an interlaboratory study.

For the present data, within the same run (i.e., experiment done on the same occasion) 17β-estradiol was tested in up to three plates. This provided us with an opportunity to investigate relative contribution of intrinsic between-plate variability and within-plate variability to overall between-run variability. For Tables 4 and 8, within-run summary was estimated from within-plate summary employing the same method used to the between-run, within-lab summary, i.e., either the traditional method or DL random effects method.

Simultaneous modeling of mean of response and log(variance) of response was performed to compare two methods of summarization using a heteroscedastic regression.

Stata statistical software (version 8) was used. A user-defined command "meta" was used for the DerSimonian-Laird random effects model and "regh" for the heteroscedastic regression.

Results

Overall between-lab variability

The results for individual runs as well as overall variability (within-lab or between-lab) are presented in Tables 6.1-11. These results based on the alternative procedures in general lead us to the same conclusions as the ones based on the original combination of procedures, i.e., the linear interpolation for run-specific summary and traditional method for between-run (lab) summary. That is, the assay demonstrated acceptable overall within-lab variability as well as between-lab variability. Overall between-lab SDs are estimated for each of the chemicals tested as follows using Hill equation-based nonlinear regression and DerSimonian-Laird random effects model (extracted from Tables 6.9 and 10). (The readers who wish to rely on results based on a combination of procedures other than this can base their decisions on corresponding overall between-lab SD values in Tables 17-20 in the body of the report or Tables 6.2, 3, 5, 6 in this appendix. Qualitative conclusion would be similar to the ones presented above.)

Summary of overall between-lab SD estimates for presumed positives

Chemical	logPC10	logPC50
17α-Estradiol	0.31	0.29
Bisphenol A	0.29	0.27
Genistein	0.31	0.15
17α-Methyltestosterone	0.21	0.21
4-tert-Octylphenol	0.15	0.18
p-tert-pentylphenol	0.30	0.08
17β-Estradiol	0.21	0.24
Arithmetic mean	0.25	0.20
Arithmetic mean*	0.26	0.20

* For all chemicals other than 17β-Estradiol

Overall between-lab SD of 0.25 means that a future parameter estimate from a lab drawn from a universe of labs like the four labs in the interlaboratory study is expected to fall in the range between 0.33 times the true value and 3.1 times the true value ($0.33 = 1/3.1 = 10^{-1.95*0.25}$) with a probability of 95%. The overall between-lab variability could be greater for some test chemicals. The observed maximum was 0.31, and this corresponds to the minimum and maximum ratios to the true value of 0.25 and 4.0, respectively. This level of variability seems satisfactorily low for the intended use of the assay.

For the rest of this Appendix, foci will be given to logPC10 and logPC50. Interpretation of logEC50 depends on the top plateau level of the observed curve, which can vary considerably across chemicals. As such, logEC50 is not as readily interpretable as logPC10 or logPC50.

Table 6.1 Estimated logPC10, logPC50 and logEC50 and their SE for 17β-estradiol based on linear interpolation by run and within- and overall between-lab variation (DL random effects model)

Test Substance	Test vial No.	Laboratory	Trial	Log_{10}[PC10(M)] Esti-mate		Mean	SD	intrinsic SDbtw-plate	homo-geneity *p*-value	Mean	SD	intrinsic SDbtw-run	homo-geneity *p*-value	Log_{10}[PC50(M)] Esti-mate		Mean	SD	intrinsic SDbtw-plate	homo-geneity *p*-value	Mean	SD	intrinsic SDbtw-run
17β-Estradiol	37	ceri	1-1	-11.89	0.12									-10.69	0.10							
			1-2	-11.73	0.05	-11.82	0.12	0.08	0.12					-10.52	0.03	-10.62	0.12	0.10	< 0.01			
			1-3	-11.91	0.08									-10.69	0.04							
			2-1	-12.42	0.04									-10.98	0.04							
			2-2	-12.65	0.09	-12.32	0.39	0.39	< 0.01	-12.11	0.33	0.29	< 0.01	-11.22	0.06	-11.02	0.14	0.13	< 0.01	-10.99	0.37	0.36
			2-3	-11.91	0.01									-10.90	0.03							
			3-1	-11.98	0.11									-11.11	0.10							
			3-2	-12.41	0.10	-12.28	0.22	0.20	< 0.01					-11.36	0.06	-11.31	0.10	0.08	0.05			
			3-3	-12.41	0.06									-11.37	0.04							
	38	sumitomo	1-1	-11.74	0.06									-11.06	0.07							
			1-2			-11.74	0.06							-10.83	0.19	-11.18	0.40	0.38	< 0.01			
			1-3											-11.59	0.05							
			2-1	-12.01	0.58									-10.51	0.05							
			2-2	-11.62	0.04	-11.60	0.06	0.00	0.39	-11.72	0.14	0.12	< 0.01	-10.54	0.02	-10.54	0.03	0.00	0.75	-10.71	0.21	0.18
			2-3	-11.50	0.09									-10.50	0.07							
			3-1	-12.12	0.41									-11.05	0.26							
			3-2	-11.82	0.16	-11.87	0.14	0.00	0.79					-10.77	0.09	-10.72	0.14	0.10	0.10			
			3-3	-11.87	0.09									-10.63	0.04							
	39	otsuka	1-1	-11.58	0.07									-10.44	0.03							
			1-2	-11.56	0.09	-11.57	0.08	0.00	0.92					-10.48	0.05	-10.45	0.03	0.00	0.48			
			1-3																			
			2-1	-11.09	0.11									-10.38	0.03							
			2-2	-11.58	0.05	-11.35	0.35	0.34	< 0.01	-11.64	0.22	0.18	< 0.01	-10.43	0.06	-10.39	0.04	0.00	0.46	-10.48	0.09	0.08
			2-3																			
			3-1	-11.84	0.08									-10.55	0.05							
			3-2	-11.64	0.14	-11.85	0.13	0.10	0.09					-10.75	0.04	-10.71	0.15	0.13	< 0.01			
			3-3	-11.95	0.06									-10.90	0.12							
	40	kaneka	1-1	-12.61	0.28									-11.08	0.06							
			1-2			-12.16	0.56	0.53	< 0.01					-11.04	0.07	-10.95	0.19	0.17	< 0.01			
			1-3	-11.80	0.04									-10.71	0.08							
			2-1	-11.88	0.12									-10.65	0.03							
			2-2	-12.23	0.38	-11.87	0.12	0.00	0.60	-11.80	0.13	0.09	0.10	-10.51	0.03	-10.57	0.07	0.06	< 0.01	-10.63	0.17	0.16
			2-3	-11.85	0.09									-10.56	0.02							
			3-1	-11.66	0.08									-10.48	0.02							
			3-2	-11.72	0.08	-11.72	0.07	0.00	0.71					-10.37	0.01	-10.45	0.08	0.08	< 0.01			
			3-3	-11.75	0.06									-10.52	0.04							
Total	MAX			-11.09	0.58	-11.35	0.56	0.53		-11.64	0.33	0.29		-10.37	0.26	-10.39	0.40	0.38		-10.48	0.37	0.36
	MIN			-12.65	0.01	-12.32	0.06	0.00		-12.11	0.13	0.09		-11.59	0.01	-11.31	0.03	0.00		-10.99	0.09	0.08
	Ave.			-11.87	0.14	-11.81	0.23	0.19		-11.70	0.20	0.17		-10.76	0.07	-10.72	0.15	0.13		-10.58	0.21	0.20

Table 6.2 Estimated logPC10 and its SE based on linear interpolation by run and within- and between-lab variation (DL random effects model

Test Substance	Test vial No.	Laboratory	Trial	Log$_{10}$ [PC10(M)]											
				Data	SE	Within-lab					Between-lab				
						Mean	SE	SD	intrinsic SDbtw-run	homogeneity p-value	Mean	SE	SD	intrinsic SDbtw-run	homogeneity p-value
Hematoxylin	1	ceri	1	-	-						-8.12	0.30	0.30	0.00	-
			2	-	-	-8.12	0.30	0.30	0.00	-					
			3	-8.12	0.30										
	2	sumitomo	1	-	-										
			2	-	-	-	-	-	-	-					
			3	-	-										
	3	otsuka	1	-	-										
			2	-	-	-	-	-	-	-					
			3	-	-										
	4	kaneka	1	-	-										
			2	-	-	-	-	-	-	-					
			3	-	-										
alpha-Estradiol	5	ceri	1	-10.49	0.27						-9.90	0.13	0.25	0.18	0.10
			2	-10.69	0.27	-10.33	0.26	0.45	0.39	< 0.01					
			3	-9.98	0.02										
	6	sumitomo	1	-10.47	0.10										
			2	-9.84	0.11	-10.05	0.20	0.35	0.34	< 0.01					
			3	-9.84	0.06										
	7	otsuka	1	-9.65	0.12										
			2	-9.33	0.10	-9.62	0.17	0.30	0.28	< 0.01					
			3	-9.85	0.05										
	8	kaneka	1	-10.06	0.09										
			2	-9.83	0.06	-9.83	0.11	0.19	0.17	< 0.01					
			3	-9.64	0.06										
Benzophenone	9	ceri	1	-	-						-7.61	0.42	0.42	0.00	-
			2	-	-	-	-	-	-	-					
			3	-	-										
	10	sumitomo	1	-	-										
			2	-	-	-	-	-	-	-					
			3	-	-										
	11	otsuka	1	-	-										
			2	-	-	-	-	-	-	-					
			3	-	-										
	12	kaneka	1	-7.61	0.42										
			2	-	-	-7.61	0.42	0.42	0.00	-					
			3	-	-										
Bisphenol A	13	ceri	1	-7.77	0.12						-7.43	0.19	0.38	0.29	0.04
			2	-7.51	0.04	-7.69	0.13	0.22	0.19	0.01					
			3	-7.92	0.18										
	14	sumitomo	1	-7.73	0.19										
			2	-7.28	0.30	-7.28	0.31	0.54	0.50	< 0.01					
			3	-6.88	0.04										
	15	otsuka	1	-10.20	0.55										
			2	-7.08	0.18	-7.80	0.51	0.88	0.82	< 0.01					
			3	-6.82	0.09										
	16	kaneka	1	-10.81	0.82										
			2	-6.97	0.07	-7.10	0.17	0.30	0.24	< 0.01					
			3	-6.92	0.03										
Diethylhexyl phthalate	17	ceri	1	-	-						-5.49	0.20	0.20	0.00	-
			2	-	-	-	-	-	-	-					
			3	-	-										
	18	sumitomo	1	-	-										
			2	-5.49	0.20	-5.49	0.20	0.20	0.00	-					
			3	-	-										
	19	otsuka	1	-	-										
			2	-	-	-	-	-	-	-					
			3	-	-										
	20	kaneka	1	-	-										
			2	-	-	-	-	-	-	-					
			3	-	-										

Table 6.2 Estimated logPC10 and its SE based on linear interpolation by run and within- and between-lab variation (DL random effects model) (Continued)

Genistein	21	ceri	1	-7.19	0.10						-7.97	0.03	0.06	0.00	0.76
			2	-8.40	0.13	-8.09	0.49	0.84	0.83	< 0.01					
			3	-8.70	0.07										
	22	sumitomo	1	-8.70	0.22										
			2	-7.84	0.07	-8.01	0.13	0.23	0.20	< 0.01					
			3	-7.84	0.04										
	23	otsuka	1	-7.94	0.06										
			2	-7.94	0.06	-7.96	0.03	0.05	0.00	0.77					
			3	-7.99	0.04										
	24	kaneka	1	-8.92	0.21										
			2	-8.05	0.31	-8.30	0.34	0.58	0.54	< 0.01					
			3	-7.95	0.02										
17α-Methyltestosterone	25	ceri	1	-7.66	0.25						-7.43	0.16	0.31	0.24	0.04
			2	-7.40	0.45	-7.81	0.18	0.31	0.13	0.30					
			3	-8.06	0.23										
	26	sumitomo	1	-7.44	0.16										
			2	-6.32	0.38	-6.83	0.37	0.64	0.59	< 0.01					
			3	-6.58	0.24										
	27	otsuka	1	-7.06	0.51										
			2	-6.74	0.43	-7.25	0.18	0.31	0.10	0.34					
			3	-7.40	0.19										
	28	kaneka	1	-7.59	0.16										
			2	-7.45	0.22	-7.50	0.12	0.21	0.00	0.51					
			3	-7.17	0.34										
4-tert-Octylphenol	29	ceri	1	-7.90	0.12						-7.90	0.03	0.05	0.01	0.38
			2	-7.96	0.01	-8.13	0.20	0.34	0.33	< 0.01					
			3	-8.51	0.07										
	30	sumitomo	1	-9.67	0.44										
			2	-7.65	0.06	-8.35	0.49	0.84	0.79	< 0.01					
			3	-7.99	0.30										
	31	otsuka	1	-7.96	0.05										
			2	-7.84	0.09	-7.92	0.04	0.07	0.00	0.46					
			3	-7.84	0.15										
	32	kaneka	1	-7.85	0.05										
			2	-8.07	0.45	-7.87	0.03	0.06	0.00	0.78					
			3	-7.89	0.04										
p-tert-pentylphenol	33	ceri	1	-7.54	0.10						-7.48	0.12	0.23	0.16	0.12
			2	-7.67	0.12	-7.71	0.12	0.21	0.18	0.02					
			3	-7.96	0.12										
	34	sumitomo	1	-9.79	0.46										
			2	-6.70	0.17	-7.62	0.48	0.83	0.79	< 0.01					
			3	-6.86	0.09										
	35	otsuka	1	-7.42	0.24										
			2	-7.34	0.20	-7.38	0.15	0.26	0.00	0.95					
			3	-7.52	0.61										
	36	kaneka	1	-7.46	0.07										
			2	-6.97	0.15	-7.30	0.13	0.23	0.20	0.01					
			3	-7.40	0.12										
17β-Estradiol	37	ceri	1	-11.82	0.07						-11.77	0.06	0.13	0.08	0.19
			2	-12.32	0.23	-12.11	0.19	0.33	0.29	< 0.01					
			3	-12.28	0.13										
	38	sumitomo	1	-11.74	0.06										
			2	-11.60	0.03	-11.72	0.08	0.14	0.12	< 0.01					
			3	-11.87	0.08										
	39	otsuka	1	-11.57	0.06										
			2	-11.35	0.24	-11.64	0.12	0.22	0.18	< 0.01					
			3	-11.85	0.08										
	40	kaneka	1	-12.16	0.40										
			2	-11.87	0.07	-11.80	0.08	0.13	0.09	0.10					
			3	-11.72	0.04										

Table 6.3 Estimated logPC50 and its SE based on linear interpolation by run and within- and between-lab variation (DL random effects model)

Test Substance	Test vial No.	Laboratory	Trial	Log_{10} [PC50 (M)]											
						Within-lab					Between-lab				
				Estimate	SE	Mean	SE	SD	intrinsic SDbtw-run	homogeneity *p*-value	Mean	SE	SD	intrinsic SDbtw-run	homogeneity *p*-value
Hematoxylin	1	ceri	1	-	-	-	-	-	-	-	-	-	-	-	-
			2	-	-										
			3	-	-										
	2	sumitomo	1	-	-	-	-	-	-	-					
			2	-	-										
			3	-	-										
	3	otsuka	1	-	-	-	-	-	-	-					
			2	-	-										
			3	-	-										
	4	kaneka	1	-	-	-	-	-	-	-					
			2	-	-										
			3	-	-										
alpha-Estradiol	5	ceri	1	-9.04	0.10	-9.34	0.06	0.11	0.10	< 0.01	-8.87	0.24	0.48	0.46	< 0.01
			2	-9.46	0.04										
			3	-9.39	0.02										
	6	sumitomo	1	-9.22	0.18	-8.82	0.11	0.19	0.17	< 0.01					
			2	-8.63	0.08										
			3	-8.79	0.04										
	7	otsuka	1	-8.33	0.07	-8.49	0.09	0.16	0.14	< 0.01					
			2	-8.46	0.04										
			3	-8.77	0.13										
	8	kaneka	1	-9.15	0.02	-8.79	0.22	0.38	0.38	< 0.01					
			2	-8.66	0.05										
			3	-8.56	0.03										
Benzophenone	9	ceri	1	-	-	-	-	-	-	-	-	-	-	-	-
			2	-	-										
			3	-	-										
	10	sumitomo	1	-	-	-	-	-	-	-					
			2	-	-										
			3	-	-										
	11	otsuka	1	-	-	-	-	-	-	-					
			2	-	-										
			3	-	-										
	12	kaneka	1	-	-	-	-	-	-	-					
			2	-	-										
			3	-	-										
Bisphenol A	13	ceri	1	-6.50	0.02	-6.58	0.05	0.08	0.07	< 0.01	-6.41	0.09	0.17	0.14	0.02
			2	-6.57	0.03										
			3	-6.76	0.08										
	14	sumitomo	1	-6.52	0.15	-6.37	0.11	0.19	0.16	0.03					
			2	-6.48	0.12										
			3	-6.22	0.04										
	15	otsuka	1	-6.14	0.08	-6.31	0.10	0.17	0.15	< 0.01					
			2	-6.46	0.05										
			3	-6.31	0.08										
	16	kaneka	1	-6.53	0.02	-6.29	0.15	0.26	0.26	< 0.01					
			2	-6.18	0.06										
			3	-6.14	0.03										
Diethylhexyl phthalate	17	ceri	1	-	-	-	-	-	-	-	-	-	-	-	-
			2	-	-										
			3	-	-										
	18	sumitomo	1	-	-	-	-	-	-	-					
			2	-	-										
			3	-	-										
	19	otsuka	1	-	-	-	-	-	-	-					
			2	-	-										
			3	-	-										
	20	kaneka	1	-	-	-	-	-	-	-					
			2	-	-										
			3	-	-										

Table 6.3 Estimated logPC50 and its SE based on linear interpolation by run and within- and between-lab variation (DL random effects model) (Continued)

Genistein			1	-6.77	0.07						-7.21	0.11	0.21	0.16	0.07
	21	ceri	2	-7.66	0.01	-7.41	0.21	0.37	0.37	< 0.01					
			3	-7.78	0.05										
			1	-7.42	0.15										
	22	sumitomo	2	-6.89	0.05	-7.03	0.08	0.14	0.12	< 0.01					
			3	-6.99	0.03										
			1	-7.23	0.05										
	23	otsuka	2	-7.27	0.05	-7.35	0.11	0.19	0.18	< 0.01					
			3	-7.54	0.03										
			1	-7.52	0.00										
	24	kaneka	2	-7.07	0.05	-7.18	0.22	0.38	0.38	< 0.01					
			3	-6.97	0.02										
17α-Methyltestosterone			1	-5.75	0.36						-5.56	0.10	0.20	0.17	< 0.01
	25	ceri	2	-5.87	0.14	-5.86	0.13	0.22	0.00	0.95					
			3	-5.90	0.39										
			1	-5.49	0.15										
	26	sumitomo	2	-5.10	0.30	-5.38	0.08	0.14	0.00	0.47					
			3	-5.35	0.11										
			1	-5.73	0.15										
	27	otsuka	2	-	-	-5.62	0.11	0.16	0.02	0.31					
			3	-5.50	0.16										
			1	-5.60	0.20										
	28	kaneka	2	-5.51	0.18	-5.45	0.10	0.18	0.00	0.50					
			3	-5.31	0.16										
4-tert-Octylphenol			1	-6.85	0.07						-6.78	0.07	0.14	0.11	< 0.01
	29	ceri	2	-7.12	0.01	-7.16	0.16	0.27	0.27	< 0.01					
			3	-7.51	0.03										
			1	-6.67	0.17										
	30	sumitomo	2	-6.68	0.08	-6.59	0.08	0.14	0.10	0.10					
			3	-6.48	0.07										
			1	-6.89	0.04										
	31	otsuka	2	-6.82	0.11	-6.81	0.07	0.12	0.10	0.04					
			3	-6.70	0.06										
			1	-6.72	0.04										
	32	kaneka	2	-6.80	0.04	-6.75	0.03	0.05	0.00	0.37					
			3	-6.75	0.06										
p-tert-pentylphenol			1	-6.08	0.12						-5.95	0.07	0.14	0.11	0.04
	33	ceri	2	-6.30	0.10	-6.39	0.22	0.38	0.37	< 0.01					
			3	-6.79	0.08										
			1	-5.91	0.10										
	34	sumitomo	2	-5.82	0.11	-5.82	0.05	0.09	0.00	0.54					
			3	-5.77	0.08										
			1	-5.89	0.19										
	35	otsuka	2	-5.87	0.35	-5.99	0.10	0.17	0.00	0.72					
			3	-6.06	0.13										
			1	-6.00	0.18										
	36	kaneka	2	-5.89	0.09	-5.95	0.06	0.11	0.00	0.72					
			3	-5.99	0.10										
17β-Estradiol			1	-10.98	0.11						-10.73	0.09	0.18	0.17	< 0.01
	37	ceri	2	-11.11	0.08	-11.11	0.04	0.07	0.00	0.45					
			3	-11.14	0.06										
			1	-10.82	0.13										
	38	sumitomo	2	-10.64	0.04	-10.67	0.02	0.03	0.00	0.39					
			3	-10.67	0.02										
			1	-10.54	0.06										
	39	otsuka	2	-10.56	0.06	-10.58	0.03	0.04	0.00	0.66					
			3	-10.60	0.03										
			1	-10.65	0.06										
	40	kaneka	2	-10.55	0.04	-10.57	0.03	0.05	0.02	0.32					
			3	-10.55	0.04										
MAX							0.22	0.38	0.38			0.24	0.48	0.46	
MIN							0.02	0.03	0.00			0.07	0.14	0.11	
Ave.							0.10	0.17	0.12			0.11	0.22	0.19	

Table 6.4 Estimated logPC10, logPC50 and logEC50 and their SE for 17β-estradiol based on Hill equation-based nonlinear regression by run and within- and overall between-lab variation (traditional analysis)

