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Report of Pre-validation and Inter-laboratory Validation Studies For Androgen Receptor (AR) Mediated Stably Transfected Transcriptional Activation (AR-STTA) Assay to Detect Androgenic and Anti-androgenic Activities of Chemicals: AR EcoScreenTM

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Series on Testing and Assessment

No. 241

REPORT OF PRE-VALIDATION AND INTER-LABORATORY VALIDATION STUDIES FOR ANDROGEN RECEPTOR (AR) MEDIATED STABLY TRANSFECTED TRANSCRIPTIONAL ACTIVATION (AR-STTA) ASSAY TO DETECT ANDROGENIC AND ANTI-ANDROGENIC ACTIVITIES OF CHEMICALS: AR ECOSCREEN™

IOMC
INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS
A cooperative agreement among FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD

Environment Directorate
ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT
Paris 2016

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or contact:

OECD Environment Directorate,
Environment, Health and Safety Division
2, rue André-Pascal
75775 Paris cedex 16
France

Fax : (33-1) 44 30 61 80

E-mail : ehscont@oecd.org

FOREWORD

This document is the report of the first and second validation studies for the Androgen Receptor Mediated Stably Transfected Transcriptional Activation (AR-STTA) Assay to Detect Androgenic and Anti-androgenic Activities of Chemicals: AR EcoScreen™. This validation report supported the development of a new Test Guideline (TG), TG 458. The project for the development of this new TG was proposed by Japan and included in the work plan of the Test Guidelines Programme in 2008.

The first part of the validation report was developed in December 2010. The peer review subsequently conducted recommended that further validation be performed, using a few more chemicals, before developing a TG. To address the recommendation of the peer review, Japan conducted additional validation studies, using additional chemicals and the second validation report was developed in 2015. This additional validation report was then reviewed by a subgroup of the Validation Management Group for Non Animal Testing (VMG NA).

The second validation report was sent to the Working Group of the National Coordinators of the Test Guidelines Programme (WNT) for review and commenting in July 2015 and in December 2015, together with the draft TG on Stably Transfected Human Androgen Receptor Transcriptional Activation Assay for Detection of Androgenic Agonist and Antagonist Activity. The validation report and the new draft TG were discussed at the meeting of the VMG NA on 1-3 December 2015 (Budapest, Hungary). No comment was received on the validation report following the second circulation of the document in December 2015 and the validation report was subsequently approved by the WNT at its 28th meeting in April 2016. The Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology agreed to declassification of the validation report on 8 July 2016.

This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology.

Report of Pre-validation and Inter-laboratory Validation Studies For Androgen Receptor (AR) Mediated Stably Transfected Transcriptional Activation (AR-STTA) Assay to Detect Androgenic and Anti-androgenic Activities of Chemicals: AR EcoScreen™

First and second validation reports

Report of the first studies:

Pre-validation and Inter-laboratory Validation Studies For Androgen Receptor (AR) Mediated Stably Transfected Transcriptional Activation (AR-STTA) Assay to Detect Androgenic and Anti-androgenic Activities of Chemicals

- The Human Androgen Receptor Mediated Reporter Gene Assay Using AR-EcoScreen™ cells-

Prepared by

Chemicals Evaluation and Research Institute, Japan

2010

TABLE OF CONTENTS

ACRONYMS

AR	Androgen Receptor
ARE	Androgen Responsive Element
CERI	Chemicals Evaluation and Research Institute (Japan)
CV	Coefficient of Variation
DCC-FBS	Dextran-Coated Charcoal-treated Fetal Bovine Serum
DHT	5α-Dihydrotestosterone
DIP	Data Interpretation Procedure
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
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
ER	Estrogen Receptor
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
hAR	Human Androgen Receptor
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods (U.S.)
IC50	Half Maximal (50%) Inhibitory Concentration
JaCVAM	Japanese Centre for the Validation of Alternative Methods
LinearIC30/ LinearIC50	The concentration of chemical estimated to cause 30% or 50% inhibition of the spiked-in (500 pM of DHT) response, respectively, on a plate by plate basis.

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)
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
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

0. EXECTIVE SUMMARY

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 a global concern regarding endocrine disruption effects resulting from chemical exposure, particularly those mediated by the estrogen receptor (ER) and androgen receptor (AR). 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.

2 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 into the OECD Conceptual Framework 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. Only the assay "Stably Transfected Transcriptional Activation (TA) using HeLa-9903 cell line for detecting estrogenic activity of chemicals" has been adopted as OECD test guideline (TG 455) in 2009. Although the need for AR *in vitro* assays are also urgent, at the present time there are no *in vitro* screening assays for androgenic 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 AR mediated **Stably Transfected Transcriptional Activation (TA) Assay (AR-STTA)** using the AR-EcoScreen^{TM} cell line for detecting the androgenic activity of chemicals mediated by the human androgen receptor (hAR) (Araki et al., 2005a; 2005b) for a level 2 screening test in the OECD Conceptual Framework for the Testing and Assessment of endocrine disrupting chemicals (EDCs).

5 In order to develop and validate a test protocol to support the development of test guidelines for the detection of chemicals possessing the potential androgenic and anti-androgenic activity mediated through human androgen receptor (hAR), a series of validation tests for the AR-STTA established in Japan were conducted under the agreement of the 1st OECD VMG-NA meeting that Japan would take lead in this assay.

6 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 hAR mediated stably transfected TA assay conducted in Japan consisted of both pre-validation and inter-laboratory validation. The pre-validation work was conducted at Otsuka Pharmaceutical Co., Ltd. under the direction of 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.