Test Substance	Test vial No.	Laboratory	Trial	Log_{10}[PC10(M)]					Log_{10}[PC50(M)]					Log_{10}[EC50(M)]					
				Estimate		Mean	SE	SD	Estimate		Mean	SE	SD	HILLSLOPE	R2	Estimate		Mean	SE
17β-Estradiol	37	ceri	1-1	-11.66	0.17				-10.74	0.08				1.07	0.99	-10.75	0.10		
			1-2	-11.56	0.20	-11.69	0.08	0.14	-10.54	0.10	-10.67	0.07	0.11	0.96	0.98	-10.57	0.13	-10.69	0.06
			1-3	-11.84	0.16				-10.73	0.08				1.04	0.99	-10.74	0.09		
			2-1	-11.80	0.24				-11.03	0.08				6.97	0.99	-10.98	0.02		
			2-2	-12.15	0.17	-11.88	0.14	0.25	-11.22	0.07	-11.07	0.08	0.14	1.55	0.99	-11.00	0.05	-10.97	0.03
			2-3	-11.68	0.14				-10.94	0.05				1.41	0.99	-10.91	0.05		
			3-1	-11.85	0.37				-11.10	0.13				1.32	0.96	-11.17	0.19		
			3-2	-11.99	0.26	-11.96	0.06	0.10	-11.27	0.10	-11.23	0.07	0.11	2.18	0.99	-11.04	0.10	-11.12	0.04
			3-3	-12.05	0.23				-11.31	0.10				1.62	0.99	-11.15	0.13		
	38	sumitomo	1-1	-11.17	0.02				-11.01	0.02				1.31	0.97	-11.12	0.14		
			1-2	-12.46	0.51	-12.29	0.60	1.04	-11.02	0.21	-11.19	0.18	0.31	1.05	0.96	-10.48	0.23	-11.03	0.30
			1-3	-13.23	0.37				-11.56	0.15				0.46	0.98	-11.49	0.54		
			2-1	-11.72	0.26				-10.50	0.12				0.83	0.98	-10.51	0.17		
			2-2	-11.37	0.23	-11.47	0.12	0.21	-10.54	0.12	-10.50	0.02	0.04	1.28	0.97	-10.46	0.16	-10.48	0.02
			2-3	-11.33	0.19				-10.47	0.09				0.96	0.99	-10.46	0.11		
			3-1	-12.23	0.28				-11.00	0.12				0.74	0.98	-10.93	0.19		
			3-2	-11.61	0.17	-11.82	0.21	0.36	-10.81	0.08	-10.82	0.10	0.17	1.32	0.99	-10.71	0.10	-10.73	0.11
			3-3	-11.61	0.11				-10.65	0.05				1.02	1.00	-10.55	0.06		
	39	otsuka	1-1	-11.36	0.10				-10.42	0.05				1.03	1.00	-10.40	0.06		
			1-2	-11.30	0.15	-11.33	0.03	0.05	-10.45	0.07	-10.43	0.02	0.02	1.08	0.99	-10.37	0.09	-10.39	0.01
			1-3	-	-				-	-				1.18	0.99	-	-		
			2-1	-11.02	0.21				-10.32	0.09				1.12	0.99	-10.42	0.10		
			2-2	-11.31	0.13	-11.17	0.14	0.20	-10.38	0.06	-10.35	0.03	0.04	1.06	0.99	-10.26	0.07	-10.34	0.08
			2-3	-	-				-	-				0.72	0.98	-	-		
			3-1	-11.46	0.22				-10.61	0.13				1.22	0.97	-10.67	0.15		
			3-2	-11.30	0.15	-11.56	0.18	0.31	-10.79	0.10	-10.77	0.09	0.15	1.68	0.98	-10.71	0.12	-10.72	0.03
			3-3	-11.91	0.20				-10.91	0.09				0.96	0.99	-10.78	0.11		
	40	kaneka	1-1	-11.89	0.19				-11.09	0.07				3.50	0.99	-10.97	0.40		
			1-2	-12.26	0.19	-11.89	0.21	0.36	-11.15	0.08	-10.99	0.13	0.23	1.64	1.00	-10.89	0.06	-10.82	0.11
			1-3	-11.53	0.11				-10.73	0.05				1.20	0.99	-10.60	0.07		
			2-1	-11.49	0.09				-10.68	0.05				1.28	1.00	-10.58	0.06		
			2-2	-11.65	0.18	-11.54	0.06	0.10	-10.53	0.08	-10.60	0.04	0.08	1.03	0.99	-10.36	0.10	-10.47	0.06
			2-3	-11.47	0.13				-10.58	0.07				1.27	0.99	-10.46	0.08		
			3-1	-11.40	0.14				-10.46	0.06				1.04	0.99	-10.35	0.08		
			3-2	-11.42	0.13	-11.43	0.02	0.04	-10.33	0.05	-10.43	0.05	0.09	1.02	1.00	-10.20	0.06	-10.32	0.06
			3-3	-11.48	0.08				-10.51	0.04				1.02	1.00	-10.40	0.05		
Total	MAX			-11.02	0.51	-11.17	0.60	1.04	-10.32	0.21	-10.35	0.18	0.31	6.9700	1.0000	-10.20	0.54	-10.32	0.30
	MIN			-13.23	0.02	-12.29	0.02	0.04	-11.56	0.02	-11.23	0.02	0.02	0.4600	0.9600	-11.49	0.02	-11.12	0.01
	Ave.			-11.67	0.20	-11.63	0.20	0.34	-10.76	0.09	-10.73	0.08	0.14	1.5567	0.9869	-10.67	0.14	-10.64	0.10

Table 6.5 Estimated logPC10 and its SE based on Hill equation-based nonlinear regression by run and within- and overall between-lab variation (traditional analysis)

Test Substance	Test vial No.	Laboratory	Trial	Log_{10} [PC10 (M)]							
				Estimate	SE	intra-Lab			inter-Lab		
						Mean	SE	SD	Mean	SE	SD
Hematoxylin	1	ceri	1	-	-	-	-	-	-4.18	-	-
			2	-	-						
			3	-	-						
	2	sumitomo	1	-	-	-4.18	-	-			
			2	-4.18	0.97						
			3	-	-						
	3	otsuka	1	-	-	-	-	-			
			2	-	-						
			3	-	-						
	4	kaneka	1	-	-	-	-	-			
			2	-	-						
			3	-	-						
alpha-Estradiol	5	ceri	1	-10.00	0.38	-10.01	0.08	0.14	-9.73	0.11	0.22
			2	-10.17	0.33						
			3	-9.88	0.33						
	6	sumitomo	1	-10.69	0.58	-10.00	0.34	0.60			
			2	-9.64	0.18						
			3	-9.67	0.11						
	7	otsuka	1	-9.36	0.20	-9.31	0.07	0.12			
			2	-9.17	0.18						
			3	-9.40	0.35						
	8	kaneka	1	-9.89	0.12	-9.58	0.17	0.30			
			2	-9.55	0.13						
			3	-9.30	0.24						
Benzophenone	9	ceri	1	-	-	-	-	-	-7.99	-	-
			2	-	-						
			3	-	-						
	10	sumitomo	1	-	-	-	-	-			
			2	-	-						
			3	-	-						
	11	otsuka	1	-	-	-	-	-			
			2	-	-						
			3	-	-						
	12	kaneka	1	-7.99	0.33	-7.99	-	-			
			2	-	-						
			3	-	-						
Bisphenol A	13	ceri	1	-7.37	0.08	-7.31	0.08	0.14	-7.16	0.21	0.41
			2	-7.14	0.10						
			3	-7.41	0.17						
	14	sumitomo	1	-7.88	0.66	-7.22	0.36	0.63			
			2	-7.14	0.23						
			3	-6.63	0.11						
	15	otsuka	1	-6.83	0.28	-7.07	0.60	1.05			
			2	-8.21	0.36						
			3	-6.16	0.01						
	16	kaneka	1	-7.31	0.17	-7.05	0.14	0.24			
			2	-7.02	0.15						
			3	-6.83	0.11						
Diethylhexyl phthalate	17	ceri	1	-	-	-	-	-	-5.14	-	-
			2	-	-						
			3	-	-						
	18	sumitomo	1	-	-	-5.14	0.18	0.26			
			2	-5.33	0.15						
			3	-4.96	0.03						
	19	otsuka	1	-	-	-	-	-			
			2	-	-						
			3	-	-						
	20	kaneka	1	-	-	-	-	-			
			2	-	-						
			3	-	-						

Table 6.5 Estimated logPC10 and its SE based on Hill equation-based nonlinear regression by run and within- and overall between-lab variation (traditional analysis) (Continued)

Genistein	21	ceri	1	-7.05	0.22				-8.52	0.20	0.40
			2	-9.02	0.26	-8.43	0.69	1.20			
			3	-9.22	0.27						
	22	sumitomo	1	-9.34	0.22						
			2	-7.99	0.24	-8.40	0.47	0.81			
			3	-7.88	0.20						
	23	otsuka	1	-8.29	0.38						
			2	-8.26	0.30	-8.51	0.24	0.41			
			3	-8.98	0.26						
	24	kaneka	1	-8.96	0.27						
			2	-8.35	0.22	-8.74	0.20	0.34			
			3	-8.91	0.15						
17α-Methyltestosterone	25	ceri	1	-7.86	0.50				-7.25	0.15	0.30
			2	-7.44	0.24	-7.67	0.12	0.21			
			3	-7.72	0.43						
	26	sumitomo	1	-7.72	0.28						
			2	-6.15	0.37	-6.76	0.48	0.84			
			3	-6.42	0.18						
	27	otsuka	1	-7.32	0.29						
			2	-6.62	0.39	-7.16	0.28	0.48			
			3	-7.54	0.29						
	28	kaneka	1	-7.49	0.37						
			2	-7.53	0.30	-7.39	0.12	0.20			
			3	-7.16	0.24						
4-tert-Octylphenol	29	ceri	1	-7.67	0.13				-8.00	0.19	0.38
			2	-7.92	0.03	-7.94	0.16	0.28			
			3	-8.23	0.16						
	30	sumitomo	1	-7.97	0.50						
			2	-7.55	0.18	-8.11	0.38	0.65			
			3	-8.83	0.28						
	31	otsuka	1	-7.81	0.09						
			2	-7.37	0.86	-7.58	0.13	0.22			
			3	-7.57	0.13						
	32	kaneka	1	-7.67	0.06						
			2	-9.56	0.43	-8.35	0.61	1.05			
			3	-7.81	0.07						
p-tert-pentylphenol	33	ceri	1	-7.46	0.19				-7.26	0.10	0.20
			2	-7.48	0.14	-7.57	0.10	0.18			
			3	-7.77	0.12						
	34	sumitomo	1	-7.39	0.24						
			2	-6.12	0.02	-6.74	0.37	0.64			
			3	-6.72	0.14						
	35	otsuka	1	-7.70	0.29						
			2	-7.19	0.28	-7.40	0.15	0.27			
			3	-7.30	0.23						
	36	kaneka	1	-7.31	0.22						
			2	-6.95	0.19	-7.34	0.23	0.40			
			3	-7.75	0.18						
17β-Estradiol	37	ceri	1	-11.69	0.08				-11.67	0.06	0.12
			2	-11.88	0.14	-11.84	0.08	0.14			
			3	-11.96	0.06						
	38	sumitomo	1	-12.29	0.60						
			2	-11.47	0.12	-11.86	0.24	0.41			
			3	-11.82	0.21						
	39	otsuka	1	-11.33	0.03						
			2	-11.17	0.14	-11.35	0.11	0.19			
			3	-11.56	0.18						
	40	kaneka	1	-11.89	0.21						
			2	-11.54	0.06	-11.62	0.14	0.24			
			3	-11.43	0.02						
MAX					0.97		0.69	1.20		0.21	0.41
MIN					0.01		0.07	0.12		0.06	0.12
Ave.					0.24		0.25	0.44		0.15	0.29

Table 6.6 Estimated logPC50 and its SE based on Hill equation-based nonlinear regression by run and within- and overall between-lab variation (traditional analysis)

Test Substance	Test vial No.	Laboratory	Trial	Log$_{10}$ [PC50 (M)]							
				Estimate	SE	intra-Lab			inter-Lab		
						Mean	SE	SD	Mean	SE	SD
Hematoxylin	1	ceri	1	-	-						
			2	-	-	-	-	-			
			3	-	-						
	2	sumitomo	1	-	-						
			2	-	-	-	-	-			
			3	-	-				-	-	-
	3	otsuka	1	-	-						
			2	-	-	-	-	-			
			3	-	-						
	4	kaneka	1	-	-						
			2	-	-	-	-	-			
			3	-	-						
alpha-Estradiol	5	ceri	1	-9.09	0.17						
			2	-9.48	0.21	-9.29	0.11	0.20			
			3	-9.31	0.15						
	6	sumitomo	1	-9.06	0.25						
			2	-8.71	0.09	-8.87	0.10	0.18			
			3	-8.83	0.05				-8.90	0.02	0.04
	7	otsuka	1	-8.36	0.12						
			2	-8.47	0.11	-8.57	0.15	0.26			
			3	-8.86	0.13						
	8	kaneka	1	-9.12	0.04						
			2	-8.71	0.06	-8.85	0.13	0.23			
			3	-8.73	0.20						
Benzophenone	9	ceri	1	-	-						
			2	-	-	-	-	-			
			3	-	-						
	10	sumitomo	1	-	-						
			2	-	-	-	-	-			
			3	-	-				-	-	-
	11	otsuka	1	-	-						
			2	-	-	-	-	-			
			3	-	-						
	12	kaneka	1	-	-						
			2	-	-	-	-	-			
			3	-	-						
Bisphenol A	13	ceri	1	-6.45	0.03						
			2	-6.48	0.05	-6.55	0.09	0.16			
			3	-6.73	0.09						
	14	sumitomo	1	-6.38	0.19						
			2	-6.35	0.08	-6.28	0.08	0.15			
			3	-6.11	0.02				-6.30	0.01	0.03
	15	otsuka	1	-6.09	0.07						
			2	-6.35	0.05	-6.16	0.09	0.16			
			3	-6.05	0.01						
	16	kaneka	1	-6.46	0.07						
			2	-6.12	0.04	-6.22	0.12	0.21			
			3	-6.09	0.03						
Diethylhexyl phthalate	17	ceri	1	-	-						
			2	-	-	-	-	-			
			3	-	-						
	18	sumitomo	1	-	-						
			2	-	-	-	-	-			
			3	-	-				-	-	-
	19	otsuka	1	-	-						
			2	-	-	-	-	-			
			3	-	-						
	20	kaneka	1	-	-						
			2	-	-	-	-	-			
			3	-	-						

Table 6.6 Estimated logPC50 and its SE based on Hill equation-based nonlinear regression by run and within- and overall between-lab variation (traditional analysis) (Continued)

Genistein	21	ceri	1	-6.53	0.11	-7.32	0.40	0.69	-7.17	0.12	0.23
			2	-7.65	0.12						
			3	-7.77	0.12						
	22	sumitomo	1	-7.28	0.13	-7.00	0.15	0.26			
			2	-6.78	0.08						
			3	-6.95	0.09						
	23	otsuka	1	-7.10	0.16	-7.21	0.09	0.16			
			2	-7.14	0.13						
			3	-7.40	0.26						
	24	kaneka	1	-7.46	0.11	-7.13	0.17	0.29			
			2	-7.00	0.08						
			3	-6.93	0.05						
17α-Methyltestosterone	25	ceri	1	-5.72	0.24	-5.79	0.04	0.07	-5.48	0.07	0.14
			2	-5.81	0.12						
			3	-5.84	0.21						
	26	sumitomo	1	-5.39	0.08	-5.45	0.15	0.26			
			2	-5.74	0.12						
			3	-5.22	0.04						
	27	otsuka	1	-5.60	0.08	-5.28	0.22	0.39			
			2	-4.85	0.15						
			3	-5.39	0.08						
	28	kaneka	1	-5.58	0.16	-5.41	0.10	0.18			
			2	-5.41	0.08						
			3	-5.23	0.06						
4-tert-Octylphenol	29	ceri	1	-6.90	0.05	-7.15	0.17	0.29	-6.84	0.05	0.10
			2	-7.07	0.01						
			3	-7.46	0.08						
	30	sumitomo	1	-6.68	0.18	-6.58	0.07	0.12			
			2	-6.61	0.07						
			3	-6.45	0.07						
	31	otsuka	1	-6.92	0.04	-6.87	0.06	0.11			
			2	-6.93	0.16						
			3	-6.74	0.07						
	32	kaneka	1	-6.72	0.03	-6.77	0.03	0.06			
			2	-6.83	0.05						
			3	-6.78	0.03						
p-tert-pentylphenol	33	ceri	1	-6.07	0.08	-6.38	0.23	0.40	-6.04	0.08	0.16
			2	-6.24	0.05						
			3	-6.83	0.05						
	34	sumitomo	1	-5.88	0.09	-5.89	0.04	0.07			
			2	-5.96	0.02						
			3	-5.82	0.05						
	35	otsuka	1	-5.88	0.13	-5.94	0.04	0.07			
			2	-5.92	0.15						
			3	-6.02	0.09						
	36	kaneka	1	-6.00	0.08	-5.96	0.03	0.06			
			2	-5.90	0.07						
			3	-5.99	0.05						
17β-Estradiol	37	ceri	1	-10.67	0.07	-10.99	0.17	0.29	-10.75	0.03	0.05
			2	-11.07	0.08						
			3	-11.23	0.07						
	38	sumitomo	1	-11.19	0.18	-10.84	0.20	0.35			
			2	-10.50	0.02						
			3	-10.82	0.10						
	39	otsuka	1	-10.43	0.02	-10.52	0.13	0.22			
			2	-10.35	0.03						
			3	-10.77	0.09						
	40	kaneka	1	-10.99	0.13	-10.67	0.17	0.29			
			2	-10.60	0.04						
			3	-10.43	0.05						
MAX					0.26		0.40	0.69		0.12	0.23
MIN					0.01		0.03	0.06		0.01	0.03
Ave.					0.09		0.13	0.22		0.05	0.11

Table 6.7 Estimated logPC50 and its SE based on Hill equation-based nonlinear regression by run and within- and overall between-lab variation (DL random effects model)

Test Substance	Test vial No.	Laboratory	Trial	Log_{10} [EC50(M)]							
				Estimate	SE	intra-Lab			inter-Lab		
						Mean	SE	SD	Mean	SE	SD
Hematoxylin	1	ceri	1	-	-	-	-	-	-6.69	1.96	1.96
			2	-	-						
			3	-	-						
	2	sumitomo	1	-	-	-6.02	-	-			
			2	-	-						
			3	-6.02	0.66						
	3	otsuka	1	-4.23	0.03	-5.15	0.93	1.31			
			2	-6.08	0.37						
			3	-	-						
	4	kaneka	1	-	-	-8.89	-	-			
			2	-8.89	0.15						
			3	-	-						
alpha-Estradiol	5	ceri	1	-9.02	0.03	-9.25	0.12	0.20	-8.89	0.13	0.26
			2	-9.37	0.33						
			3	-9.37	0.19						
	6	sumitomo	1	-	-	-8.82	0.01	0.01			
			2	-8.83	0.10						
			3	-8.82	0.06						
	7	otsuka	1	-8.48	0.14	-8.64	0.12	0.21			
			2	-8.57	0.12						
			3	-8.88	0.16						
	8	kaneka	1	-9.04	0.04	-8.85	0.10	0.17			
			2	-8.69	0.08						
			3	-8.84	0.14						
Benzophenone	9	ceri	1	-4.37	0.04	-6.82	1.25	2.16	-7.62	0.50	0.71
			2	-8.41	0.75						
			3	-7.69	0.61						
	10	sumitomo	1	-	-	-	-	-			
			2	-	-						
			3	-	-						
	11	otsuka	1	-7.90	0.75	-7.90	-	-			
			2	-	-						
			3	-	-						
	12	kaneka	1	-7.44	0.36	-8.15	0.58	1.00			
			2	-9.29	0.99						
			3	-7.72	0.36						
Bisphenol A	13	ceri	1	-6.25	0.04	-6.31	0.08	0.13	-6.10	0.07	0.14
			2	-6.22	0.03						
			3	-6.46	0.11						
	14	sumitomo	1	-	-	-5.99	0.00	0.00			
			2	-5.99	0.10						
			3	-5.99	0.02						
	15	otsuka	1	-6.00	0.08	-6.07	0.06	0.10			
			2	-6.18	0.06						
			3	-6.03	0.04						
	16	kaneka	1	-6.15	0.02	-6.03	0.06	0.10			
			2	-5.97	0.05						
			3	-5.98	0.03						
Diethylhexyl phthalate	17	ceri	1	-	-	-9.00	-	-	-7.76	3.05	3.05
			2	-9.00	0.02						
			3	-	-						
	18	sumitomo	1	-4.31	0.07	-4.29	0.02	0.02			
			2	-	-						
			3	-4.27	0.04						
	19	otsuka	1	-	-	-	-	-			
			2	-	-						
			3	-	-						
	20	kaneka	1	-9.99	0.43	-9.99	-	-			
			2	-	-						
			3	-	-						

Table 6.7 Estimated logPC10 and its SE based on Hill equation-based nonlinear regression by run and within- and overall between-lab variation (DL random effects model) (Continued)

Genistein	21	ceri	1	-5.95	0.05	-5.72	0.20	0.34	-4.77	0.74	1.49
			2	-5.33	0.37						
			3	-5.88	0.28						
	22	sumitomo	1	-	-	-5.62	0.34	0.49			
			2	-5.27	0.44						
			3	-5.96	0.12						
	23	otsuka	1	-5.00	0.79	-2.57	2.55	4.41			
			2	-5.24	0.46						
			3	2.52	0.66						
	24	kaneka	1	-5.21	0.37	-5.18	0.20	0.35			
			2	-4.82	0.54						
			3	-5.51	0.13						
17α-Methyltestosterone	25	ceri	1	-	-	-6.06	-	-	-5.13	0.92	1.30
			2	-	-						
			3	-6.06	0.48						
	26	sumitomo	1	-5.73	0.45	-3.65	2.03	3.51			
			2	-5.63	0.97						
			3	0.41	0.88						
	27	otsuka	1	-5.53	0.74	-5.69	0.13	0.23			
			2	-5.95	0.68						
			3	-5.59	0.71						
	28	kaneka	1	-	-	-	-	-			
			2	-	-						
			3	-	-						
4-tert-Octylphenol	29	ceri	1	-6.93	0.06	-7.08	0.15	0.27	-6.72	0.17	0.34
			2	-6.93	0.02						
			3	-7.39	0.09						
	30	sumitomo	1	-6.25	0.20	-6.27	0.05	0.08			
			2	-6.36	0.11						
			3	-6.20	0.12						
	31	otsuka	1	-6.80	0.05	-6.85	0.11	0.19			
			2	-7.06	0.08						
			3	-6.70	0.08						
	32	kaneka	1	-6.58	0.04	-6.67	0.05	0.08			
			2	-6.69	0.06						
			3	-6.75	0.05						
p-tert-pentylphenol	33	ceri	1	-5.92	0.25	-6.21	0.30	0.52	-5.36	0.61	1.22
			2	-5.90	0.17						
			3	-6.80	0.07						
	34	sumitomo	1	0.81	0.78	-3.56	2.19	3.78			
			2	-5.61	0.42						
			3	-5.88	0.10						
	35	otsuka	1	-5.71	0.43	-5.95	0.14	0.23			
			2	-6.18	0.30						
			3	-5.98	0.12						
	36	kaneka	1	-5.55	0.45	-5.73	0.09	0.16			
			2	-5.84	0.13						
			3	-5.80	0.17						
17β-Estradiol	37	ceri	1	-10.69	0.06	-10.92	0.13	0.22	-10.67	0.10	0.20
			2	-10.97	0.03						
			3	-11.12	0.04						
	38	sumitomo	1	-11.03	0.30	-10.75	0.16	0.28			
			2	-10.48	0.02						
			3	-10.73	0.11						
	39	otsuka	1	-10.39	0.01	-10.48	0.12	0.21			
			2	-10.34	0.08						
			3	-10.72	0.03						
	40	kaneka	1	-10.82	0.11	-10.53	0.15	0.26			
			2	-10.47	0.06						
			3	-10.32	0.06						
MAX *				-10.43	0.05	-	-	-	-	-	-
MIN *					0.01		0.00	0.00		0.07	0.14
Ave. *					0.22		0.37	0.64		0.39	0.71

* Excepting for Hematoxylin, Benzophenone and Diethylhexyl phthalate

Table 6.8 Estimated logPC10, logPC50 and logEC50 and their SE for 17β-estradiol based on Hill equation-based nonlinear regression by run and within- and overall between-lab variation (DL random effects model)

Test Substance	Test vial No.	Laboratory	Trial	Log_{10}[PC10(M)] Estimate	SE	Within-run Mean	Within-run SD	Within-run intrinsic SDbtw-plate	Within-run homogeneity *p*-value	Within-lab Mean	Within-lab SD	Within-lab intrinsic SDbtw-run	Within-lab homogeneity *p*-value
17β-Estradiol			1-1	-11.66	0.17								
			1-2	-11.56	0.20	-11.70	0.17	0.00	0.51				
			1-3	-11.84	0.16								
			2-1	-11.80	0.24								
	37	ceri	2-2	-12.15	0.17	-11.87	0.28	0.21	0.09	-11.82	0.15	0.08	0.27
			2-3	-11.68	0.14								
			3-1	-11.85	0.37								
			3-2	-11.99	0.26	-11.99	0.27	0.00	0.90				
			3-3	-12.05	0.23								
			1-1	-11.17	0.02								
			1-2	-12.46	0.51	-12.25	1.31	1.26	< 0.01				
			1-3	-13.23	0.37								
			2-1	-11.72	0.26								
	38	sumitomo	2-2	-11.37	0.23	-11.43	0.22	0.00	0.46	-11.59	0.24	0.14	0.21
			2-3	-11.33	0.19								
			3-1	-12.23	0.28								
			3-2	-11.61	0.17	-11.73	0.26	0.19	0.11				
			3-3	-11.61	0.11								
			1-1	-11.36	0.10								
			1-2	-11.30	0.15	-11.34	0.12	0.00	0.72				
			1-3										
			2-1	-11.02	0.21								
	39	otsuka	2-2	-11.31	0.13	-11.21	0.20	0.11	0.24	-11.34	0.12	0.02	0.36
			2-3										
			3-1	-11.46	0.22								
			3-2	-11.30	0.15	-11.54	0.32	0.26	0.05				
			3-3	-11.91	0.20								
			1-1	-11.89	0.19								
			1-2	-12.26	0.19	-11.87	0.39	0.36	< 0.01				
			1-3	-11.53	0.11								
			2-1	-11.49	0.09								
	40	kaneka	2-2	-11.65	0.18	-11.51	0.12	0.00	0.71	-11.51	0.11	0.07	0.19
			2-3	-11.47	0.13								
			3-1	-11.40	0.14								
			3-2	-11.42	0.13	-11.45	0.11	0.00	0.85				
			3-3	-11.48	0.08								
Total	MAX			-11.02	0.51	-11.21	1.31	1.26		-11.34	0.24	0.14	
	MIN			-13.23	0.02	-12.25	0.11	0.00		-11.82	0.11	0.02	
	Ave.			-11.67	0.20	-11.62	0.41	0.30		-11.44	0.18	0.09	

Test Substance	Test vial No.	Laboratory	Trial	Log_{10}[PC50(M)] Estimate	SE	Within-run Mean	Within-run SD	Within-run intrinsic SDbtw-plate	Within-run homogeneity *p*-value	Within-lab Mean	Within-lab SD	Within-lab intrinsic SDbtw-run	Within-lab homogeneity *p*-value
17β-Estradiol			1-1	-10.74	0.08								
			1-2	-10.54	0.10	-10.68	0.10	0.06	0.22				
			1-3	-10.73	0.08								
			2-1	-11.03	0.08								
	37	ceri	2-2	-11.22	0.07	-11.06	0.15	0.14	< 0.01	-11.00	0.32	0.32	< 0.01
			2-3	-10.94	0.05								
			3-1	-11.10	0.13								
			3-2	-11.27	0.10	-11.25	0.11	0.00	0.39				
			3-3	-11.31	0.10								
			1-1	-11.01	0.02								
			1-2	-11.02	0.21	-11.19	0.32	0.29	< 0.01				
			1-3	-11.56	0.15								
			2-1	-10.50	0.12								
	38	sumitomo	2-2	-10.54	0.12	-10.50	0.11	0.00	0.88	-10.79	0.30	0.27	< 0.01
			2-3	-10.47	0.09								
			3-1	-11.00	0.12								
			3-2	-10.81	0.08	-10.80	0.16	0.14	0.01				
			3-3	-10.65	0.05								
			1-1	-10.42	0.05								
			1-2	-10.45	0.07	-10.42	0.06	0.00	0.72				
			1-3										
			2-1	-10.32	0.09								
	39	otsuka	2-2	-10.38	0.06	-10.37	0.07	0.00	0.58	-10.52	0.18	0.17	< 0.01
			2-3										
			3-1	-10.61	0.13								
			3-2	-10.79	0.10	-10.79	0.14	0.09	0.15				
			3-3	-10.91	0.09								
			1-1	-11.09	0.07								
			1-2	-11.15	0.08	-10.98	0.24	0.23	< 0.01				
			1-3	-10.73	0.05								
			2-1	-10.68	0.05								
	40	kaneka	2-2	-10.53	0.08	-10.62	0.08	0.05	0.22	-10.64	0.19	0.17	< 0.01
			2-3	-10.58	0.07								
			3-1	-10.46	0.06								
			3-2	-10.33	0.05	-10.43	0.10	0.08	0.03				
			3-3	-10.51	0.04								
Total	MAX			-10.32	0.21	-10.37	0.32	0.29		-10.52	0.32	0.32	
	MIN			-11.56	0.02	-11.25	0.06	0.00		-11.00	0.18	0.17	
	Ave.			-10.76	0.09	-10.73	0.15	0.11		-10.61	0.25	0.23	

Test Substance	Test vial No.	Laboratory	Trial	Log_{10} [EC50(M)] Estimate	SE	Within-run Mean	Within-run SD	Within-run intrinsic SDbtw-plate	Within-run homogeneity *p*-value	Within-lab Mean	Within-lab SD	Within-lab intrinsic SDbtw-run	Within-lab homogeneity *p*-value
17β-Estradiol			1-1	-10.75	0.10								
			1-2	-10.57	0.13	-10.71	0.10	0.00	0.51				
			1-3	-10.74	0.09								
			2-1	-10.98	0.02								
	37	ceri	2-2	-11.00	0.05	-10.98	0.03	0.00	0.45	-10.93	0.16	0.15	< 0.01
			2-3	-10.91	0.05								
			3-1	-11.17	0.19								
			3-2	-11.04	0.10	-11.09	0.12	0.00	0.73				
			3-3	-11.15	0.13								
			1-1	-11.12	0.14								
			1-2	-10.48	0.23	-10.94	0.47	0.38	0.04				
			1-3	-11.49	0.54								
			2-1	-10.51	0.17								
	38	sumitomo	2-2	-10.46	0.16	-10.47	0.14	0.00	0.97	-10.61	0.18	0.13	0.10
			2-3	-10.46	0.11								
			3-1	-10.93	0.19								
			3-2	-10.71	0.10	-10.68	0.17	0.13	0.08				
			3-3	-10.55	0.06								
			1-1	-10.40	0.06								
			1-2	-10.37	0.09	-10.39	0.07	0.00	0.82				
			1-3										
			2-1	-10.42	0.10								
	39	otsuka	2-2	-10.26	0.07	-10.32	0.11	0.07	0.20	-10.48	0.21	0.19	< 0.01
			2-3										
			3-1	-10.67	0.15								
			3-2	-10.71	0.12	-10.73	0.12	0.00	0.82				
			3-3	-10.78	0.11								
			1-1	-10.97	0.40								
			1-2	-10.89	0.06	-10.77	0.23	0.18	< 0.01				
			1-3	-10.60	0.07								
			2-1	-10.58	0.06								
	40	kaneka	2-2	-10.36	0.10	-10.48	0.11	0.08	0.10	-10.50	0.18	0.16	< 0.01
			2-3	-10.46	0.08								
			3-1	-10.35	0.08								
			3-2	-10.20	0.06	-10.32	0.11	0.09	0.04				
			3-3	-10.40	0.05								
Total	MAX			-10.20	0.54	-10.32	0.47	0.38		-10.48	0.21	0.19	
	MIN			-11.49	0.02	-11.09	0.03	0.00		-10.93	0.16	0.13	
	Ave.			-10.67	0.14	-10.62	0.18	0.11		-10.52	0.19	0.17	

Table 6.9 Estimated logPC10 and its SE based on Hill equation-based nonlinear regression by run and within- and overall between-lab variation (DL random effects model)

Test Substance	Test vial No.	Laboratory	Trial	Log_{10} [PC10(M)]											
				Data	SE	Within-lab					Between-lab				
						Mean	SE	SD	intrinsic SDbtw-run	homogeneity *p* -value	Mean	SE	SD	intrinsic SDbtw-run	homogeneity *p* -value *
Hematoxylin	1	ceri	1	-	-	-	-	-	-	-	-4.18	0.97	0.97	0.00	-
			2	-	-										
			3	-	-										
	2	sumitomo	1	-	-	-4.18	0.97	0.97	0.00	-					
			2	-4.18	0.97										
			3	-	-										
	3	otsuka	1	-	-	-	-	-	-	-					
			2	-	-										
			3	-	-										
	4	kaneka	1	-	-	-	-	-	-	-					
			2	-	-										
			3	-	-										
alpha-Estradiol	5	ceri	1	-10.00	0.38	-10.01	0.20	0.34	0.00	0.83	-9.63	0.15	0.31	0.26	< 0.01
			2	-10.17	0.33										
			3	-9.88	0.33										
	6	sumitomo	1	-10.69	0.58	-9.72	0.14	0.25	0.15	0.21					
			2	-9.64	0.18										
			3	-9.67	0.11										
	7	otsuka	1	-9.36	0.20	-9.27	0.12	0.22	0.00	0.72					
			2	-9.17	0.18										
			3	-9.40	0.35										
	8	kaneka	1	-9.89	0.12	-9.62	0.16	0.28	0.23	0.03					
			2	-9.55	0.13										
			3	-9.30	0.24										
Benzophenone	9	ceri	1	-	-	-	-	-	-	-	-7.99	0.33	0.33	0.00	-
			2	-	-										
			3	-	-										
	10	sumitomo	1	-	-	-	-	-	-	-					
			2	-	-										
			3	-	-										
	11	otsuka	1	-	-	-	-	-	-	-					
			2	-	-										
			3	-	-										
	12	kaneka	1	-7.99	0.33	-7.99	0.33	0.33	0.00	-					
			2	-	-										
			3	-	-										
Bisphenol A	13	ceri	1	-7.37	0.08	-7.30	0.09	0.15	0.10	0.16	-7.18	0.09	0.17	0.07	0.32
			2	-7.14	0.10										
			3	-7.41	0.17										
	14	sumitomo	1	-7.88	0.66	-6.99	0.27	0.47	0.38	0.03					
			2	-7.14	0.23										
			3	-6.63	0.11										
	15	otsuka	1	-6.83	0.28	-7.02	0.56	0.96	0.93	< 0.01					
			2	-8.21	0.36										
			3	-6.16	0.01										
	16	kaneka	1	-7.31	0.17	-7.03	0.14	0.24	0.19	0.06					
			2	-7.02	0.15										
			3	-6.83	0.11										
Diethylhexyl phthalate	17	ceri	1	-	-	-	-	-	-	-	-5.11	0.18	0.18	0.00	-
			2	-	-										
			3	-	-										
	18	sumitomo	1	-	-	-5.11	0.18	0.25	0.23	0.02					
			2	-5.33	0.15										
			3	-4.96	0.03										
	19	otsuka	1	-	-	-	-	-	-	-					
			2	-	-										
			3	-	-										
	20	kaneka	1	-	-	-	-	-	-	-					
			2	-	-										
			3	-	-										

Table 6.9 Estimated logPC10 and its SE based on Hill equation-based nonlinear regression by run and within- and overall between-lab variation (DL random effects model) (Continued)

Genistein	21	ceri	1	-7.05	0.22						-8.64	0.14	0.29	0.00	0.86
			2	-9.02	0.26	-8.43	0.73	1.26	1.24	< 0.01					
			3	-9.22	0.27										
	22	sumitomo	1	-9.34	0.22										
			2	-7.99	0.24	-8.40	0.47	0.82	0.79	< 0.01					
			3	-7.88	0.20										
	23	otsuka	1	-8.29	0.38										
			2	-8.26	0.30	-8.55	0.26	0.44	0.32	0.13					
			3	-8.98	0.26										
	24	kaneka	1	-8.96	0.27										
			2	-8.35	0.22	-8.74	0.19	0.33	0.26	0.08					
			3	-8.91	0.15										
17α-Methyltestosterone	25	ceri	1	-7.86	0.50						-7.36	0.11	0.22	0.02	0.39
			2	-7.44	0.24	-7.56	0.19	0.33	0.00	0.69					
			3	-7.72	0.43										
	26	sumitomo	1	-7.72	0.28										
			2	-6.15	0.37	-6.77	0.46	0.80	0.75	< 0.01					
			3	-6.42	0.18										
	27	otsuka	1	-7.32	0.29										
			2	-6.62	0.39	-7.22	0.25	0.43	0.29	0.16					
			3	-7.54	0.29										
	28	kaneka	1	-7.49	0.37										
			2	-7.53	0.30	-7.34	0.16	0.29	0.00	0.56					
			3	-7.16	0.24										
4-tert-Octylphenol	29	ceri	1	-7.67	0.13						-7.83	0.07	0.14	0.05	0.35
			2	-7.92	0.03	-7.92	0.12	0.21	0.17	0.03					
			3	-8.23	0.16										
	30	sumitomo	1	-7.97	0.50										
			2	-7.55	0.18	-8.11	0.46	0.80	0.73	< 0.01					
			3	-8.83	0.28										
	31	otsuka	1	-7.81	0.09										
			2	-7.37	0.86	-7.72	0.08	0.15	0.05	0.33					
			3	-7.57	0.13										
	32	kaneka	1	-7.67	0.06										
			2	-9.56	0.43	-7.98	0.19	0.33	0.28	< 0.01					
			3	-7.81	0.07										
p-tert-pentylphenol	33	ceri	1	-7.46	0.19						-7.36	0.14	0.29	0.21	0.07
			2	-7.48	0.14	-7.60	0.11	0.19	0.12	0.18					
			3	-7.77	0.12										
	34	sumitomo	1	-7.39	0.24										
			2	-6.12	0.02	-6.71	0.34	0.58	0.56	< 0.01					
			3	-6.72	0.14										
	35	otsuka	1	-7.70	0.29										
			2	-7.19	0.28	-7.38	0.15	0.26	0.00	0.41					
			3	-7.30	0.23										
	36	kaneka	1	-7.31	0.22										
			2	-6.95	0.19	-7.34	0.24	0.42	0.37	< 0.01					
			3	-7.75	0.18										
17β-Estradiol	37	ceri	1	-11.70	0.10						-11.56	0.11	0.21	0.19	< 0.01
			2	-11.87	0.16	-11.82	0.09	0.15	0.08	0.27					
			3	-11.99	0.16										
	38	sumitomo	1	-12.25	0.75										
			2	-11.43	0.13	-11.59	0.14	0.24	0.14	0.21					
			3	-11.73	0.15										
	39	otsuka	1	-11.34	0.08										
			2	-11.21	0.14	-11.34	0.07	0.12	0.02	0.36					
			3	-11.54	0.19										
	40	kaneka	1	-11.87	0.23										
			2	-11.51	0.07	-11.51	0.07	0.11	0.07	0.19					
			3	-11.45	0.06										
MAX							0.97	1.26	1.24	#		0.97	0.97	0.26	
MIN							0.07	0.11	0.00	#		0.07	0.14	0.00	
Ave.							0.26	0.41	0.27			0.23	0.31	0.08	

* Heterogeneity *p* -value could not be calculated when summarizing just one estimate.

Table 6.10 Estimated logPC50 and its SE based on Hill equation-based nonlinear regression by run and within- and overall between-lab variation (DL random effects model)

Test Substance	Test vial No.	Laboratory	Trial	Log_{10} [PC50 (M)]											
						Within-lab					Between-lab				
				Data	SE	Mean	SE	SD	intrinsic SDbtw-run	homogeneity *p*-value	Mean	SE	SD	intrinsic SDbtw-run	homogeneity *p*-value
Hematoxylin	1	ceri	1	-	-	-	-	-	-	-	-	-	-	-	-
			2	-	-										
			3	-	-										
	2	sumitomo	1	-	-	-	-	-	-	-					
			2	-	-										
			3	-	-										
	3	otsuka	1	-	-	-	-	-	-	-					
			2	-	-										
			3	-	-										
	4	kaneka	1	-	-	-	-	-	-	-					
			2	-	-										
			3	-	-										
alpha-Estradiol	5	ceri	1	-9.09	0.17	-9.28	0.10	0.18	0.06	0.32	-8.89	0.14	0.29	0.26	< 0.01
			2	-9.48	0.21										
			3	-9.31	0.15										
	6	sumitomo	1	-9.06	0.25	-8.81	0.05	0.09	0.04	0.30					
			2	-8.71	0.09										
			3	-8.83	0.05										
	7	otsuka	1	-8.36	0.12	-8.56	0.14	0.25	0.22	0.01					
			2	-8.47	0.11										
			3	-8.86	0.13										
	8	kaneka	1	-9.12	0.04	-8.87	0.17	0.29	0.27	< 0.01					
			2	-8.71	0.06										
			3	-8.73	0.20										
Benzophenone	9	ceri	1	-	-	-	-	-	-	-	-	-	-	-	-
			2	-	-										
			3	-	-										
	10	sumitomo	1	-	-	-	-	-	-	-					
			2	-	-										
			3	-	-										
	11	otsuka	1	-	-	-	-	-	-	-					
			2	-	-										
			3	-	-										
	12	kaneka	1	-	-	-	-	-	-	-					
			2	-	-										
			3	-	-										
Bisphenol A	13	ceri	1	-6.45	0.03	-6.52	0.06	0.11	0.09	0.01	-6.30	0.10	0.19	0.17	< 0.01
			2	-6.48	0.05										
			3	-6.73	0.09										
	14	sumitomo	1	-6.38	0.19	-6.25	0.10	0.18	0.15	< 0.01					
			2	-6.35	0.08										
			3	-6.11	0.02										
	15	otsuka	1	-6.09	0.07	-6.16	0.10	0.18	0.17	< 0.01					
			2	-6.35	0.05										
			3	-6.05	0.01										
	16	kaneka	1	-6.46	0.07	-6.21	0.09	0.16	0.15	< 0.01					
			2	-6.12	0.04										
			3	-6.09	0.03										
Diethylhexyl phthalate	17	ceri	1	-	-	-	-	-	-	-	-	-	-	-	-
			2	-	-										
			3	-	-										
	18	sumitomo	1	-	-	-	-	-	-	-					
			2	-	-										
			3	-	-										
	19	otsuka	1	-	-	-	-	-	-	-					
			2	-	-										
			3	-	-										
	20	kaneka	1	-	-	-	-	-	-	-					
			2	-	-										
			3	-	-										

Table 6.10 Estimated logPC50 and its SE based on Hill equation-based nonlinear regression by run and within- and overall between-lab variation (DL random effects model) (Continued)

Genistein	21	ceri	1	-6.53	0.11	-7.32	0.41	0.71	0.70	< 0.01	-7.11	0.07	0.13	0.00	0.71
			2	-7.65	0.12										
			3	-7.77	0.12										
	22	sumitomo	1	-7.28	0.13	-6.98	0.13	0.23	0.21	< 0.01					
			2	-6.78	0.08										
			3	-6.95	0.09										
	23	otsuka	1	-7.10	0.16	-7.16	0.10	0.16	0.00	0.60					
			2	-7.14	0.13										
			3	-7.40	0.26										
	24	kaneka	1	-7.46	0.11	-7.11	0.14	0.24	0.23	< 0.01					
			2	-7.00	0.08										
			3	-6.93	0.05										
17α-Methyltestosterone	25	ceri	1	-5.72	0.24	-5.80	0.10	0.17	0.00	0.92	-5.49	0.12	0.25	0.21	< 0.01
			2	-5.81	0.12										
			3	-5.84	0.21										
	26	sumitomo	1	-5.39	0.08	-5.42	0.13	0.22	0.21	< 0.01					
			2	-5.74	0.12										
			3	-5.22	0.04										
	27	otsuka	1	-5.60	0.08	-5.31	0.17	0.30	0.28	< 0.01					
			2	-4.85	0.15										
			3	-5.39	0.08										
	28	kaneka	1	-5.58	0.16	-5.37	0.09	0.16	0.13	0.05					
			2	-5.41	0.08										
			3	-5.23	0.06										
4-tert-Octylphenol	29	ceri	1	-6.90	0.05	-7.13	0.11	0.18	0.18	< 0.01	-6.81	0.09	0.18	0.16	< 0.01
			2	-7.07	0.01										
			3	-7.46	0.08										
	30	sumitomo	1	-6.68	0.18	-6.55	0.07	0.12	0.07	0.20					
			2	-6.61	0.07										
			3	-6.45	0.07										
	31	otsuka	1	-6.92	0.04	-6.86	0.07	0.12	0.09	0.06					
			2	-6.93	0.16										
			3	-6.74	0.07										
	32	kaneka	1	-6.72	0.03	-6.77	0.03	0.05	0.04	0.10					
			2	-6.83	0.05										
			3	-6.78	0.03										
p-tert-pentylphenol	33	ceri	1	-6.07	0.08	-6.38	0.23	0.40	0.40	< 0.01	-5.96	0.04	0.08	0.05	0.20
			2	-6.24	0.05										
			3	-6.83	0.05										
	34	sumitomo	1	-5.88	0.09	-5.90	0.06	0.10	0.08	0.02					
			2	-5.96	0.02										
			3	-5.82	0.05										
	35	otsuka	1	-5.88	0.13	-5.96	0.07	0.11	0.00	0.61					
			2	-5.92	0.15										
			3	-6.02	0.09										
	36	kaneka	1	-6.00	0.08	-5.97	0.03	0.06	0.00	0.49					
			2	-5.90	0.07										
			3	-5.99	0.05										
17β-Estradiol	37	ceri	1	-10.98	0.08	-11.08	0.02	0.04	0.00	0.50	-10.75	0.12	0.24	0.24	< 0.01
			2	-11.08	0.05										
			3	-11.09	0.03										
	38	sumitomo	1	-10.82	0.10	-10.75	0.02	0.04	0.00	0.61					
			2	-10.72	0.05										
			3	-10.75	0.03										
	39	otsuka	1	-10.55	0.08	-10.60	0.03	0.05	0.00	0.62					
			2	-10.58	0.07										
			3	-10.62	0.04										
	40	kaneka	1	-10.67	0.08	-10.56	0.03	0.04	0.01	0.35					
			2	-10.56	0.04										
			3	-10.54	0.03										
MAX							0.41	0.71	0.70			0.14	0.29	0.26	
MIN							0.02	0.04	0.00			0.04	0.08	0.00	
Ave.							0.10	0.18	0.14			0.10	0.19	0.16	

Table 6.11 Estimated logEC50 and its SE based on Hill equation-based nonlinear regression by run and within- and overall between-lab variation (DL random effects model)

Test Substance	Test vial No.	Laboratory	Trial	Log$_{10}$ [EC50(M)] Data	SE	Within-lab Mean	Within-lab SE	Within-lab SD	Within-lab intrinsic SDbtw-run	Within-lab homogeneity p-value	Between-lab Mean	Between-lab SE	Between-lab SD	Between-lab intrinsic SDbtw-run	Between-lab homogeneity p-value
			1	-	-										
	1	ceri	2	-	-	-	-	-	-	-					
			3	-	-										
			1	-	-										
	2	sumitomo	2	-	-	-6.02	0.66	0.66	0.00	-					
Hematoxylin			3	-6.02	0.66						-6.78	1.31	2.27	2.18	< 0.01
			1	-4.23	0.03										
	3	otsuka	2	-6.08	0.37	-5.11	0.93	1.31	1.28	< 0.01					
			3	-	-										
			1	-	-										
	4	kaneka	2	-8.89	0.15	-8.89	0.15	0.15	0.00	-					
			3	-	-										
			1	-9.02	0.03										
	5	ceri	2	-9.37	0.33	-9.17	0.14	0.25	0.19	0.10					
			3	-9.37	0.19										
			1	-	-										
	6	sumitomo	2	-8.83	0.10	-8.82	0.05	0.07	0.00	0.87					
alpha-Estradiol			3	-8.82	0.06						-8.85	0.09	0.18	0.14	0.03
			1	-8.48	0.14										
	7	otsuka	2	-8.57	0.12	-8.63	0.11	0.19	0.13	0.15					
			3	-8.88	0.16										
			1	-9.04	0.04										
	8	kaneka	2	-8.69	0.08	-8.86	0.13	0.23	0.21	< 0.01					
			3	-8.84	0.14										
			1	-4.37	0.04										
	9	ceri	2	-8.41	0.75	-6.76	1.49	2.58	2.52	< 0.01					
			3	-7.69	0.61										
			1	-	-										
	10	sumitomo	2	-	-	-	-	-	-	-					
Benzophenone			3	-	-						-7.74	0.30	0.52	0.00	0.79
			1	-7.90	0.75										
	11	otsuka	2	-	-	-7.90	0.75	0.75	0.00	-					
			3	-	-										
			1	-7.44	0.36										
	12	kaneka	2	-9.29	0.99	-7.75	0.34	0.59	0.35	0.21					
			3	-7.72	0.36										
			1	-6.25	0.04										
	13	ceri	2	-6.22	0.03	-6.26	0.04	0.07	0.05	0.12					
			3	-6.46	0.11										
			1	-	-										
	14	sumitomo	2	-5.99	0.10	-5.99	0.02	0.03	0.00	0.99					
Bisphenol A			3	-5.99	0.02						-6.09	0.07	0.14	0.14	< 0.01
			1	-6.00	0.08										
	15	otsuka	2	-6.18	0.06	-6.07	0.06	0.10	0.08	0.05					
			3	-6.03	0.04										
			1	-6.15	0.02										
	16	kaneka	2	-5.97	0.05	-6.04	0.06	0.11	0.11	< 0.01					
			3	-5.98	0.03										
			1	-	-										
	17	ceri	2	-9.00	0.02	-9.00	0.02	0.02	0.00	-					
			3	-	-										
			1	-4.31	0.07										
	18	sumitomo	2	-	-	-4.28	0.03	0.05	0.00	0.67					
Diethylhexyl phthalate			3	-4.27	0.04						-7.74	1.93	3.34	3.33	< 0.01
			1	-	-										
	19	otsuka	2	-	-	-	-	-	-	-					
			3	-	-										
			1	-9.99	0.43										
	20	kaneka	2	-	-	-9.99	0.43	0.43	0.00	-					
			3	-	-										

Table 6.11 Estimated logEC50 and its SE based on Hill equation-based nonlinear regression by run and within- and overall between-lab variation (DL random effects model) (Continued)

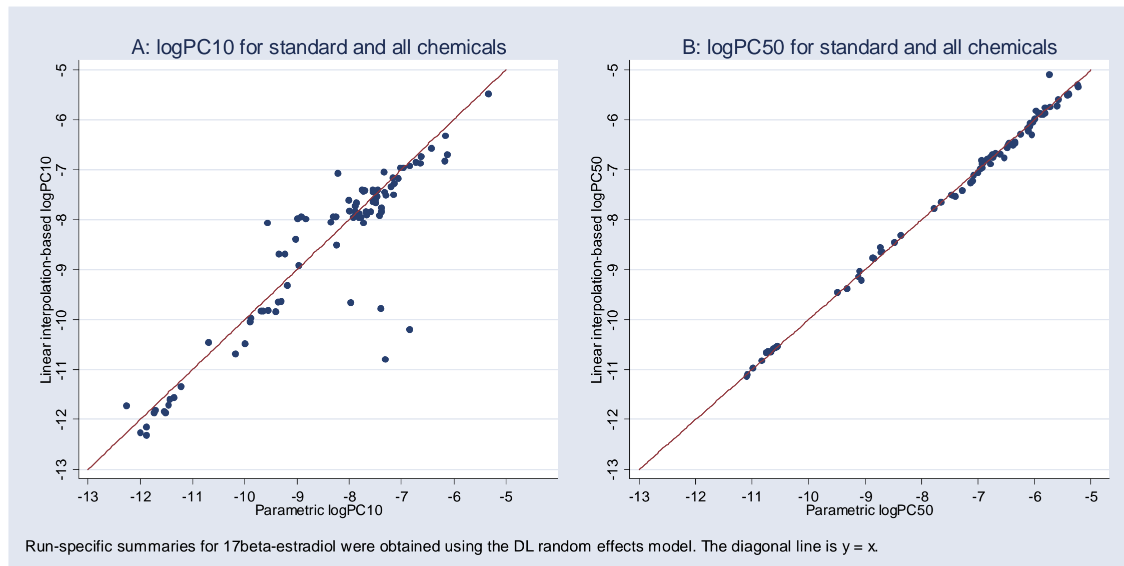
Genistein	21	ceri	1	-5.95	0.05	-5.87	0.14	0.24	0.16	0.23	-5.65	0.17	0.34	0.23	0.08
			2	-5.33	0.37										
			3	-5.88	0.28										
	22	sumitomo	1	-	-	-5.74	0.32	0.45	0.37	0.13					
			2	-5.27	0.44										
			3	-5.96	0.12										
	23	otsuka	1	-5.00	0.79	-2.57	2.55	4.42	4.37	< 0.01					
			2	-5.24	0.46										
			3	2.52	0.66										
	24	kaneka	1	-5.21	0.37	-5.44	0.13	0.22	0.04	0.36					
			2	-4.82	0.54										
			3	-5.51	0.13										
17α-Methyltestosterone	25	ceri	1	-	-	-6.06	0.48	0.48	0.00	-	-5.80	0.31	0.53	0.00	0.46
			2	-	-										
			3	-6.06	0.48										
	26	sumitomo	1	-5.73	0.45	-3.68	1.93	3.35	3.26	< 0.01					
			2	-5.63	0.97										
			3	0.41	0.88										
	27	otsuka	1	-5.53	0.74	-5.70	0.41	0.71	0.00	0.90					
			2	-5.95	0.68										
			3	-5.59	0.71										
	28	kaneka	1	-	-	-	-	-	-	-					
			2	-	-										
			3	-	-										
4-tert-Octylphenol	29	ceri	1	-6.93	0.06	-7.06	0.10	0.18	0.17	< 0.01	-6.71	0.15	0.30	0.29	< 0.01
			2	-6.93	0.02										
			3	-7.39	0.09										
	30	sumitomo	1	-6.25	0.20	-6.29	0.07	0.13	0.00	0.58					
			2	-6.36	0.11										
			3	-6.20	0.12										
	31	otsuka	1	-6.80	0.05	-6.85	0.09	0.16	0.15	< 0.01					
			2	-7.06	0.08										
			3	-6.70	0.08										
	32	kaneka	1	-6.58	0.04	-6.67	0.05	0.09	0.08	0.03					
			2	-6.69	0.06										
			3	-6.75	0.05										
p-tert-pentylphenol	33	ceri	1	-5.92	0.25	-6.23	0.36	0.63	0.60	< 0.01	-5.91	0.12	0.23	0.14	0.18
			2	-5.90	0.17										
			3	-6.80	0.07										
	34	sumitomo	1	0.81	0.78	-3.69	1.34	2.32	2.27	< 0.01					
			2	-5.61	0.42										
			3	-5.88	0.10										
	35	otsuka	1	-5.71	0.43	-5.99	0.11	0.18	0.00	0.66					
			2	-6.18	0.30										
			3	-5.98	0.12										
	36	kaneka	1	-5.55	0.45	-5.81	0.10	0.18	0.00	0.83					
			2	-5.84	0.13										
			3	-5.80	0.17										
17β-Estradiol	37	ceri	1	-10.71	0.06	-10.93	0.09	0.16	0.15	< 0.01	-10.64	0.11	0.22	0.19	< 0.01
			2	-10.98	0.02										
			3	-11.09	0.07										
	38	sumitomo	1	-10.94	0.27	-10.61	0.11	0.18	0.13	0.10					
			2	-10.47	0.08										
			3	-10.68	0.10										
	39	otsuka	1	-10.39	0.05	-10.48	0.12	0.21	0.19	< 0.01					
			2	-10.32	0.08										
			3	-10.73	0.07										
	40	kaneka	1	-10.77	0.13	-10.50	0.11	0.18	0.16	< 0.01					
			2	-10.48	0.06										
			3	-10.32	0.06										
MAX *					0.97		2.55	4.42	4.37			0.31	0.53	0.29	
MIN *					0.02		0.02	0.03	0.00			0.07	0.14	0.00	
Ave. *					0.22		0.34	0.57	0.48			0.15	0.28	0.16	

* Excepting for Hematoxylin, Benzophenone and Diethylhexyl phthalate

Estimation of logPC10/50 (linear interpolation vs. Hill equation-based nonlinear regression)

The choice of estimation method for logPC10 made considerable difference in the logPC10 estimates (see Graph 6.1.A). logPC10 values based on linear interpolation tended to be much smaller than those based on parametric estimation from Hill equation-based nonlinear regression. As mentioned earlier, this was expected because the concave curvature likely present around the response level of 10% was ignored in the linear interpolation. All linear interpolation-based logPC10 values for 17β-estradiol were smaller than the parametric counterparts. For other test compounds, linear interpolation sometimes generated an estimate greater (more than one log10 unit in the most extreme case) than the parametric method. Such overestimation is more problematic than underestimation since overestimation of logPC10 actually means underestimation of the chemical's potential to induce estrogenic response. It is notable that these interpretations are based on the assumption that the parametric logPC10 values are more accurate than the linear interpolation-based counterparts. That is, our results actually does not provide any direct information as to whether a linear interpolation-based estimate is biased downward or a parametric counterpart is biased upward when the former is smaller than the latter.

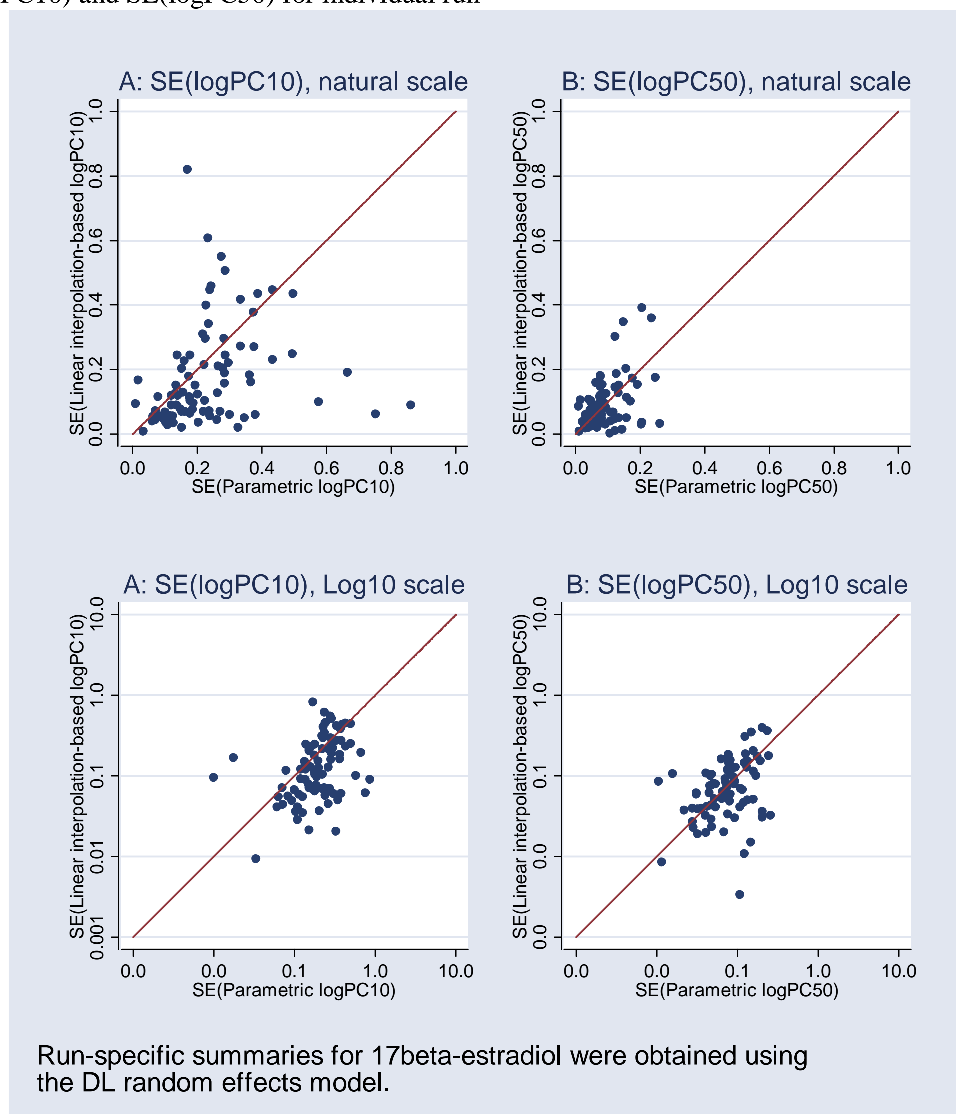
Graph 6.1 Comparison between linear interpolation and Hill equation-based nonlinear regression: logPC10 and logPC50



As can be seen in Graph 6.1.B, the choice of these two estimation methods made little difference for the estimated logPC50 values. Results from the two methods are similar presumably because underlying true curve has much lower level of curvature in the range where response is closer to the midpoint of the entire curve. An exception was a point at the upper right hand corner, which was

noticeably above the y = x line, indicating the Hill equation-based estimate was greater than the interpolation-based estimate. In general this kind of difference may arise when an underlying response curve at around logPC50 is convex (i.e., the curve is reaching to its plateau). Graph 6.2 shows agreement of SE (parameter estimate for individual run) across the two analytical procedures in absolute as well as logarithmic scales.

Graph 6.2 Comparison between linear interpolation and Hill equation-based nonlinear regression: SE(logPC10) and SE(logPC50) for individual run



As mentioned earlier, there were some reasons to speculate that SE of linear interpolation-based estimates of logPC10 and logPC50 could be greater than those from Hill equation-based nonlinear regression. Contrary to this speculation, there was some evidence that linear interpolation resulted in smaller SEs (Table 6.12).

Nonetheless, SE of logPC10 and logPC50 based on the linear interpolation varied more as seen by greater SD of log(SE(estimate)) in Table 6.12 (logarithm of SEs are summarized since they were right-skewed and log-transformation would make the resultant distribution more normal).

Table 6.12 Comparison between linear interpolation and nonlinear regression: analytical within-run variability.

Variable	Method	Mean	SD	*p*-value for difference*
log(SE(logPC10) (N = 86)	Linear interpolation	-0.91	0.39	$5*10^{-6}$
	Nonlinear regression	-0.72	0.31	
log(SE(logPC50) (N = 83)	Linear interpolation	-1.18	0.37	0.31
	Nonlinear regression	-1.14	0.29	

* Based on paired t-test ignoring within-lab and within-chemical correlation.

It is possible that SEs of the linear interpolation-based parameter estimates do not reflect the true level of variability in the estimates since SEs are calculated ignoring the uncertainly due to the probabilistic selection of data points used for the interpolation. The true level of variability could be greater than the expected level given the selected adjacent log(concentration)-response pairs. The Hill equation-based nonlinear regression used the whole data set without selection, introducing no uncertainty of this sort.

For the data sets on 17β-Estradiol from "Sumitomo" lab trials 1-2 and 1-3 and "Kaneka" lab trial 1-2, attempts to estimate logPC10 using the interpolation method failed since the lowest concentration-specific mean response was greater than 10%. The nonlinear regression, on the other hand, successfully generated a logPC10 estimate for these data sets. These examples illustrate another advantage of the nonlinear regression, i.e., its capacity to generate a logPC10 estimate for the data set with the lowest concentration-specific mean response greater than 10% as long as the data overall have a monotonously increasing pattern. This can be considered a cost saving feature since additional experiments to be conducted using lower concentrations of the test chemical may be omitted.

For other positive chemicals, failure to report either interpolation- or Hill equation-based logPC10 estimate did not occur. On the other hand, in some runs for negative chemicals the interpolation- and/or Hill equation-based procedure reported logPC10. It may appear possible that random fluctuation in response may be detected as monotonous increase by the linear interpolation. The nonlinear regression is less susceptible to the consequence of such random fluctuation because the rest of the data would tend to inform the absence of monotonous increase. Of note are data for Diethylhexyl phthalate (a negative) from Sumitomo lab where logPC10 was reported for each of three runs. A spurious gradient in background response across wells with increasing concentrations may have resulted in this. Other than this, the frequency of reporting logPC10 for a negative

chemical was not much different between the interpolation and Hill equation-based nonlinear regression.

The *observed* within-(lab and chemical combination) variability of logPC10 and logPC50 is compared across estimation methods in Table 6.13. There is no noticeable across-method difference either in means of within-lab SD or SD of log(within-lab SD).

Table 6.13 Comparison between linear interpolation and nonlinear regression: observed within-lab, between-run variability.

Variable	Method	Mean (variable)	SD (variable)	*p*-value for difference* in Mean	*p*-value for difference* in SD
Summarized by traditional method					
log(within-lab SD(logPC10)) N = 28	Linear interpolation	-0.50	0.43	0.54	0.13
	Nonlinear regression	-0.45	0.29		
log(within-lab SD(logPC50)) N = 30	Linear interpolation	-0.75	0.26	0.24	0.27
	Nonlinear regression	-0.74	0.37		
Summarized by DL random effects method					
log(within-lab SD(logPC10)) N = 28	Linear interpolation	-0.55	0.32	0.50	0.77
	Nonlinear regression	-0.49	0.27		
log(within-lab SD(logPC50)) N = 28	Linear interpolation	-0.85	0.29	0.80	0.63
	Nonlinear regression	-0.85	0.31		

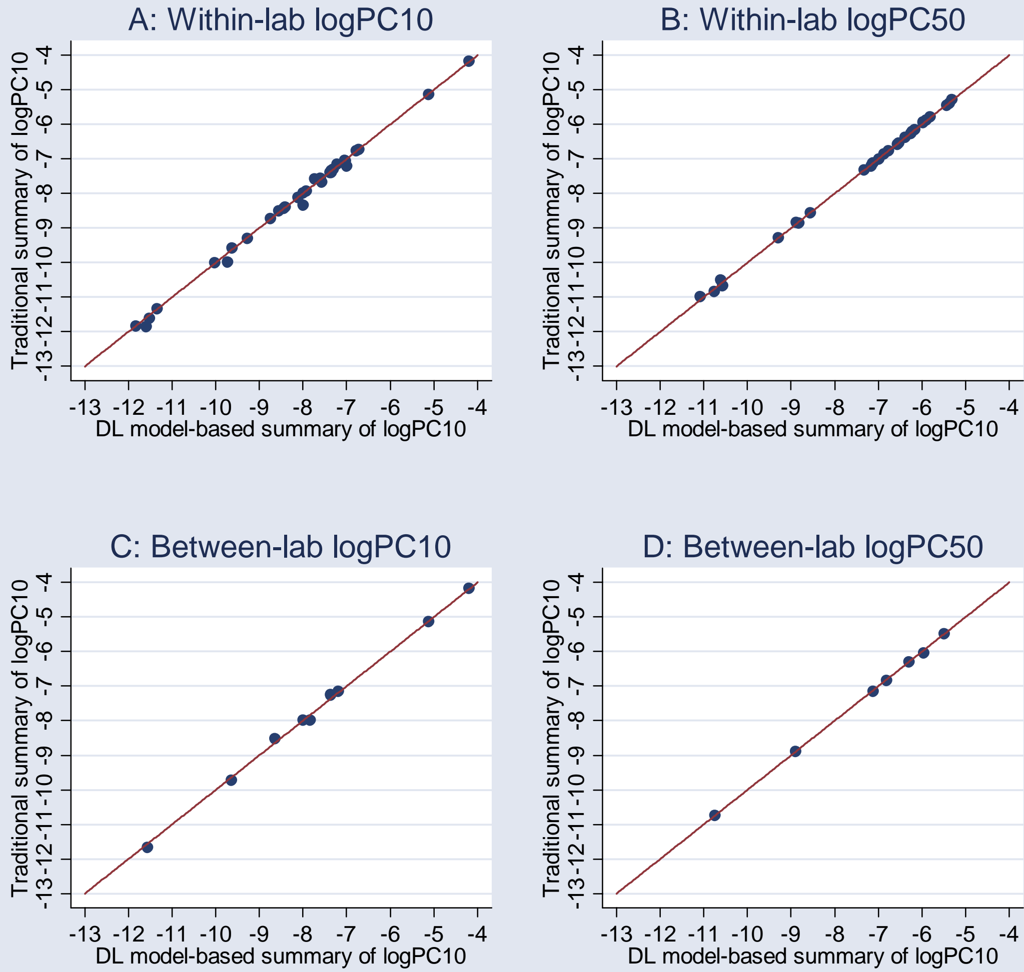
*** Based on a heteroscedastic regression model, which models mean and SD (logarithm of variance) simultaneously considering dependence within laboratory. These could be a function not only of the summarization method (traditional or DL) but potentially also of test chemical and laboratory, which were not included in the model for simplicity.**

Overall, it seems desirable to estimate logPC10 and logPC50 using Hill equation-based nonlinear regression rather than linear interpolation.

Traditional vs. DerSimonian Laird for summarizing estimates

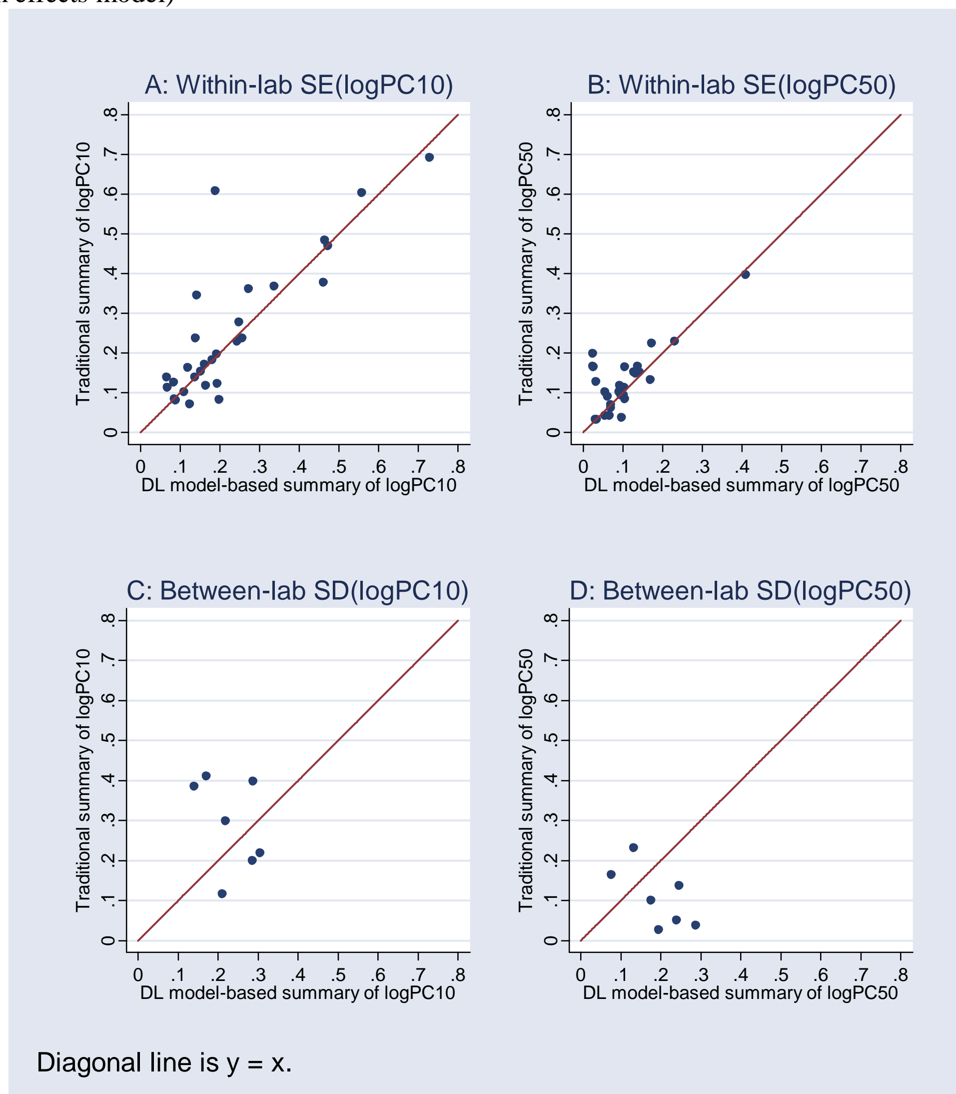
Lab-specific summaries for logPC10 and logPC50 estimates are shown in Graph 6.3, panels A and B which indicate the choice of summarization procedures (traditional vs. DL random effects model) did not make any material difference. Between-lab summaries also show good agreement across two procedures (panels C and D).

Graph 6.3 Comparison of point estimates across summary methods (traditional vs. DL random effects model)



On the other hand, estimates of overall between-variability for logPC10 and logPC50 differed much more across summarization procedures (Graph 6.4). (Please note that the use of within-lab *SE* and between-lab *SD* are intentional: the former is the within-component of the latter on a common scale, i.e., the square of the former plus the square of intrinsic between-lab SD equals the square of the latter.)

Graph 6.4 Comparison of overall within-lab (= between-run) and between-lab variability estimates for nonlinear regression-based logPC10 and logPC50 across summary methods (traditional vs. DL random effects model)



In order to examine whether estimates of overall between-run variability systematically differed across summarization methods, its mean and spread were modeled by heteroscedastic regression. As seen in Table 6.14, overall between-run variability for logPC10 were similar across summarization methods. For logPC50, overall between-run variability was smaller and less variable when the DL method was applied. Nonetheless, the magnitude of the difference was not substantial.

Table 6.14 Comparison between traditional method vs. DL random effects model: observed within-lab, between-run variability.

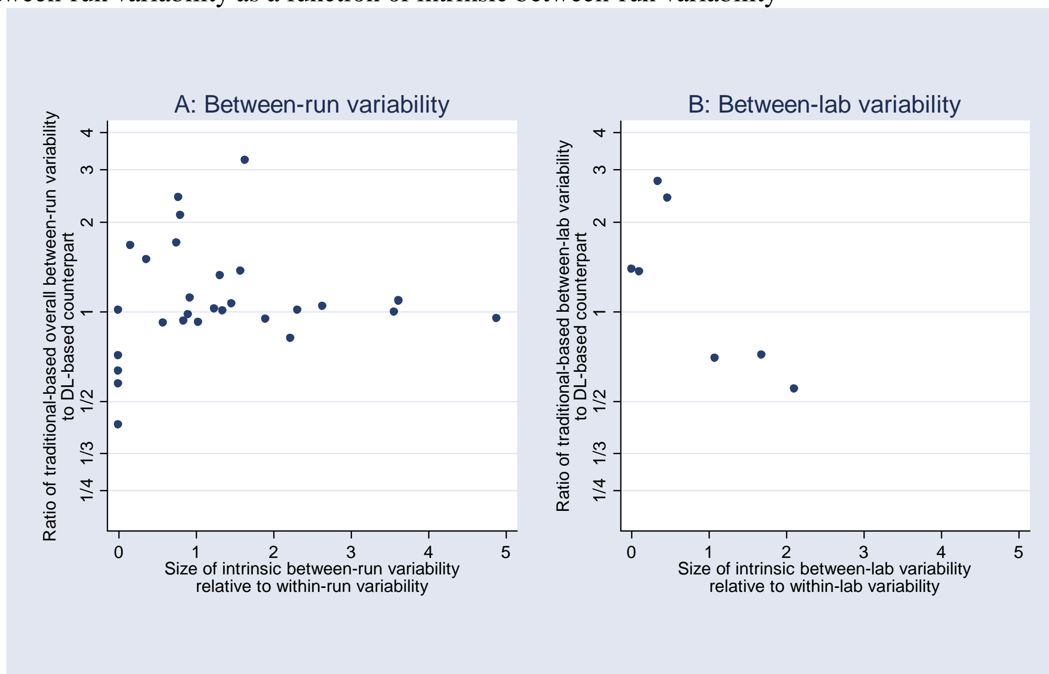
Variable	Summarization Method	Mean (var-iable)	SD (var-iable)	*p*-value for difference* in Mean (variable)	*p*-value for difference* in SD (variable)
Linear interpolation					
log(within-lab SD(logPC10)) N = 28	Traditional	-0.50	0.43	0.43	0.003
	DL random effects	-0.55	0.33		
log(within-lab SD(logPC50)) N = 28	Traditional	-0.75	0.27	0.02	0.05
	DL random effects	-0.85	0.29		
Nonlinear regression					
log(within-lab SD(logPC10)) N = 29	Traditional	-0.46	0.29	0.36	0.35
	DL random effects	-0.49	0.28		
log(within-lab SD(logPC50)) N = 28	Traditional	-0.74	0.27	<0.01	<0.01
	DL random effects	-0.85	0.31		

*** Based on a heteroscedastic regression model, which models mean and SD (logarithm of variance) simultaneously considering dependence within laboratory. These could be a function not only of the summarization method (traditional or DL) but potentially also of test chemical and laboratory, which were not included in the model for simplicity.**

Some simulation results performed to date (not shown) indicated that when intrinsic between-unit variability is small relative to within-unit variability the traditional method tended to underestimate the overall between-unit variability. Such tendency could be seen for the overall between-run variability estimated for the current data as shown in Graph 6.5. This graph shows how the relative size of the traditional method-based overall between-run variability compared to the DL-based counterpart changed according to the size of the observed intrinsic between-run variability, which is expressed in relation to the within-run variability. The vertical axis value of 1 means the traditional overall between-run variability estimate was equal to the DL overall between-run variability. Small horizontal axis values mean small intrinsic between-run variability in relation to within-run variability. The horizontal axis value of zero means the intrinsic between-variability was estimated to be zero. Note that in the panel A the vertical axis values at the horizontal axis value of zero are mostly below 1, meaning the traditional overall between-run variability estimates were consistently smaller than the DL counterparts. The aforementioned simulation indicated that the tendency for the traditional method to underestimate overall between-unit variability diminishes rapidly as intrinsic between-run variability increases proportionally, and the pattern seen in the panel A is consistent

with such simulation results. No such pattern, though, was seen for overall between-lab variability (panel B).

Graph 6.5 Difference between "traditional" overall between-run variability and DL-based overall between-run variability as a function of intrinsic between-run variability



As discussed earlier, intrinsic between-unit variability estimate and its contribution to the overall between-unit variability is potentially useful in investigating sources of variation. Quantification of intrinsic between-plate variability in the data for 17β-estratiol is an example of such use. In the current data intrinsic between-plate variability tended to be estimated to be zero or small compared to overall between-plate variability of logPC10 and logPC50 (Table 6.8). For logPC10, intrinsic between-run variability also was small or at least evidence against run-to-run homogeneity was weak (i.e., relatively large within-lab, between-run homogeneity *p*-values of 0.27, 0.21, 0.36, and 0.19 for four labs). Taken together, within-plate variability, i.e., analytic variability expressed as SE(plate-specific logPC10), was a predominant source of variation for logPC10. On the other hand, for logPC50, intrinsic between-run variability also was relatively large and contributed to the majority of the overall between-run variability. As such, relative contributions of the sources of variation were different for logPC10 and logPC50.

Conclusions

The assay appears to have acceptably low between-lab variation. It is recommended that Hill equation-based nonlinear regression be used for estimating logPC10 because it has advantage over

linear interpolation in terms of accuracy and precision. For logPC50, there is not much difference between the two methods. While a thorough comparison of the CERI method vs. DL random effects model based solely on this single data set cannot be made, minor, relatively unimportant drawbacks of the CERI method were noted. For a full comparison, detailed analyses preferably based on simulation would be necessary.

Appendix 7 Report of the preliminary validation assessment panel of the 'Japanese multi-laboratories validation study of a stably transfected ER alpha mediated reporter gene assay in Japan'

REPORT OF THE PRELIMINARY VALIDATION ASSESSMENT PANEL OF THE 'JAPANESE MULTI-LABORATORIES VALIDATION STUDY OF A STABLY TRANSFECTED ER ALPHA MEDIATED REPORTER GENE ASSAY IN JAPAN'.

Final version 29 June 2006

ACCRONYMS

AR Androgen Receptor

CERI Chemicals Evaluation and Research Institute (Japan)

CV coefficient of variation

DIP Data interpretation procedure

ECVAM European Centre for the Validation of Alternative Methods

EDTA (OECD) Task Force on Endocrine Disruptor Testing and Assessment

ER Estrogen Receptor

ERE Estrogen Responsive Element

GD 34 Guidance Document 34

GLP Good Laboratory Practice

ICCVAM Interagency coordinating Committee on the Validation of Alternative Methods (US)

JaCVAM Japanese Centre for the Validation of Alternative Methods

NICEATM National Toxicology Program (NTP) Interagency Centre for the Evaluation of Alternative Toxicological Methods (US)

NIEHS National Institute of Environment and Health Sciences (US)

NIHS National Institute of Health Sciences (Japan)

PC_{50}/PC_{10} The concentration of chemical estimated to cause 50% or 10%, respectively, of activity of the positive control response on a plate by plate basis.

PM Prediction Model

QA Quality Assurance

SOP Standard Operating Procedure

SPSF Standard Project Submission Form

TA Transcriptional Activation

US EPA US Environmental Protection Agency

VMG-NA Validation Management Group for Non –Animal Testing

WNT (OECD) Working Group of the National Coordinators for the Test Guidelines Programme

1. INTRODUCTION AND BACKGROUND

1.1 At the present time, there is global concern regarding endocrine disruption effects, particulary mediated by the estrogen receptor (ER) resulting from chemical exposure. Several *in vitro* ER binding and transfected cell line assay methods are currently or imminently being (pre) validated at national, regional and international levels, but are some way away from completion and full assessment of their validation status.

1.2 A screening test method is a rapid, usually simple test performed for the purposes of prioritizing or grouping substances in general categories of potential modes of action (*e.g., in vitro* binding to the oestrogen receptor). The results from screening tests are generally used for preliminary decision making and to set priorities for additional and more complex tests. Although the results from screening tests, alone, may not be sufficient for risk assessment purposes, there may be circumstances where such results may be combined with other test results in a tiered testing approach to provide in the hazard/risk assessments (GD34).

1.3 Currently, no *in vitro* screening assay for ER activity that can be used for OECD regulatory purposes has been peer reviewed for potential test guideline development, although the need is urgent. Recognizing this urgency, Japan has made an extensive effort to establish and domestically validate a new *in vitro* pre-screening procedure, the **hER-HeLa-9903 Estrogen Receptor (ER) Transcriptional Activation (TA) Test** for detecting the estrogenic activity of chemicals for a level 2 screening test in the OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals.

1.4 The with-in Japan multi-laboratory validation process of Japanese ER TA Assay was completed as an activity of the Validation Management Group (Non -Animal)(VMG-NA) and the results were presented at the 3rd VMG-NA held in November 2005.

1.5 The assay is based on an estrogen reactive stable human cervical tumor cell line, hER-HeLa- 9903, which was developed by the Sumitomo Chemical Company in Japan. An initial test protocol of the assay system was developed and optimized by the Chemicals Evaluation and Research Institute (CERI). Using this optimized protocol, a pre-validation of the test system was conducted by CERI as an initial assessment exercise in order to identify the reliability, relevance and performance (accuracy) of the assay system. Following this first assessment, CERI, led an inter-laboratory validation involving four participating laboratories, all of which used coded chemicals under GLP compliance conditions. The data produced indicated good reproducibility and technical transference between laboratories. The data compared favourably and showed good concordance with that reported for the immature rat uterotrophic assay (80%) and summarised by ICCVAM (85%), (ICCVAM 2003), with an overall low false positive rate of 9%.

1.6 Following this presentation, the VMG-NA agreed to create a panel with the task of assisting the Japanese in assessing the readiness of the validation study for independent scientific peer review and supporting additional requirements that might be deemed necessary. The panel activities were informal and unofficial, as member countries did not make official nominations for panel membership, and the panel members participated on a voluntary basis.

1.7 Using GD 34 criteria as a basis, the primary tasks or charges of the panel were to assist the Japanese in a transparent manner in assessing whether there is sufficient information on the domestic validation to submit a report for scientific review, with the independent review procedure to be agreed by the Japanese. This report, which is based upon the three teleconference discussions of the panel held over six months, provides the first step in this process and will be made available to the VMG-NA. **All the points and discussions documented herein were agreed by the panel during their preliminary validation assessment activities or charge.** These teleconferences were conducted under the auspices of the individual expertise of the participants, and therefore the teleconference minutes and this report reflect their expert opinion, and not that of the organisations in which the experts are employed.

1.8 The report outlines the panel discussions, and each meeting is summarised in this report. Through the teleconference process the steps taken in the preliminary validation assessment are identified, and also included as appendices are the summary statements from participants. Subsequent activities include writing of a comprehensive validation report, or peer charge, using the preliminary validation assessment report as a basis. Following this next report the Japanese will decide how to go ahead with the formal peer review process. Routes for the organisation of independent peer review were identified and discussed at the 9th EDTA and 18th WNT via a contract house or by a member country competent authority, as follows: 'The Secretariat drew the attention of the WNT on Document ENV/JM/TG(2006)5 including two examples of approaches proposed to address the peer review of validated methods: a proposal made by the United States and a proposal made by Japan. It proposed to initially address peer reviews on a case-by-case basis until experience is gained and after a certain time, possibly consider a more comprehensive guidance on the processes for peer review. The United States introduced Annex 1 of Document ENV/JM/TG(2006)5 , which does not apply to a specific assay. The Secretariat brought Information Document [INF.6] to the attention of the meeting, as a collation of comments received from members of the VMG-eco on the Annex 1 of Document ENV/JM/TG(2006)5. The WNT agreed that the document describes a plausible approach, but does not provide standard procedures.' (Paragraph 36 of the Draft Summary record of the 18th meeting of the WNT, Bern Switzerland 16-18 May 2006.)

1.9 Should the assay be ultimately considered by the Japanese to be appropriate for submission to the OECD, the Japanese will be required to submit a SPSF to the OECD Secretariat for consideration by the WNT. On 1 June 2006, in response to queries from CERI, and discussions and recommendations at the 18th WNT with the Japanese National Coordinator and chair, the Secretariat recommended that the Japanese submit an SPSF soon, so that the project could be added to the rolling work plan.

Background information: What is a reporter gene assay?

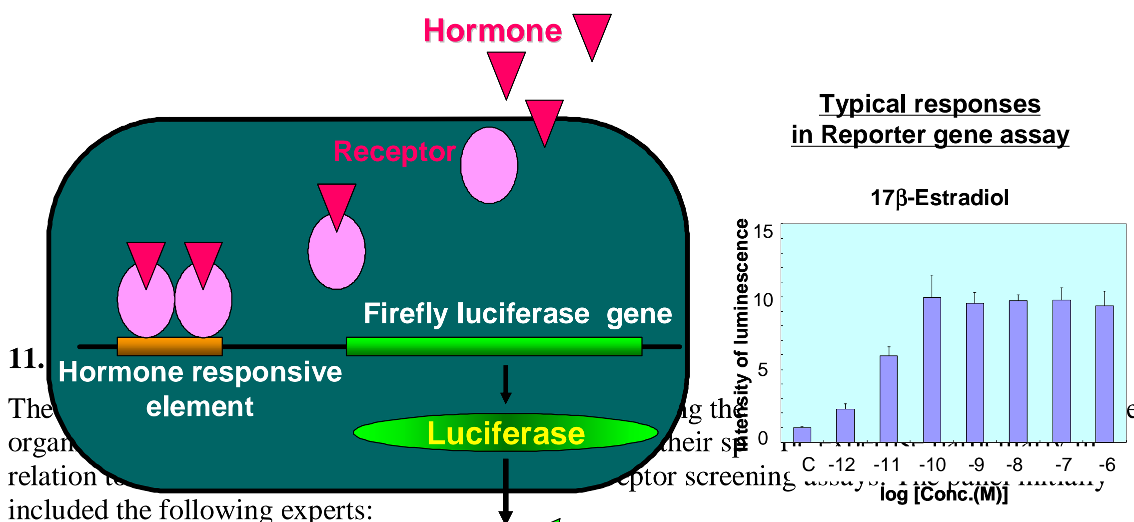
1.10 The Reporter gene assay method is an *in vitro* tool that allows the identification of promoters and enhancers together with an assessment of the correlations between their activities and conformations by measurement of the reporter proteins that are expressed from reporter genes. The promoters 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 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 (See Figure 7-1).

1.11 Thus the reporter gene assay technique may be suitable for detecting hormonal activity of chemicals, because it has been used to detect enhancers and promoter activity of genes. The reporter gene assay system may also provide a powerful tool to screen for endocrine disrupting chemicals (Takeyoshi et al., 2002; Yamasaki et al., 2002, and has also been developed for use in other cell lines, e.g. CALUX (Sonneveld et al 2006).

1.12 The assay used for this validation study 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.

Figure 7-1. Diagram showing the principle of the reporter gene assay



11.

The ... g the ... e
organ... heir sp...
relation t... eptor screening ...
included the following experts:

1. Dr. Masahiro Ta...
2. Dr. Yumi Akahori (CERI)
3. Prof. Daniel Dietrich (on behalf ... ECVAM)
4. Dr. Susan Laws (US EPA)
5. Mr. Gary Timm (US EPA)
6. Dr. Yutaka Aoki (ASPH Fellow at US EPA)
7. Dr.Tim Schrader (Health Canada)
8. Dr. Bill Stokes (NIEHS/NICEATM, ICCVAM)
9. Dr. Ray Tice (NIEHS/NICEATM, ICCVAM)
10. Ms. Patricia Ceger (ILS. Inc./NICEATM, ICCVAM)
11. Mr. Frank Deal (ILS. Inc./NICEATM, ICCVAM)
12. Dr. Miriam Jacobs (OECD call leader)

There were alterations in participation of the panel activities: Following the first teleconference,

13. Dr. Jun Kanno (NIHS) and

14. Dr. Hajime Kojima (JaCVAM)
were invited to join, to improve the Japanese representation and expertise.
Although a panel member, Prof Bob Combes did not participate in any of the teleconferences, but did submit written comments at a later date.

1.14 Following the second teleconference it became apparent that the panel had become a little unbalanced with respect to numbers of persons with validation expertise representing different bodies. The Secretariat therefore recommended that the numbers of such persons for each of the different participating bodies, during the teleconferences, is reduced and/or maintained at two persons to improve the balance in representation across the participating bodies and improve manageability of the teleconference.

1.15 Further consultation with experts outside the panel was sought where panel members felt it useful. Dr Ray Tice requested further consultation on statistical matters from the Statistical expert consultant to ICCVAM, Dr. Joe Haseman. The ECVAM computational toxicology and statistical expert Dr. Sebastian Hoffman was also consulted. Dr. Jean-Claude Nicholas was consulted with respect to the possibility of induction of non receptor mediated effects that might be observed at higher concentrations that might impact upon and increase the chemical luminescence.

Steps undertaken during preliminary validation assessment process

1.16 The steps taken during the preliminary validation assessment at the request of the panel included (in chronological order):

- Teleconference 2

i. CERI conducted a comparison of the draft report submission with the guidelines provided in the OECD Guidance Document 34 and *ICCVAM Evaluation of In Vitro Test Methods for Detecting Potential Endocrine Disruptors* (NIH Pub. No. 03-4503) and stated their rationale for deviations from these guidelines.

ii. CERI provided further information on cell line characterisation, methods of cytotoxicity evaluation and ER alpha antagonist TA testing (See also appendix 7-1).

iii. CERI provided raw fold induction data for the positive controls and for the chemicals assessed under the (pre) validation stage from data generated by the CERI laboratory. The provision of such data from the other laboratories was not possible. The panel required this information to assess the extent of the variation in fold induction over time.

iv. Dr. Yutaka Aoki (US EPA) provided information on proposed methods for between- and within-variation estimation to the whole group (Appendix 7-2) and consulted directly with CERI on how to proceed.

v. CERI conducted an internal audit of data transcribed.

vi. CERI provided raw data on edge effects from the CERI laboratory.

- Teleconference 3

vii. CERI submitted the antagonist assay protocol (SOP) and raw data for consideration by the panel. (See appendix 7-1).

viii. For the negative substances used, information and justification was provided by CERI on solubility and the maximum concentration used.

ix. Data analysis proposal from Dr Yutaka Aoki and subsequent discussion from and response to NICEATM consultant statistician Dr Joe Haseman, and Dr Sebastian Hoffman (ECVAM). (Appendix 7-3)

x. Assistance from Dr Aoki to CERI in conducting statistical estimations of between- and within-run (laboratory) variation (provisionally in June 2006).

TELECONFERENCE SUMMARIES

2. THE FIRST TELECONFERENCE WAS HELD ON 6 FEBRUARY 2006.

The meeting opened with a presentation from CERI summarising the validation of the reporter gene assay using the hER-HeLa-9903 cell line to detect estrogenic activity. During the presentation there were a number of queries, to which the following clarifications were given:

2.1 Coefficients of variation (CV) analysis to evaluate intra- and inter-laboratory reproducibility were based on log EC_{50} values, not EC_{50} values.

2.2 Requests were made for clarification as to the nature of the PC_{50} and PC_{10} values, how they are calculated, why there was no CV for the PC_{10} of 17β estradiol (E2), and whether PC_{50} and PC_{10} values were calculated within or across experiments. The PC_{50} and PC_{10} values are defined as the concentration of chemical estimated to cause 50% or 10%, respectively, of activity of the positive control response on a plate by plate basis. This measure is not the same as % maximum induction of the positive control, and is not the same as an EC_{50}. It was not always possible to calculate EC_{50} values. 100pM E2 was the single positive control for both PC_{50} and PC_{10} values. No CV could be calculated for the PC_{10} of E2 due to the fact that the lowest concentration tested was 10^{-12} M, at which concentration ERα activation was still high. CERI did not try to increase the concentration of the chemicals for which an EC_{50} could not be obtained at a dose range from 10pM to 10µM, to obtain an EC_{50} value.

2.3 When selecting substances from the ICCVAM List of Reference Substances, CERI excluded substances that had excessive cost or limited commercial availability.

2.4 During prevalidation testing, a historical database was established using three substances, E2, bisphenol A (positive) and methyl testosterone (negative) which were tested 13 times over a four month period.

2.5 The first phase of the inter-laboratory testing study used two substances, E2 and bisphenol A to determine assay transferability. In this phase, it was determined that the sensitivity of the luminometer could be a limiting factor in a laboratory's ability to duplicate the results of other laboratories. This problem was overcome by the use of a more sensitive luminescence system in some of the participating laboratories.

2.6 Of the 10 substances used in the inter-laboratory validation phase, seven were selected because they were positive in the uterotrophic assay and three were selected because they were negative. 10 chemicals were tested to keep within the cytotoxicity and solubility range for each chemical. Of the 46 substances used by CERI to examine concordance between CERI uterotrophic and ICCVAM data, 10 compounds were problematic in terms of cytotoxicity or limited solubility. Further discussion as to the nature of PC_{10} and PC_{50} values ensued.

2.7 In general practice, substances are determined to be positive by CERI based on their PC_{50} values, with a substance being considered positive if a PC_{50} could be calculated. However, for the examination of concordance between CERI and ICCVAM data, CERI considered substances to be positive if a PC_{10} could be calculated for that substance. Concern was expressed that the PC_{10} value could give rise to many false positives. Potential metabolism issues also need to be addressed with respect to the metabolism of substances such that they do not reach the cellular target.

2.8 Participants requested that CERI send additional copies of raw data for this assay for examination. Raw data spreadsheets were sent out to the original panel of participants, prior to the meeting and to ICCVAM subsequently. Additional raw data was required to assess fold activation/induction, so as to clarify the variation of fold induction and enable comparison with fold induction data from comparable assays. Assessment of this data was favourable, and the variation observed was considered acceptable by the panel.

2.9 The following questions were asked regarding the calculation of PC_{10} and PC_{50} values:

i. Why is only one concentration (100 pM) used for the calculation of PC_{10} and PC_{50} values?.

ii. Might it be more statistically valid to use at least three concentrations (for instance, 10 pM, 100 pM and 1 nM) for this calculation? This would define a range of acceptability which could include historical and concurrent data which one could use to tease out performance criteria.

2.10 The comment from the participating statistician was that a single point would probably be sufficient for making this evaluation, but that ideally, this single concentration should be run in several additional wells, to stabilise the titrations and thus improve precision of the calculation. Also, an option worth considering is to introduce a relative index comparing a test chemical to a standard. In this approach one would calculate a ratio of (PC_{10} for the standard) to (PC_{10} for the test chemical) utilizing the data concurrently obtained for the standard. Similar to this ratio is relative binding affinity (RBA), which is already in use for receptor binding assays. Intuitively the use of relative index of this sort would result in more efficient cancellation of day-to-day (batch-to-batch) variation common to the standard and test chemical. Appendix 7-1 includes the statistical evaluation advice and discussion provided to the Panel by Dr Yutaka Aoki.

2.11 Additional questions were raised regarding the table from the presentation showing five to 15-fold induction of 100 pM E2 over a four-month period:

i. Are the hER-HeLa-9903 cells stable for longer than the four-month period used by CERI?

ii. Why is there so much variability in fold induction?

iii. Is there a risk of "false positives" showing up when the induction is 15 fold that would not appear when the induction was five fold?

iv. Are there upper and lower limit "cut-offs" for fold induction?

In response, CERI stated that the cells are stable for longer than the four month period, but that they do not use the cells longer than this period. The lower cut-off for induction is five fold for 100 pM E2, but there is no upper limit of induction used as a cut-off.

2.12 This led to the question of controlling for cell number. In particular, it was asked whether knowledge of cell number would allow for normalisation of induction. The conclusion was that although this could be done, it would not necessarily prove to be of any use. Luciferase reporter gene systems normally have varied degrees of response (i.e., varying fold inductions) that are not related to cell number in a linear fashion (i.e., on some days, the cells just respond better than on other days). In particular, there should not be any risk of seeing an

increase in “false positives” on days where there is a higher than usual induction because what usually happens in these cases is that the response is elevated for all cells. However, it was also decided that this issue would require additional thought and consideration.

2.13 Information on the test cell line characterisation was requested. As a cervical carcinoma cell line it is possible that there may be intrinsic metabolism occurring via for example P450, other receptors such as the Progesterone receptor and the Pregnane X receptor and cellular transporters such as Pgp.

2.14 Cytotoxicity evaluation was conducted by examining baseline induction. If a substance causes luciferase activity to fall below baseline, the substance is considered to be cytotoxic. The panel were concerned that this method was open to confounding, because if a substance is an antagonist, it could suppress luciferase activity below basal levels, without killing cells. A request was made for CERI to provide more information on this issue and QA controls generally (see paragraphs 3. 7 and 4.11).

2.15 It was recommended that CERI compare their submission to the guidelines in the OECD Guidance Document 34 and *ICCVAM Evaluation of In Vitro Test Methods for Detecting Potential Endocrine Disruptors* (NIH Pub. No. 03-4503).

3. THE SECOND TELECONFERENCE MEETING WAS HELD ON 17 MARCH 2006.

3.1 This meeting opened with a presentation from CERI summarising where the validation principles in OECD GD34 were met, partially met, or not met (Table 7-3.1) and whether the validation principles from the Minimum Standard Procedure recommended by ICCVAM were met, partially met, or not met (Appendix 7-4, Table 7-4.1). Table 7-3.2 gives the 10 core coded compounds tested in the inter- laboratory testing phase of the validation study.

Table 7-3.1. Checklist to assess whether the validation principles in OECD GD34 were met, partially met, or not met by the Japanese multi-laboratories validation study of a stably transfected ER alpha mediated reporter gene assay in Japan.

Principles	Met/Not met	Explanation and Justification
a) The rationale for the test method should be available.	MET	The proposed test method is used to provide mechanistic information and used for the purposes of prioritizing or grouping substances that has a potential estrogenic activity mediated estrogen receptor alpha.
b) The relationship between the test method's endpoint(s) and the (biological) phenomenon of interest should be described.	MET	The endpoint is a luciferase activity that is produced as a result of transcriptional activation of the reporter gene. Stimulation of reporter gene expression in response to ER agonists, is thought to be mediated by direct binding where E2-liganded ER binds directly to estrogen responsive element (ERE) and interacts directly with coactivator proteins and components of the RNA polymerase II transcription initiation complex resulting in enhanced transcription.
c) A detailed protocol for the test method should be available.	MET	This is provided in the draft report appendices. Further statistical discussions on data analysis and decision criteria are provided in paragraphs 3.11 and 4.10 and appendices 2 and 3.
d) The intra-, and inter-laboratory reproducibility of the test method should be demonstrated.	MET	Demonstrated.
e) Demonstration of the test method's performance should be based on the testing of reference chemicals representative of the types of substances for which the test method will be used. A sufficient number of the reference chemicals should have been tested under code to exclude bias.	NOT FULLY MET	Reference chemicals are necessary to establish the relevance and reliability of the proposed test and should include a minimum number of chemicals possessing expected range of response (strong, moderate, weak and negative). There was not consensus that this requirement was met. A minority view expressed concerns that the requirements specified by the ICCVAM ED to test 78 specified chemicals were not met. This opinion was attached as appendix 7-1 in the summary of the third teleconference and is attached to this report as appendix 4. 10 coded chemicals (Table 7-3.2) possessing expected ranges of response were tested under the inter-laboratory validation, and relevance and reliability were demonstrated. However while a sufficient number of chemicals were not tested in all participating laboratories, according to ICCVAM recommendations, data were collected at the lead laboratory for further comparison with 46 chemicals selected from the ICCVAM list, and these data give a strong indication of relevance of the proposed test method.

		While the ICCVAM list of 78 chemicals does span a broad range of chemical classes and, for that reason, may be useful for identifying the limitations of the assay it also states that EC50 and IC50 data area available for 18 (23%) and 10 (13%) of these 78 recommended substances for agonism and antagonism, respectively. Qualitative data are available for 27 (35%) and 10 (13%) of these 78 recommended substances for agonism and antagonism, respectively. Thus, there is incomplete information regarding how all 78 of the recommended substances will respond in *in vitro* ER TA agonism and antagonism assays utilizing mammalian cell reporter gene systems. In which case testing only 10 of the 78 substances in multiple laboratories and the remainder in the lead laboratory is not a significant flaw in this validation effort. The limitations of the assay can be adequately determined by testing the remainder of the 78 chemicals in one or more laboratory/s. This could be considered to be consistent with ECVAM's proposed modular approach to validation (Hartung et al 2004), where core, better characterised coded sets of chemicals are tested in all participating laboratories, but further chemicals being tested for the prediction model are split or staggered between the three different laboratories. Such an approach is intended to improve the efficiency, reduce costs and speed up the validation process to meet pressing European and international regulatory requirements.
f) The performance of the test method should have been evaluated in relation to relevant information from the species of concern, and existing relevant toxicity testing data.	MET	Relevant information obtained from the ICCVAM ED list, and results for selected chemicals were compared with this list. All data used for this comparison were produced at the lead laboratory. Additionally a data comparison was conducted with the proposed test method and the hERalpha Binding assay (and data from the immature rat uterotrophic assay) with good concordance.
g) Ideally, all data supporting the validity of a test method should have been obtained in accordance with the principles of GLP.	NOT FULLY MET	The pre-validation and data collection for comparison with ICCVAM list or hERalpha binding assay were not conducted to GLP. However the inter laboratory validation was conducted to GLP. There was consensus from the panel that although GLP is ideal, for practical purposes, the fact that components of this validation and data comparison was not always to GLP was acceptable.

h) All data supporting the assessment of the validity of the test method should be available for expert review.	MET	A detailed test protocol is available, and data is available for independent review (including that prepared by this pre-peer review). Benchmark: The responses of positive control (E2) and vehicle control (DMSO) wells in each assay plate act as a benchmark such that reproducible results can be obtained when generating PC_{10} and PC_{50} values normalized by the positive control response.

Table 7-3.2. The 10 coded chemicals possessing expected ranges of response tested under the inter-laboratory validation.

Chemical Name	CAS	Category	Chemical Class
17b-Estradiol	50-28-2	Strong ER and AR agonist; AR antagonist	Steroid, phenolic, Estrene
17a-Estradiol	57-91-0	ER agonist	Steroid, phenolic, Estrene
Genistein	446-72-0	Weak ER agonist and antagonist	Flavonoid; Isoflavone; Phenol
Bisphenol A	80-05-7	ER agonist	Diphenylalkane; Bisphenol; Phenol
17a-Methyltestosterone	58-18-4	ER and AR agonist	Steroid, non-phenolic; Androstene
4-*tert*-Octylphenol	140-66-9	ER agonist	Alkylphenol; Phenol
p-tert-Pentylphenol	80-46-6		Alkylphenol; Phenol
Hematoxylin	517-28-2	Negative	
Di(2-ethylhexyl)phthalate	117-81-7	Negative. ER binder	Phthalate
Benzophenone	119-61-9	Negative	Benzophenone

3.2 Cell line Characterization -hER-HeLa-9903. The host cell line was checked for the following nuclear receptors, Estrogen Receptors α and β (ERα, ERβ respectively), Thyroid Receptors α and β (TRα and TRβ respectively) and the Androgen Receptor (AR). This was confirmed by a mock transfection assay with each hormone responsive reporter construct. No mycoplasma infection was detected.

3.3 It was emphasised that although there might be further applications, the assay was primarily designed to provide mechanistic information. There was also concern expressed by CERI with respect to the number of chemicals tested, and whether these were sufficient or not, and that the triplicate tests were not always repeated for the same chemicals.

3.4 Discussion began first with concerns regarding the number of chemicals tested. For statistical purposes it was recommended that in order to a get good grasp of assay reliability for each class of chemicals, a minimum of three chemicals is required for each class. Also that ‘difficult’ chemicals should be included (but were not), to address for example, solubility and cytotoxicity responses. A total of 10 chemicals were tested, therefore with 2 to 3 chemicals for each *class* of chemicals for 4 classes. Dr. Akahori pointed out that 5 chemicals classified as "weak" were tested and so were 3 "negatives." While for these particular classes the number of chemicals satisfies the minimum requirement, for the remaining classes, the chemical class-specific information on reliability is somewhat limited. It is therefore suggested that the best course of action at this point is to assess the repeatability of each chemical class by obtaining the chemical class-specific estimation of between- and within-laboratory variations, and examining any statistical evidence that they differ across chemical classes. If they do not, then estimate the variations common to all applicable chemical classes, assuming the true levels of variations are comparable across chemical classes. Before doing this, the intra (within-lab) and inter (between-lab) laboratory variations require reassessment as currently they are overestimated in the draft report.

3.5 The use of the concordance, sensitivity and specificity. It was recommended that sensitivity and specificity be the primary endpoints, and that the use of concordance as a summary measure of the sensitivity and specificity should be avoided unless a caveat is included stating that here the term concordance is used to mean the weighted average of

sensitivity and specificity, with weights being the prevalence of the substances being evaluated for sensitivity and specificity, and that prevalence is not a well defined concept in this example. The reason for this is that here true positives and true negatives have been chosen arbitrarily, so prevalence does not have real meaning. As such, the concordance here is a function of an arbitrary number (prevalence). Sensitivity and specificity values are far more accurate terms to use, as they are not influenced by an arbitrary level of prevalence.

3.6 Maximum concentrations that can be realistically tested in this test method. ICCVAM's expert panel recommend a maximum concentration of 1mM which is extremely high for cellular systems, and it was agreed that for practicality, one does not really need such a high response if a full dose response curve is obtained at a lower dose, or depending upon the reasons for conducting the assay or if practical reasons such as solubility/cytotoxicity preclude it. However it was agreed that for substances that tested negative in the assay (where negative is defined as no observed transcription), information and or justification should be provided on solubility and the maximum concentration used. This is because in some instances, higher concentrations have been shown to be positive in other cellular assay systems. This should therefore help explain where negative data is discordant with that published in the literature (as seen with nonylphenol for example, which is positive at higher concentrations) and identify limitations of the test, or possibly the literature. Further testing with the antagonist ICI 182 780 which is used to inhibit effects seen, would be useful to verify the ER alpha mediated mechanism.

3.7 Cytoxicity. Questions were raised about the cytotoxicity tests, and whether the control cells were the same as those used for assay purposes. It was explained that the same basal cell line had been used to develop both the ER responsive cell line and that used to evaluate cytotoxicity, and that the cytotoxicity test was not conducted at the same time as the ER test. Concern was expressed with respect to reproducibility of the cytotoxicity assay is when conducted at a different time and using a different (but related) cell line. Cytotoxicity was further discussed during the third teleconference, see paragraphs 2. 15, 4.11.).

3.8 From the summary information provided on the ERα antagonist TA assay (also see appendix 7-1 for the SOP), it was noted that the vehicle and positive controls were placed on the far edge of the plate. The question was therefore raised about assessment of edge effects, by dosing test plates with all vehicle controls and another with all positive controls to assess any variation for both controls. CERI informed the participants that the plate layout was different for the ERα agonist TA assay. CERI confirmed that they had assessed edge effects and it was not a concern, however this was discussed further at the final teleconference, see paragraph 5.6.

3.9 **GLP.** Although preferable, GLP and GCCP were not considered to be an issue of major concern, so long as the laboratory practice was clear and transparent. However an internal audit was requested, such that all data transcription is double checked by an additional operator (as with QA in GLP), to ensure error reduction.

3.10 Discussion with respect to the Prediction Model (PM) and the Data Interpretation Procedure (DIP). The applicability of the Prediction Model (PM) concept, that is the relevance of the assay to the applicability domain(s) of the chemical universe that the test can be applied to, was not planned for this domestic validation at the outset, and does not appear to be possible on the basis of the chemicals selected for testing in this reporter gene assay domestic validation. This may have implications for similar reporter gene assays that may be taken forward for validation assessment at the OECD level. While GD 34 is the OECD validation guidance for the panel, concern was expressed at setting a precedent that did not comply with the more stringent PM validation requirements considered by both ICCVAM and ECVAM to be an essential

component of a successful formal validation exercise. Relevance with respect to the DIP, can however be established on the mechanistic knowledge of the broadly-defined "estrogenic effects", as proposed by CERI, although the panel felt that some supplemental analyses on relevance based on comparison of this assay to other "semi-gold standard" assays would be useful, particulary with reference to sensitivity and specificity.

3.11 Issues regarding log EC50 were presented for information and discussion, see appendix 7-2. It was agreed that there was a need to continue the discussion on the potential usefulness of logPC_{10} and logPC_{50} over logEC50, relative potency measures and definition of a positive chemical based on these or other measures including a traditional LOAEL. Preliminary suggestions on the best approach for the relative induction potency, is to use difference between logPC10 for estradiol and logPC_{10} for a test chemical, which is similar to logIC50-based RBA for the estrogen receptor (ER). Further statistical concerns included the size of error bars and classification of positives and magnitude of response. CERI confirmed a positive to be a PC_{10} value (three fold increase above vehicle control). It was agreed that this required further exploration, to achieve consensus on an agreed statistical uniformity/consistency with particular respect to reporter gene assays (beyond this study) between countries and individual regulatory bodies, and that this might be the sole subject of a future teleconference.

4. THE THIRD TELECONFERENCE MEETING HELD ON 19 MAY 2006.

4.1 This meeting opened with update from Secretariat on the 9th EDTA and 18th WNT regarding the activities of this panel and the preparation of the preliminary validation assessment report. This was followed by an Update from CERI regarding outstanding action points from second teleconference.

4.2 For the negative substances used in the validation study, information and justification on solubility and the maximum concentration used was provided, See Table 7-4.1.

Table 7-4.1. Information and justification on solubility and the maximum concentration used for three negative compounds.

	Hematoxylin	Di(2-ethylhexyl)phthalate	Benzophenone
Preparable max. conc. in DMSO	>1 M	100 mM	>1 M
Solubility in assay medium	>1 mM	Not soluble at 100 µM	>1 mM
Control cell assay	Decrease of luminescence more than 100 µM.	-	Not cytotoxic at 1 mM
Overall conclusion of testable concentration	10 µM	10 µM	Could be tested up to 1 mM

Discussion followed with concern again expressed that for some substances classified as negative; they had not been tested at concentrations up to 1mM (solubility depending), so that very weak agonists might not be detected. A counter argument was that such doses may be unrealistic for physiological purposes, even in an extreme exposure situation, as the medium is considered to be equivalent to the *in vivo* situation. In the protocol it could be indicated that it may be possible, and in some situations desirable to test at concentrations higher than 10µM.

4.3 To what extent one needs to identify very weak agonists or antagonists was discussed further. By testing at higher doses, the EC50 can be measured, and is particularly appropriate for prioritizing for testing. However it was pointed out that the US EPA (and other regulatory authorities and agencies) would never prioritize based on just one assay, rather on the basis of a battery of tests.

4.4 It was further pointed out that one cannot control what the test might be used for, and that it would be constructive to consider more long term planning, particularly with the 3R's in mind. A robust and broad testing strategy would be ideal. While this assay falls under level 2 in the EDTA conceptual framework and US EPA ED screening program tier 1 screening, identifying substances for further testing, provision of data evaluating the ability of the test method to predict *in vivo* ED effects would be of great prospective value. It would allow better characterisation of the ability of this test method, and this might potentially lead to a reduction in animal use for ED testing.

4.5 Non-receptor mediated effects upon chemical luminescence. Concern was raised that some substances could also be inducing other non receptor mediated effects at higher concentrations that might impact upon and increase the chemical luminescence. This has been reported for some phytoestrogens (e.g. Escade et al., 2006) and has also been found to be the case in QA contract work conducted by the US EPA. Dr. Nicholas of INSERM reported that they are working on a new cell line containing two reporter genes, one responding to the hormone and a control in order to identify these non-specific effects. All three cell lines are HeLa cells lines, where one is the control and the other two are controlled by the ERα or ERβ.

Escade, et al., state… 'Moreover, at a concentration higher than 1µM, we noticed an over activation of the luciferase reporter gene by genistein, daidzein and biochanin A which was observed not only in HELN-ERα and HELN-ERβ cells but also in the parental HELN cell

line….This effect, which was previously reported for genistein (Kuiper, et al., 1998), indicated that luciferase expression obtained at high concentrations of phytoestrogens needs to be examined carefully.'

4.6 Edge effects. CERI provided CERI laboratory data on edge effects (assessed by tested a single concentration of estradiol in all 96 wells) conducted after the 2nd teleconference. CERI considered that there was no edge effect affecting the final results. Data from the other participating laboratories was not available. This was discussed further as follows;

At the edge of the plate the wells may suffer from humidity effects and evaporative loss, and the conditions of incubation at the CERI lab are the same as that generally found in other laboratories. Dr Yutaka Aoki assessed the data and noted higher signals by 3.5% among the edge wells, compared with the inner wells, although it was agreed with CERI that these differences were likely to be trivial, and unlikely to affect the final result. For this reason it would be preferable to document the overall CV, and even conduct a formal analysis to see that these results do not affect the final data. It was agreed that as long as the CV for the whole plate is small, say less than 10%, in a plate with common positive control in all well on one hand, and with clear dose response in a plate with test chemical(s) and standard on the other, then the edge effects could be considered not to affect the final data for practical purposes, and that this should be clearly stated in the protocol and monitored by individual laboratories.
Further, there can be a number of plate effects one might usefully consider, for example:

- There can be effects due to cell respiration and metabolism that can be affected by the buffering capacity of the medium and cell number in each well, such that the greater the cell density required by a protocol, the more unhealthy or depleted the cells in central wells might be due to limited gas exchange, compared to those at the edge.

- Optical differences in position of the different wells of the plates can affect the luminosity readings by a plate reader (as well as observation by the naked eye).

- Stacking of plates: effects on cell metabolism have been observed in plates at the bottom of the pile of stacked plates when a large number, i.e. more than 5 plates have been stacked on top of one another in the incubator in some cells.

- Over spraying of ethanol before placing plates in the incubator.

4.7 Provision of antagonist data. CERI provided the SOP (appendix 7-1) and presented antagonist data on three substances tested nine times each in-house. It was noted that a concurrent positive control was not included in these experiments. The Secretariat reminded the conference call participants that the focus of the validation effort was on the agonist assay and that more antagonist testing data existed. These data are currently available on the CERI website in Japanese; CERI offered to prepare and make these data available to the panel and for independent scientific review. Although there was no assessment of interlaboratory reproducibility for the antagonist assay, CERI indicated that as the assay is almost identical to the agonist protocol, extrapolation might be possible by consideration of that validated protocol.

4.8 Concern was expressed that from a regulatory standpoint not having the antagonist data would mean that a substance that was negative for agonist activity would need to be tested in, for example, a binding assay to demonstrate that the substance was not an antagonist. A compromise was suggested such that at a later time point the currently validated protocol could be updated and extended in a catch-up manner, with the validated antagonist protocol as and

when such a protocol might be supported and made available (within a year or so). However for the present, the progression of this test should continue, as other similar assays are not so close to being validated and independently scientifically reviewed. It was generally preferred that there is no delay with moving the assay forward now. However, provision of the range of antagonist data in the report for independent peer review submission, which shows that the antagonist assay is working well, would be of great value to the reviewers.

4.9 Internal audit of data transcribed. This was done according to GLP; one error was identified in Table 13, which has now been corrected. A modified Table 13 will be attached in the final report for independent scientific review.

4.10 Statistical data analyses: Proposed methods for estimation of between- and within-run (laboratory) variation. Agreement on future plans on the revision of and addition to the analysis. Dr Yutaka Aoki (US EPA) gave a presentation with a focus particulary on a weighted average approach for assessing between- and within-run (laboratory) variation and the calculation of standard deviation (SD), with a view to refine the estimates of the various sources of variability that contribute to differences in response. Two macros were also included for the panel participants to experiment with. Appendices 2 and 3 provide information on this approach and further discussion which is presently ongoing.

4.11 Cytotoxicity queries: Provision of the criteria for when cytotoxicity is evaluated and how the data are interpreted, together with the provision of such data with respect to the reproducibility of the cytotoxicity assay when conducted at a different time and using a different (but related) cell line (see paragraph 4.7). CERI explained that generally, when the cell viability is below 80% of the solvent control, the test concentration is regarded as a cytotoxic concentration and the data at that concentration is excluded from the antagonist data analysis. CERI does not have data on the reproducibility of the cytotoxicity assay at this point.

5. DISCUSSION

5.1 Overall, the feeling from the Japanese participants for the domestic validation of this ERα reporter gene assay is that they consider that the current status of the assay is sufficient to be taken forward for official independent scientific peer review with respect to pre-screening for ERα mediated ED effects. This recommendation was therefore made to the WNT meeting in May 2006, and endorsed by the ED Task Force and WNT. With the assistance of the Secretariat, the Japanese are therefore now preparing a report for submission for independent scientific peer review.

5.2 Queries with respect to protocol optimisation, chemical selection, data analyses with sufficient statistical power for the assay, and relatively minor and non essential questions regarding inter (or between) laboratory assessment of making up the chemicals in stock solution have or are in the process of being addressed as far as reasonably possible. From a retrospective point of view, taking the validation data generated together with the extensive data set conducted by CERI in-house using this assay (which is generally in concordance with that from other published ERα mediated in vitro assays), the majority view was that this assay was robust. The minority view (Dr Tice, Dr Stokes and Prof. Combes) was attached as an appendix to the Summary of teleconference 3 and is presented in this report as appendix 7-4.

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Appendix 7-1

Detection of anti-estrogenic activity using reporter gene assay

Description: This document provides a methodology for detecting anti-estrogenic activity of chemicals by reporter gene assay technique using hER-HeLa-9903 cell line.

Materials and methods

1. Test chemicals

Test chemicals should be dissolved in dimethylsulfoxide (DMSO) at a concentration of 10 mM.

2. Competitive substance

17β-Estradiol (E2)

3. Vehicle for chemical stock solutions

Dimethylsulfoxide (DMSO) should be used for the vehicle.

4. Test system and operating procedures

4.1 Cell lines

hERα-*HeLa*-9903 stable cell line (Sumitomo Chemicals Co.) will be used for the assay and 9903-control cell which consistently express firefly luciferase by the RSV promoter without stimulation will be used for evaluating cell-toxic effect of chemicals when anti-estrogenic like effect is observed.

4.2 Cell culture (See support protocols No.1 – No. 4)

Cells should be maintained in Eagle's Minimum Essential Medium (EMEM) without phenol red, supplemented with 10% dextran-coated-charcoal (DCC)-treated fetal bovine serum (DCC-FBS), in a CO_2 incubator (5% CO_2) at 37˚C.

4.3 Preparation of chemicals

All chemicals will be dissolved in DMSO at a concentration of 10 mM, and the solutions will be serially diluted with the same solvent at a common ratio of 1:10 to prepare stock solutions with concentrations of 1 mM, 100 µM, 10 µM, 1 µM, 100 nM and 10 nM.

4.4 Preparation of cells

Assay plate will be prepared according to the support protocol No.5

4.5 Reagents for luciferase assay

Commercial luciferase assay reagent, Steady-Glo Luciferase Assay System (Promega, E2510 and its equivalents) or standard luciferase assay system (Promega, E1500 and its equivalents) will be used in this study. A bottle of Luciferase Assay Substrate is dissolved with the Luciferase Assay Buffer. Dissolved substrate should be used immediately or stored below -20 C.

In the case of using the standard luciferase assay system, Cell Culture Lysis Reagent (Promega, E1531) should be used before adding the substrate.

4.7 Chemical exposure

Each test chemical diluted in DMSO will be added to the wells to final concentrations of 10 µM, 1 µM, 100 nM, 10 nM, 1 nM, 100 pM, and 10 pM (10^{-11}-10^{-5}M) for test in triplicate.

Exact 1.5 µl of 10 mM chemical stock and 6 working solutions will be diluted in serum-free EMEM (500 µl) containing 75 pM of E2.

Then 50 µl of the diluted test samples will be added to each well of assay plate according

to the assignment table shown in Figure 1.

Reference control wells (n=6) treated with 25 pM of E2 without any other chemicals and vehicle control wells (n=6) treated with DMSO alone at concentration of 0.2% will be prepared on every assay plate. After adding the chemicals, the assay plates will be incubated in as CO_2 incubator for 20-24 h to induce the reporter gene product.

Figure 1.1 Typical assignment of assay plate for antagonist assay

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

VC: Vehicle control (DMSO only), RC: Reference control (25 pM E2 only)

In the case that the anti-estrogenic like effect or downward trends in transcriptional activity are noted, cytotoxicity of chemicals should be examined by using HeLa-9903 control cell. Cytotoxicity of chemicals will be evaluated by luciferase activity under existence of test chemicals. The assay will be performed in the same manner to the above mentioned assay procedure except using HeLa-9903 control cell. The plate format should be as shown Figure 2.

Figure 1.2 Typical assignment of assay plate for cytotoxicity

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

VC: Vehicle control (DMSO only)

4.8 Luciferase assay (See support protocol No. 6)

Luciferase activity will be measured with the luciferase assay reagent and a luminometer according to the manufacturer's instructions.

5. Analysis of data

The luminescence signal data will be processed, and the average and standard deviation for

the vehicle control wells will be calculated. The integrated value for each test well will be divided by the average integrated value of the vehicle control wells to obtain individual relative transcriptional activity. Then the average transcriptional activity will be calculated for each concentration of the test chemical. Then 50% inhibitory concentration against mean transcriptional activity induced by reference wells (25 pM of E2), will be calculated, and used for evaluating anti-estrogenic activity of chemicals.

Calculation described above will be made by the commercial software with the Hill's logistic equation showing below;

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

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

In the cytotoxicity test, the luminescence signal data will be also processed, and the average of vehicle control wells will be calculated. The integrated value for each test well will be divided by the average integrated value of the vehicle control wells to obtain individual relative transcriptional activity. When transcriptional activity are reduced less than 80% of the mean transcriptional activity of vehicle control wells, the concentration should be regarded as cytotoxic concentration and excluded for evaluation of anti-estrogenic effect.

6/8/2005

SUPPORT PROTOCOLS

No.1 Preparation of medium

Reagents

- *Eagle's Minimal Essential medium without Neutral red (Nissui Pharmaceutical Co.)*
- *10% Sodium bicarbonate ($NaHCO_3$)*

  Dissolve 10 grams of $NaHCO_3$ to a final volume of 100 mL with water. Then the solution should be sterilized using vacuum-driven bottle-top sterilization filter unit and stored in room temperature.
- *3% Glutamine*

  Dissolve 3 grams of glutamine to a final volume of 100 mL with water. Then the solution should be sterilized using vacuum-driven bottle-top sterilization filter unit. Prepared 3% Glutamine should be stored in aliquots under -20℃.
- *Dextran-corted charcoal (DCC)-treated Fetal bovine serum (DCC-FBS)*

  Prepared and provided by CERI-Japan.

Preparation of EMEM*

Add following reagent into a 1-L conical glass flask and then make to 1 liter with Milli-Q water.

・9.4 grams of pre-made powder medium
・18 mL of 10% Sodium bicarbonate
・12 mL of 3% Glutamine

Preparation of EMEM containing 75pM of E2

Add 75nM E2 to EMEM at proportion of 1:1000 just prior to use.

Preparation of 10%FBS-EMEM*

Add 56 mL of dextran-corted charcoal (DCC)-treated Fetal bovine serum (DCC-FBS) to 500mL EMEM.

**EMEM and 10%FBS-EMEM should be stored in a refrigerator after sterilized with vacuum-driven bottle-top sterilization filter unit.*

6/8/2005

SUPPORT PROTOCOLS

No. 2. Reconstitute of cell from the frozen stock

1. Remove vial from Liquid Nitrogen or freezer and immediately transfer to 37°C water bath.
2. While holding the tip of the vial, gently agitate the vial.
3. When completely thawed, transfer the cell stock into 5 mL pre-warmed 10%FBS-EMEM in 15 mL conical tube.
4. Centrifuge the tube at 1100 rpm (200-300 x g) for 5min, and remove the supernatant carefully.
5. Resuspend the cell with 10 mL of 10%FBS-EMEM and place to 90 mm culture dish.
6. Incubate the cell in 5% CO_2 incubator at 37°C.

SUPPORT PROTOCOLS

No. 3. Propagation

1. Remove the medium from the culture dish with sterile pipette or sucker.
2. Rinse the cell with 5 mL of PBS.
3. Remove PBS with sterile pipette or sucker.
4. Add 2 mL of Trypsin-EDTA solution (0.25% Trypsin + 0.02%EDTA/PBS) to cover the bottom of the culture dish and then remove the excess.
5. Allow to stand Trypsin treated cell for ca. 3 min in 5% CO_2 incubator at 37°C.
   (Monitor cells under microscope. Cells are beginning to detach when they appear rounded)
6. Tap the dish gently.
7. Wash to remove the adherent cells with 5 mL of 10%FBS-EMEM.
8. Count cell number.
9. Dilute the cell suspension with 10%FBS-EMEM to 0.4-1.0 x 10^5 cells/mL.
10. Place 10 mL of cell suspension to 90 mm culture dish.
11. Incubate the cell in 5% CO_2 incubator at 37°C.

SUPPORT PROTOCOLS

No. 4. Preparation of frozen stock

1. Remove the medium from the culture dish with sterile pipette or sucker.
2. Rinse the cell with 5 mL of PBS.
3. Remove PBS with sterile pipette or sucker.
4. Add 2 mL of Trypsin-EDTA solution to cover the bottom of the culture dish and then remove the excess.
5. Allow to stand Trypsin treated cell for ca. 3 min in 5% CO_2 incubator at 37°C.
   (Monitor cells under microscope. Cells are beginning to detach when they appear rounded)
6. Tap the dish gently.
7. Wash to remove the adherent cells with 5 mL of 10%FBS-EMEM.
8. Count cell number.
9. Centrifuge the tube at 1100 rpm (200-300 x g) for 5min, and remove the supernatant carefully.
10. Add Cell-Banker* (Juji Field Inc.) and resuspend the cell at density of ca 1 x 10^4 cells/mL.
11. Make 1 mL aliquots of cell stock.
12. Freeze and store the cell stock below -80°C**.

*Conventional freeze medium (90% FBS/10% DMSO) can be used in place of Cell-Banker.
**Storage in liquid nitrogen would be preferable for long-term storage (more than 3 months).

5/2/2006

SUPPORT PROTOCOLS

No. 5 Preparation of assay plate

Prepare a dish of cultured hERα-HeLa-9903 cell

1. Remove the medium from the culture dish with sterile pipette or sucker.
2. Rinse the cell with 5 mL of PBS.
3. Remove PBS with sterile pipette or sucker.
4. Add 2 mL of Trypsin-EDTA solution to cover the bottom of the culture dish and then remove the excess.
5. Allow to stand Trypsin treated cell for ca. 3 min in 5% CO_2 incubator at 37°C. (Monitor cells under microscope. Cells are beginning to detach when they appear rounded)
6. Tap the dish gently.
7. Wash to remove the adherent cells with 5 mL of 10%FBS-EMEM and transfer the cell suspension to a centrifuge tube.
8. Count cell number.
9. Centrifuge the tube at 1100 rpm (200-300 x g) for 5min, and remove the supernatant carefully.
10. Resuspend the cell with 10%FBS-EMEM to obtain a final cell density of 1×10^5 cells/mL.
11. Add 100 μL of cell suspension into each well of 96 well assay plate (Nunc #136102 or equivalents).
12. Incubate the cell in 5% CO_2 incubator at 37°C for 3h
13. Proceed to chemical exposure.

SUPPORT PROTOCOLS

No. 6-1. Chemiluminescence Detection with standard luciferase reagent

Reagents

Cell lysis reagent (4.5x): Dilute 10 mL of 5×Cell Culture Lysis Reagent (CCLR, #E1531) with 45 mL of distilled water.

Luciferase Assay Reagent: Add 1 vial 105 mL of Luciferase Assay buffer (Promega, #E4550) into a vial containing Luciferase Assay Substrate (Promega, #E4550), and dissolve the substrate thoroughly. Store the substrate below -20°C if necessary.

Chemiluminescence Detection

1. Flick and drain off the contents of the assay plate.
2. Add 100 µl of PBS to the well to wash the plate.
3. Flick and drain off the contents of the assay plate.
4. Add 100µl of PBS to the well to wash the plate again.
5. Flick and drain off the contents of the assay plate.
6. Add 15 µL of Cell lysis reagent (4.5x) to wells.
7. Incubate for 10 min at room temperature.
8. Add 50µL of Luciferase Assay Reagent to wells.
9. Read plates on a Chemiluminescence plate reader.

SUPPORT PROTOCOLS

No. 6-2. Chemiluminescence Detection with luciferase reagent using Steady-Glo Luciferase Assay System

Reagents

Luciferase Assay Reagent: Add 1 vial (100 mL) of Luciferase Assay buffer into a vial containing Luciferase Assay Substrate (Promega, #E2520), and dissolve the substrate thoroughly. Store the substrate below -20°C if necessary.

Chemiluminescence Detection

1. Remove 50 µL of assay medium from all wells of assay plate.
2. Add 100 µL of Luciferase Assay Reagent to wells.
3. Allowed to stand for 5 min.
4. Read plates on a Chemiluminescence plate reader

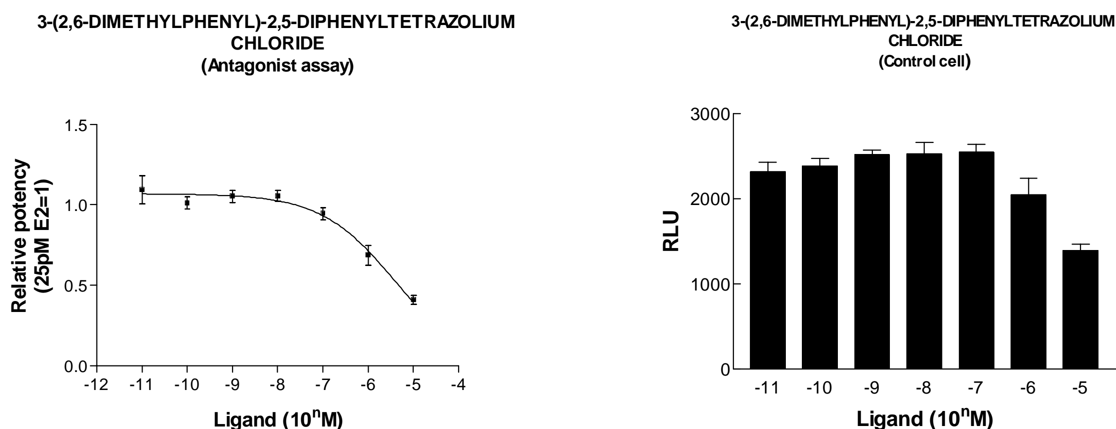
Monitoring of cytotoxic effect of chemicals in reporter gene assay

March 15, 2006
Masahiro Takeyoshi, CERI-Japan

Cytotoxicity is the quality of being toxic to cells caused by toxic agents (chemical substance). In general, cytotoxicity can be measured by the MTT assay or other conventional methods (Alamer dye method etc.). Reporter gene assay is an analysis method that allows the identification of promoters and enhancers and the study of the correlations between their activities and conformations by checking the amount of the reporter proteins that are expressed from reporter genes. And the endpoint of hER-HeLa-9903 cell based reporter gene assay is a luciferase activity that is produced as a result of the transcriptional activation of the reporter gene. Cytotoxic effect of chemicals may lead misunderstanding of the results of this assay system, especially in reporter gene assay for antagonist activity of chemicals.

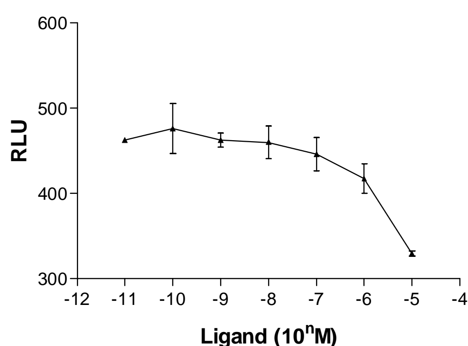
In our system, cytotoxicity detection system using control cell, which constantly produces firefly luciferase by the RSV promoter without any stimulation, is already established for antagonist assay system (Please refer to the document entitled "***Outline of ERα Antagonist assay using hER-HeLa-9903***" dated March 15, 2006).

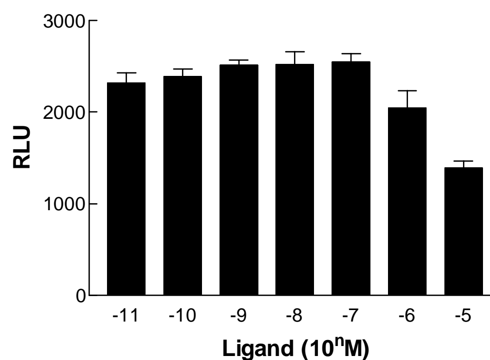
In this system, cytotoxicity of chemical is clearly detectable as shown below;



In this case, the antagonist like effect observed in the antagonist assay is concluded as negative because of its cytotoxicity.

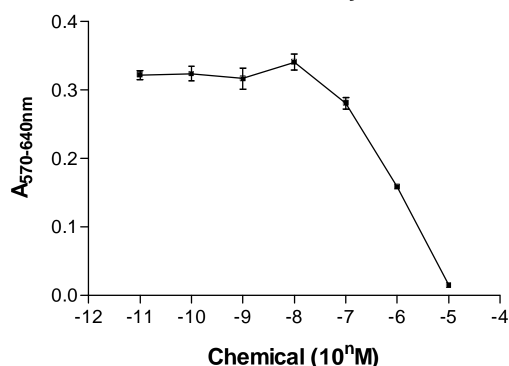
The cytotoxic effect of chemical also causes reduction of basic transcriptional activity in agonist assay (See below).

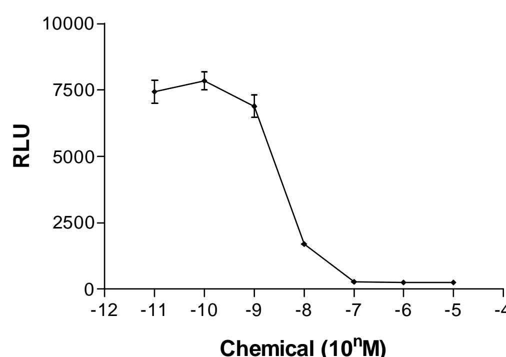




This result indicates that monitoring of basic TA activity in agonist assay can provide the cytotoxic effects of chemical.

In some laboratory, MTT assay may be employed for monitoring cytotoxic effect of chemicals. MTT assay is a general experimental technique for measuring cellular proliferation (cell growth). In this assay, the amount of yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) oxidised to purple formazan is measured spectrophotometrically. This oxidation takes place when mitochondrial reductase enzymes are active, and thus conversion is directly related to the number of viable cells, another way of saying it is related to the number of cells possessing active mitochondrial reductase enzymes.
However, an endpoint of the reporter gene assay is luciferase activity resulting from the transcriptional activation, and is not a mitochondrial reductase activity.
For this reason, clear discrepancy is noted between the cytotoxicity measured by MTT and that monitored by luciferase activity.
Figures below shows cytotoxic effects of tripropyl-tin measured by both two methods.





Although the transcriptional activity measured by luciferase, this means cytotoxic effect on cellular transcriptional activity, was definitely reduced at least 10^{-8}M of tri-propyl tin, no effect was noted in MTT assay at the same concentration (10^{-8}M of tri-propyl tin).
This suggests that cytotoxicity in the reporter gene assay should be monitored with luciferase activity of control cell or basic transcriptional activity of agonist assay rather than MTT assay.

Appendix 7-2

See Appendix 6 in the validation report

Appendix 7-3

Subject: FW: Statistical approach for intra- and interlaboratory variability

------ Forwarded Message
From: <Hasemanjk@aol.com>
Date: Thu, 25 May 2006 10:56:50 -0400
To: "Deal, Frank H (NIH/NIEHS) [C]" <dealf@niehs.nih.gov>
Cc: "Tice, Raymond (NIH/NIEHS) [E]" <tice@niehs.nih.gov>, "Ceger, Patricia (NIH/NIEHS) [C]" <cegerp@niehs.nih.gov>, "Blackard, Brad (NIH/NIEHS) [C]" <blackard@niehs.nih.gov>, "Charles, Jeffrey (NIH/NIEHS) [C]" <CharlesJ2@niehs.nih.gov>
Conversation: Statistical approach for intra- and interlaboratory variability
Subject: Re: Statistical approach for intra- and interlaboratory variability

Frank-

I have examined Dr. Aoki's PowerPoint slides, and I believe I understand his concerns.

The examples used to illustrate his concerns involve data from four labs with three runs per lab. These 12 data points (logPC50s) are apparently each based on estimates from a Hill equation analysis. However, regardless of how the estimates are obtained, each of the logPC50s is an estimate and has an associated SE of the estimate. One of Dr. Aoki's objections is that these standard errors associated with the estimation process are typically ignored in the data evaluation process.

For example, the typical approach for computing the mean response for each lab is to simply average the three runs. Dr. Aoki prefers instead a weighted average approach that weights each estimate inversely with the associated variability (i.e., the less variable estimate gets weighted more heavily in the averaging process). In my opinion, this is a reasonable option, and I suspect that a statistical purist would likely prefer the weighted average approach to the unweighted average. However, it could also be argued that since each run was carried out under identical conditions, the runs should be given equal weight, regardless of variability.

Thus, I disagree with Dr. Aoki that it is 'naïve' and 'inappropriate' to work with unweighted means, which provide unbiased estimates of the underlying parameter and typically are similar to the weighted means in any case. For example, in one of Dr. Aoki's examples, the unweighted mean is -6.94; the weighted mean is -6.93. I suspect that this is typical of what would be found in practice, especially since there are 'validity check' safeguards built in that will minimize the likelihood that the underlying variability estimates will differ greatly from run to run. From a practical point of view, it is unlikely in our area of application that the choice of weighted vs. unweighted means will have any noticeable impact on the overall interpretation of a study.

I note also that in Dr. Aoki's Slide 6, the lab and run columns are mislabelled and should be reversed.

A second related concern of Dr. Aoki is the calculation of an SD. For example, the variation in response among the three runs at a given lab in theory represents two distinct sources of variability: (i) the variability associated with the estimation process itself; and (ii) the additional variability that might be due to factors that are different from run to run. The SD that is normally calculated does not distinguish between these two sources of variability, but Dr. Aoki feels that this distinction is important and that by subtracting out (i) and focusing strictly on (ii), one obtains better 'estimates'.

Better estimates of what? I agree that his approach provides better estimates of Source of Variability (ii), but I would argue that the primary variability of interest is the actual observed variability among runs, which reflects both (i) and (ii). It should not matter if this variability is due entirely to the estimation process (as was the case in three of the four labs in his example) or if both (i) and (ii) contribute to this variability. The end result is what matters.

Similar comments apply when combining the lab means to produce an overall average. Once again, one could either use a weighted average (-7.15 in Dr. Aoki's example) or an unweighted average (-7.13). Generally, the two will agree very closely.

The variability observed among the lab means is due to a combination of three sources of variability: (i) and (ii) as noted above and (iii) additional variability introduced by factors that differ among labs. Here again, Dr. Aoki recommends 'subtracting out' (i) and (ii) to obtain a 'pure' estimate of (iii). I would once again argue that it is the overall variability that is important, regardless of the contribution of the three individual components.

Although weighted versus unweighted means will very likely have little or no impact on the final interpretation of a study, the same may not be true for an evaluation of variability. In Dr. Aoki's 'fake data' example, he concludes that the much better SD's are essentially all zero. What does this mean from the standpoint of assessing the reproducibility of the assay? I worry that a naive investigator may assume that this means that the assay is extremely reproducible (after all, it has zero SD's), but this may not be the case at all. It may simply mean that the variability associated with the estimation process is so great that it can totally account for the overall variability in response observed among runs and among labs. The magnitude of this variability may or may not be cause for concern, but I still would argue that quantifying the specific sources of the variability is not nearly as important as evaluating the magnitude of the resulting variability itself, as assessed in the 'traditional' (and not 'inappropriate') way.

Dr. Aoki states in Slide 15 that the statistical programs used to produce the Hill equation estimates of the logEC50 do not provide associated SE estimates, but I do not believe that this is the case. Doesn't Prism produce them routinely? If so, then this information can be used in the manner suggested by Dr. Aoki.

Importantly, in the final analysis, one must decide if the purpose of these studies is to refine our estimates of the various sources of variability that contribute to differences in response, or is it to determine whether or not an assay has acceptable reproducibility. Dr. Aoki's presentation focuses on the former, but in my opinion, the latter should be our goal. Thus, if I am trying to determine whether or not an assay is acceptably reproducible, I would want to focus on the observed variability in the actual EC50 estimates across and within labs regardless of the factors that contributed to the variability.

For example, suppose I observed a coefficient of variation of 50%, that in normal circumstances would be unacceptable. However, using Dr. Aoki's approach, it is not this variability that is important, but the relative contribution of the factors that produced it.

This high variability might be due to the estimation process, differences among runs, differences among labs, or a combination of these three factors. In my opinion, quantifying these sources of variability and determining which is the primary contributor should not be our focus. For example, one extreme possibility is that the Hill equation model fit is so poor (and the resulting SE's of the estimated EC50's so high) that Source of Variability (i) can account for essentially all the variability in response, and as a result all the better estimate SD's computed by Dr. Aoki for Sources of Variability (ii) and (iii) are close to zero. Would Dr. Aoki consider such an assay to have acceptable reproducibility since the estimated SD's are all close to zero? I would not.

If assessing the individual components contributing to the overall variability is viewed as a critical matter, then you could carry out a nested ANOVA to examine quantitatively the relative effects of variability among labs and variability among runs within labs on the overall response (e.g., the logEC50).

I could find nothing in Dr. Aoki's presentation to suggest how his approach could be used in a real world setting to determine whether or not an assay had acceptable reproducibility. One exercise that would be of interest would be to take a real world example and assess whether or not the assay has acceptable reproducibility in the usual way (considering CV's, etc.), and then ask Dr. Aoki and his colleagues to take the same data and make a similar 'bottom line' judgment based on his more complex assessment of weighted means, extracting sources of variability, etc. I strongly suspect that the same conclusion will be reached after considerably more work.

As a general rule, if a new complex statistical procedure is proposed to replace a 'less rigorous' one, then it should be demonstrated empirically how the old method fails and the advantages of the new approach in terms of the goal of the study, which in this case is accessing whether or not the assay has acceptable reproducibility. Until this is done, and concrete examples can be presented demonstrating the superiority of this more complex data assessment process, I see no need to make major changes in what is currently done.

Regarding Appendix 2, I strongly agree with Dr. Aoki that it makes no sense to calculate a CV based on log-transformed data. Surely, no one is recommending this (are they?). If so, this should be abandoned, and I agree with Dr. Aoki that the measure of variability to use in this case is the SD, not the CV. I further agree with his assertion that 'In general, CV is a good measure of variation where SD of a variable increases (linearly) with the mean of the variable. '

Dr. Aoki then states that 'there seems to be no reason to believe that the SD increases with the mean'. It is unclear if he is referring to the SD associated with the log transformed data (in which case I agree with him) or the untransformed data (in which case I disagree).

For example, toxic compounds with very low EC50's may have three runs with estimated EC50 values of (e.g.) 0.01, 0.03, and 0.05, while a non-toxic compound may have EC50 values of 1000, 3000, and 5000, In such cases, the SD's of the EC50's are quite different, but the SD's of the log transformed data are identical. This is what generally happens in practice. Thus, in terms of the EC50 I would use CV; in terms of the logEC50 I would use SD. I suspect that Dr. Aoki would agree with this.

Joe Haseman
5-25-06

------ End of Forwarded Message

June 20, 2006
Yutaka Aoki, ASPH Fellow at USEPA
aoki.yutaka@epa.gov

I share with Dr. Haseman the view that our primary goal is to evaluate whether the overall variability of the parameter estimate of scientific/regulatory interest from the assay is acceptably low. In the case of the transcriptional activation studies, for example, we are interested in whether the overall variability of the logPC10 across laboratories is acceptably low. In addition to this goal, it is often useful to have the capacity to evaluate the contributions of various sources of variability. In such cases it makes sense to have an estimate of intrinsic between-unit variability, not only overall (total) between-unit variability. (Please note that in my presentation I used the term "true between-run (lab)

variation" to refer to what I am calling "intrinsic between-run (lab) variation" in this document.) In general, the overall (total) variability consists of two components: intrinsic between variability and overall within variability. That is, the following relationships hold:[3]

Overall (total) within-lab variability =
Overall (total) between-run variability = intrinsic between-run variability
+ overall within-run variability

and

Overall (total) between-lab variability = intrinsic between-lab variability
+ overall within-lab variability

Please note that the term "between-run (lab) variability" appears on both sides of the equations with different descriptors ("overall" vs. "intrinsic"). Hence there are two alternative interpretations for the term "between-lab variability," which appears in various assay validation guidelines as a standard component to be estimated in interlaboratory studies. I took the between-lab variation to mean intrinsic, not overall, variability, and applied the general, widely-used procedure for its estimation (i.e., the DerSimonian Laird random effects model). However, I realized from Dr. Haseman's comments that the term "between-lab variability" could be taken to mean "overall between-lab variability". What Dr. Haseman calls the "traditional procedure" is the natural procedure that ensues from this interpretation. Using one of these interpretations results in preference for a particular kind of between-lab variability estimate, the "overall" or "intrinsic".

There are a few potential uses for the complementary pair of estimates of intrinsic between-unit variability and within-unit variability as opposed to a single estimate of overall (total) between-unit variability alone. For instance, the pair of variability estimates are useful at a pre-validation stage when one is trying to identify specific sources of variation as a target of variability reduction. High variability in radioactive count measurement, for example, would tend to increase within-run variation, not intrinsic between-run variation. Inappropriate preparation of a stock standard solution for each run, from which appropriate serial dilution can be made reliably, would result in increase in intrinsic between-run variation, not in within-run variation. For an instance of post-validation use of the complementary variability estimates, suppose the overall between-lab variability for an assay has been found to be unacceptably high under a specified design and we would like to know how much an increase in the number of runs (or, rarely, labs) might reduce the variability to the desired level. Only with the estimates of intrinsic between-lab variability and overall within-lab variability (which is a function of the number of runs), would easy calculation of the necessary number of runs be possible.

As an additional benefit, the proposed procedure gives rise to a good estimator of overall variability, which in certain circumstances performs considerably better than the counterpart for the traditional method: the latter underestimates overall variability when intrinsic between-unit variation is small compared to within-unit variability. This difference arises because the two procedures handle standard errors (SEs) of estimates differently: our proposed procedure takes SEs of estimates (either run-specific summaries or lab-specific summaries that are to be further summarized) into account while the traditional method ignores them. The advantage of the proposed procedure was clearly noted in simulations I performed. In the case of the transcriptional activation data, for example, the overall between-laboratory variability would be more accurately estimated by the new procedure if the variability within each lab were large relative to the variability between labs. Underestimation of the overall variability is problematic since it gives a false sense of reproducibility to the user.

[3] The relationships hold in terms of variance under the assumption of independence between the underlying components for the two right-hand side terms.

When deriving estimates of overall variability, which both Dr. Haseman and I regard as the most relevant variability measures, I obtain an estimate of intrinsic between-variability, and then combine it with within-variability estimates. This is done by taking into account the experimental design (i.e., how many runs and laboratories are actually used).

Although the new procedure may be more difficult to grasp conceptually than the traditional method of estimating overall variability, it is quite simple to implement. We consider the computational cost associated with our proposed procedure small, and particularly so when compared to potential benefits we gain by using it.

It is likely this response lacks the level of details that some readers would desire. I omitted many details for the sake of simplicity, but I am happy to provide more detailed information or answer questions upon request.

Appendix 7-4

Table 7-4.1 Summary of criteria that were not met according to ICCVAM Minimum Standard Procedures (ICCVAM 2003)

Minimum Standard Procedure	Met/ Not Met	Explanation and Justification
The stability of the test substances should be demonstrated prior to testing. In the absence of stability information, the stock solution should be prepared fresh prior to use.	NOT MET but resolvable retrospectively	The stabilities of test substances were not confirmed, however empirically stable substances were used. The stock solution was not freshly prepared. Under the inter-laboratory validation, the stock solution was prepared at the lead laboratory and then distributed to the participating laboratories. All stock solutions were stored at -20 C at each laboratory. The capabilities of the participating laboratories to make up stock solutions accurately were assumed, and the lead laboratory did not consider it necessary to include this as part of the validation process at the time. Should it be absolutely necessary for the purposes of the independent peer review, the participating laboratories could be requested to make up the stock solutions individually and then be subsequently assessed.
Studies should be performed in compliance with GLP guidelines.	NOT FULLY MET	The pre-validation was not to GLP, the inter-laboratory validation was under GLP, and the data collection for comparison with the ICCVAM list and hERa binding assay was not to GLP standards.
In a validation study, repeat studies would be conducted to evaluate intra-laboratory repeatability and reproducibility. In contrast, in screening studies, repeat studies are not conducted, except to clarify equivocal results.	NOT FULLY MET	The pre-validation and inter-laboratory validation was repeated but the data collection for comparison with ICCVAM list or hERa binding assay was not always repeated.

It should be noted that major deviation from the ICCVAM and ECVAM validation requirements could mean that the assay may not be considered by these validation bodies as correctly and formally validated for regulatory use.

Comments received from Drs Bill Stokes and Ray Tice (NICEATM) on Studies Conducted by CERI to Support the Validation of the hER-HeLa-9903 Estrogen Receptor (ER) Transcriptional Activation (TA) Test Method

Our comments are based on information CERI has provided in their report entitled, "Draft Pre-Validation and Inter-Laboratory Validation Report of the Human Estrogen Receptor Mediated Reporter Gene Assay", and other supporting materials, including those used to present information that CERI has provided at the request of the OECD Preliminary Validation Assessment Panel. Our assessment of the provided information is based on relevant information provided in Section VII of OECD Guidance Document No. 34, which recommends and defines the components of a new test method submission. Our assessment of the hER-HeLa-9903 ER TA test method protocol is based on the minimum procedural standards (we now call these essential test method components) recommended by ICCVAM[4] and based on the deliberations of an ICCVAM international expert panel on ER and androgen receptor binding and TA assays that met in May of 2002. Our evaluation of the substances used to evaluate the accuracy and reliability of the hER-HeLa-9903 ER TA test method is based on the ICCVAM list of recommended reference substances for ER binding or TA test methods[5].

Our comments are organized under the major headings in Section VII of OECD Guidance Document No. 34 as follows:

Introduction and Rationale for the Proposed Test Method

Reports and supporting materials address the rationale for the CERI ER TA test method, as specified in this section of the Guidance Document, but discussions regarding the specific limitations of the test method could be usefully expanded.

Test Method Protocol Components

A test method protocol has been provided, as specified in this section of the Guidance Document, but this is the protocol that was used for the experiments that involved multiple laboratories only. It is stated in the text that the in-house protocol was similar but the protocol followed throughout and any modifications and the rationale for those modifications needs to be included. For example, in the interlaboratory study, estradiol was tested over multiple concentrations but in the in-house studies, it was tested at only a single concentration. The rational for this difference should be provided.

In addition, in terms of the test method protocol, the highest concentration of substance tested was 10 µM, not the 1 mM recommended by the ICCVAM international expert panel and ICCVAM (see footnote 1). We appreciate that not all substances can be tested up to this concentration (due to solubility or excessive cytotoxicity) but the purpose for using this limit dose is to detect even very weak ER agonists or antagonists. Thus, at least some of the substances classified as negative by CERI have not been adequately tested (this was demonstrated in the data set provided by CERI for the last conference call) while others may have been adequately tested if solubility or cytotoxicity

[4] "ICCVAM Evaluation of *In Vitro* Test Methods For Detecting Potential Endocrine Disruptors: Estrogen Receptor and Androgen Receptor Binding and Transcriptional Activation Assays" (available at http://iccvam.niehs.nih.gov/methods/endocrine.htm).

[5] "ICCVAM Evaluation of *In Vitro* Test Methods For Detecting Potential Endocrine Disruptors: Estrogen Receptor and Androgen Receptor Binding and Transcriptional Activation Assays" and the 2006 Addendum to this report (available at http://iccvam.niehs.nih.gov/methods/endocrine.htm).

data can be provided to support the highest concentration tested.

There seems to be a lack of information in regard to the rationale/justification, criteria for use, and reliability for the cytotoxicity evaluation, which were conducted using the same basal cell line but with a different plasmid construct as a separate experiment. From verbal discussions, it appears that CERI does not feel a cytotoxicity evaluation is needed for the agonist tests. This issue needs to be formally discussed in their submission.

For use as a screening assay for ER or AR activity, it is critical that a TA test method evaluate for antagonist as well as agonist activity. Except for the intralaboratory repeat testing of three substances, an evaluation of the ability of the CERI ER TA test method to identify ER antagonists has not been provided. Furthermore, the antagonist protocol used in the testing of these three substances had no concurrent positive control, and did not use a reference standard with a full dose response curve as is done in the CERI agonist protocol. We appreciate the desire to move ahead with the agonist version of the test method independent of the antagonist version but wish to point out that a negative ER agonist study is virtually worthless without knowing whether or not the test substance binds to the ER and/or demonstrates antagonist activity. We do not agree with CERI's premise, stated in the most recent OECD teleconference, that the antagonist protocol is similar enough to the agonist protocol to be considered as validated in the same manner. We urge that the current ER antagonist protocol be modified to include appropriate positive controls and that further validation studies using this protocol be completed before peer review.

The protocol needs to include a discussion about potential "edging effects", and how to identify if the outside wells on the 96-well plate can be used because such effects are not detected under the experimental conditions used by a specific laboratory.

Characterisation and Selection of Substances Used for Validation of the Proposed Test Method

To facilitate validation of ER TA assay, ICCVAM compiled a list of 78 recommended reference substances. ICCVAM recommends that these substances be tested in a phased manner, with a minimum of 53 substances being tested across at least three laboratories. The remaining 25 substances are recommended for testing once in one laboratory or divided among two or more laboratories.

Our evaluation of the data submitted indicates that CERI tested a total of 56 substances, although only 10 were tested across multiple laboratories. Seven of these 10 substances are on the ICCVAM list and the remaining three have similar ER activities to other ICCVAM substances recommended for interlaboratory testing and could be considered as replacements for these.

Therefore, to meet ICCVAM recommendations, 43 additional substances from the ICCVAM recommended list or their equivalents would require further interlaboratory testing.

CERI tested 12 of the remaining 25 substances on the ICCVAM list that do not require interlaboratory testing at least once, leaving an additional 13 substances from the list or their equivalents that would require further testing.

Also, substances are not classified according to product class and only the 10 substances tested across multiple laboratories are classified by chemical class. These 10 substances represent 6 chemical classes compared to the 15 chemical classes represented by those substances recommended for interlaboratory testing by ICVAM (a total of 22 chemical classes are represented by the ICCVAM recommended list of 78).

***In Vivo* Reference Data Used to Assess the Accuracy of the Proposed Test Method**

The comparison of experimentally derived results from ER TA agonist and immature rat uterotrophic studies conducted at CERI using 50 substances adequately supports the accuracy of the proposed ER TA agonist test method.

Testing all 78 reference substances would not only allow for a better characterization of the reliability and comparative sensitivity of the CERI test method versus other Tier 1 assays but also increase the likelihood that *in vitro* tests might be developed that could be used to reduce animal use in endocrine disruptor (ED) testing.

Test Method Data and Results

Results and data from prevalidation and interlaboratory studies conducted by CERI to support the validation of their hER-HeLa-9903 ER TA agonist assay have been provided, but much of this was not provided in the CERI draft validation report but rather at the request from the OECD preliminary validation assessment panel. It is assumed that the requested results and data will be included as appropriate in the appendices of the final validation report from CERI.

Test Method Relevance (Accuracy)

Because this test method is to be used as a Tier 1 screening assay (at least in the United States), there is no need for an evaluation of the ability of the test method to predict in *vivo* endocrine disruptor effects. However, such data are welcome and would allow better characterization of the ability of *in vitro* test methods such as this to reduce animal use in ED testing. The comparison of CERI derived ER TA results with ICCVAM published ER TA results for 46 substances is appropriate.

Test Method Reliability (Repeatability/Reproducibility)

In terms of intra- and inter-laboratory reproducibility, 10 substances (two strongly active positives, four moderately active positives, one weakly active positive, and three negatives) were tested three times in each of three laboratories. All tests were conducted using stock solutions provided by CERI (i.e., the full test method protocol was not evaluated). Furthermore, substances that posed potential problems in testing due to their physico-chemical characteristics (i.e., poor solubility) or because they were overtly cytotoxic were not tested. Thus, this is not an adequate evaluation of the intra- or inter-interlaboratory reproducibility of this test method. In its international evaluation of another ER TA test method, NICEATM/ICCVAM is proposing 12 substances to evaluate intralaboratory reproducibility in three labs (testing 3 times in each lab) and another 41 substances to be tested once in each of three labs to adequately evaluate interlaboratory reproducibility. These substances cover the range of anticipated agonist and antagonist responses, include a wide variety of chemical classes, and include substances with varied physico-chemical properties and cytotoxicity properties.

Also, in their interlaboratory evaluation, the reference substance, estradiol, was tested over its complete concentration response range. In contrast, for other substances, CERI tested estradiol at a single concentration. The former is recommended by the ICCVAM International ED Expert Panel and by ICCVAM for all experiments.

Test Method Data Quality

Interlaboratory studies testing 10 substances were conducted using GLP guidelines, but none of the

pre-validation studies were conducted in this manner. At the last OECD preliminary validation assessment panel teleconference, CERI representatives indicated that a data audit has been recently conducted on the prevalidation studies and stated that non-compliance with GLP guidelines had no impact on data quality. We recommend that a specific discussion regarding data quality and non-compliance be included in the CERI report.

Animal Welfare Considerations (Refinement, Reduction and Replacement)

Our evaluation of the validation report and supporting materials indicate that specific discussions on how the proposed test method will refine, reduce, or replace animal use if used in a battery of tests to detect potential endocrine disruptors were not provided.

Practical Considerations

We recommend the inclusion of considerations such as the cost and time required to conduct the assay and report results. Considering the concerns about "edging effects", we also recommend expanding the discussion of necessary equipment and supplies, and the required level of training, expertise and demonstrated proficiency needed by study personnel.

Late Comments received on 3 June 2006 from Prof. Combes (member of the panel, but did not participate in the teleconferences or discussions prior to 3 June 2006).

Dear All,

Thanks for all the summaries which I have now had a chance to read in some detail, although I am afraid that I still have not had the opportunity to look at all the raw data.

My impression is that there has been an awful lot of work done on this assay and those involved deserve congratulations for their efforts and for getting us to the stage we are at.

Having said that, I have several overall concerns about the readiness of the work that has been done for peer review, since I am unsure as to the ability of the interlaboratory validation study to transparently and unequivocally demonstrate reliability and relevance of the assay for its stated purpose. In this regard, I share many of the concerns that have been raised in the NICEATM comments raised during the last teleconference as presented in Appendix 1 of the latest set of minutes.

Due to the large amount of information and data, I am unclear as to exactly where we are now and welcome the suggestion that there should be an overall report. This could well serve as the document for eventual peer review, but this decision should not be taken until we have all seen the document and agreed on its status. The last thing we would want is for the peer review report to be controversial (as indeed is the report for the Uterotrophic assay) as this would undermine the validation process and give the assay a bad name, when it could all be avoided by being less hasty and ensuring that the validation study is as good as possible.

I personally remain unconvinced that the stuies are ready yet for peer review for the following main reasons:

1. the raw data are not as transparent as they should be
2. there is a need to agree on how the data are transformed and statistically analyzed (personally I prefer the presentation of straightforward error bars)
3. it appears to me that the validation has only been performed in Japan, when for it should be assessed in other countries (this is no criticism of Japanese laboratories, merely it is necessary to ensure that reliability extends to other countries
4. there have been claims for the deviation of the studies from accepted OECD, ECVAM and ICCVAM validation criteria - these need to be discussed in more detail.

With regard to other matters, I think that it would be good to have more detail concerning what was discussed in relation to the assay at the recent WNT meeting. In addition, I am unhappy with the vagueness of what is stated regarding the potential arrangements for peer reviewing the assay, as stated in the minutes of the last teleconference.

A peer review of a validation study should not be contracted out to a laboratory, for goodness sake!

I am also very concerned that the OECD might be asked to organise a peer review, in view of the debacle over the review of the uterotrophic assay. Peer review of new in vitro methods should be left to those with experience and authority with undertaking them in conjunction with relevant legislative authorities; namely ICCVAM and the ECVAM Scientific Advisory Committee. In fact, my suggestion would be for a joint peer review organised by ECVAM, ICCVAM and the newly-formed JACVAM. This would be an excellent opportunity to initiate a world-wide peer review study and to capitalise on the existence of these centres. However, I re-iterate that no peer review should be undertaken until it can be
ensured that the validation study meets all the necessary criteria.

I apologise if I seem rather over-critical, but I am not trying to be - I am very impressed by the work achieved on the assay, but I think we should be cautious in going too fast and losing the opportunity to build on the excellent foundation that we have. I am as keen as anyone to see these types of assays on the books to augment and eventually replace the in vivo methods. But we must get it right, ensure it meets international criteria, and check that everything is independent and transparent.

I hope all this helps, with best wishes,

Bob Combes

Appendix 8 Summary of queries from PVAP and corresponding answers

No.	Queries from PVAP	Corresponding answers
i.	CERI conducted a comparison of the draft report submission with the guidelines provided in the OECD Guidance Document 34 and *ICCVAM Evaluation of In Vitro Test Methods for Detecting Potential Endocrine Disruptors* (NIH Pub. No. 03-4503) and stated their rationale for deviations from these guidelines.	See Table 7-3.2 in appendix 7
ii.	CERI provided further information on cell line characterisation, methods of cytotoxicity evaluation and ER alpha antagonist TA testing	・ Cell line characterization → See Paragraph 3.2 in appendix 7 ・ Method of cytotoxicity evaluation → See Paragraph 3.7 and 4.11 in appendix 7 ・ ER alpha antagonist TA testing → See Paragraph 4.7 in appendix 7
iii.	CERI provided raw fold induction data for the positive controls and for the chemicals assessed under the (pre) validation stage from data generated by the CERI laboratory. The provision of such data from the other laboratories was not possible. The panel required this information to assess the extent of the variation in fold induction over time.	The raw fold induction data was provided.
iv.	Dr. Yutaka Aoki (US EPA) provided information on proposed methods for between- and within-variation estimation to the whole group (Appendix 7-2) and consulted directly with CERI on how to proceed.	See Appendix 7-2
v.	CERI conducted an internal audit of data transcribed.	See Paragraph 4.10 in appendix 7
vi.	CERI provided raw data on edge effects from the CERI laboratory.	See Paragraph 4.6 in appendix 7
vii.	CERI submitted the antagonist assay protocol (SOP) and raw data for consideration by the panel. (See appendix 7-1).	See Appendix7-1
viii.	For the negative substances used, information and justification was provided by CERI on solubility and the maximum concentration used.	See Paragraph 4.2 in appendix 7
ix.	Data analysis proposal from Dr Yutaka Aoki and subsequent discussion from and response to NICEATM consultant statistician Dr Joe Haseman, and Dr Sebastian Hoffman (ECVAM). (Appendix 7-3)	See Appendix 3
x.	Assistance from Dr Aoki to CERI in conducting statistical estimations of between- and within-run (laboratory) variation (provisionally in June 2006).	See Appendix 6 "Independent statistical analyses for inter-laboratory validation study" in the validation report