7 The overall goal of the validation efforts for the AR-STTA assay using the AR-EcoScreen™ 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 androgenic activity through hAR.

8 In the pre-validation study, forty compounds recommended by the ICCVAM were tested for AR agonist and antagonist activity using the proposed AR-STTA assay (three-run in triplicate at a single laboratory; developer of the AR-EcoScreen™ system). This trial revealed the highly reproducible outcomes (Araki et al., 2005a).

9 Accordingly, AR-EcoScreen™ method was optimized to be ready to proceed to the phase II pre-validation study assessing the inter-laboratory variability and transfer of the protocol.

10 Also, the results obtained by the AR-STTA assay and the information given in the ICCVAM report (2003) were compared with regard to 40 chemicals. The assay performance parameters for the proposed AR-STTA assay (AR-EcoScreen™), concordance, sensitivity and specificity, were 91.2%, 88.9% and 93.8% for agonist assay and 97.0%, 83.3% and 100% for antagonist assay, respectively.

11 Additionally, in order to evidence the relevance of the assay with the AR mediated effects, the results

from AR-STTA (AR-EcoScreen™) and AR binding assay data were compared using 31 chemicals that was also listed in ICCVAM list, since chemical binding to AR is an event to trigger the transcriptional activation. The rates of concordance, sensitivity and specificity were 77.4%, 91.7% and 28.6%, respectively. It should be noted the following points;

- ✓ An AR binding assay cannot distinguish AR agonist and antagonist,
- ✓ Test concentration range was not same between AR-STTA and AR binding assays,
- ✓ This AR binding assay has not been validated yet,
- ✓ AR binding assay is non-cell based assay and therefore no metabolism can be expected

12 The inter-laboratory validation study was performed with the four participating laboratory using 5 same chemicals for both androgenic and anti-androgenic activities. All chemicals were tested 3 times in triplicates.

13 As for the results of the inter-laboratory validation study, statistical analysis using five coded test chemicals revealed that the variation within four participating laboratories of this assay system appeared to be acceptable for both androgenic and anti-androgenic activities. The results showed that the AR-STTA assay using AR-EcoScreen™ test system is highly reliable and reproducible, so the test system and protocol used in this study is adequately transferable for practical use.

14 Accordingly, the overall assay performance of the stably transfected TA assay system using AR-EcoScreen™ cell line was deemed satisfactory for practical use.

15 The AR-STTA validation study is considered sufficient to meet the requirements under the OECD guidance document 34 (GD34) though the GD34 was published after this validation study. The essences of the validation were considered fulfilled with the combination of the pre-validation study and multi-laboratory validation though the number of chemicals used under multi-laboratory validation study may not be sufficient.

16 A Japanese human AR mediated stably transfected TA assay system using AR-EcoScreen™ 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 androgenic/anti-androgenic activities mediated through hAR. The assay is therefore a promising method to use in the prescreening process of an endocrine disruptor screening strategy.

1. INTRODUCTION

17 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 and AR. 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).

18 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.

19 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.

20 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.

21 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.

22 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.

23 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 androgen receptor is well characterized and well known as a major mediator of androgenic/anti-androgenic effects. Androgenic/anti-androgenic effects may be observed at very low concentrations; therefore a highly sensitive assay method is necessary for hazard assessment.

24 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.

25 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.

26 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 quantify the gene expression induced by

receptor-ligand interaction.

27 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).

28 Prior to the ARTA assay, validation management group have been made extensive effort to establish new OECD test guideline of ERTA assay using HeLa-9903 cell line and the assay system, and it has been approved as OECD TG455 "The Stably Transfected Human Estrogen Receptor-α Transcriptional Activation Assay for Detection of Estrogenic Agonist-Activity of Chemical". This assay system will be expected to contribute many courtiers to have needs for screening endocrine modulating chemical.

29 Validation studies on several *in vitro* TA and ER binding assay methods are currently in progress and some of them are in close to completion or full assessment of their validation process. Although the need for ARTA assay system is also urgent, at the present time there are no *in vitro* screening assays for androgenic/anti-androgenic activities that have been peer reviewed for potential test guideline development, to enable use for OECD regulatory purposes.

30 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 AR-EcoScreen^{TM} cell line for detecting the androgenic/anti-androgenic activities 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.

31 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.

2. OBJECTIVES

32 The overall goal of the validation efforts for the stably transfected TA assay using AR-EcoScreen™ 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 androgenic and anti-androgenic activity through human androgen receptor (hAR).

33 The data obtained from TA assays for agonistic and antagonistic effects are typically analysed to derive the EC50 and IC50 values, respectively, as a biological parameter. These parameters (EC50 and IC50) are calculated by applying an appropriate model equation, such as a logistic equation. For the use of such model equations to calculate the EC50 and IC50 values, 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 using the traditional EC50 and IC50 for agonistic and antagonistic activities, respectively, is not possible. The quantitative explanation is important for providing information about the strength of the potential activity of a test chemical. Therefore, we also employ the PC50 and PC10 values as in the case of ERTA assay using HeLa-9903 cell line other than EC50 and Lin IC30 were also investigated within this validation work.

34 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 and IC50 for agonistic and antagonistic activities, respectively.

3. VALIDATION DESIGN

35 The work of validating the stably transfected TA assay using AR-EcoScreen™ cell line to detect androgenic/anti-androgenic activities consisted of both pre-validation and inter-laboratory validations. The pre-validation work was conducted at Otsuka Pharmaceutical Co., Ltd. under the direction of 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), Japan.

36 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.

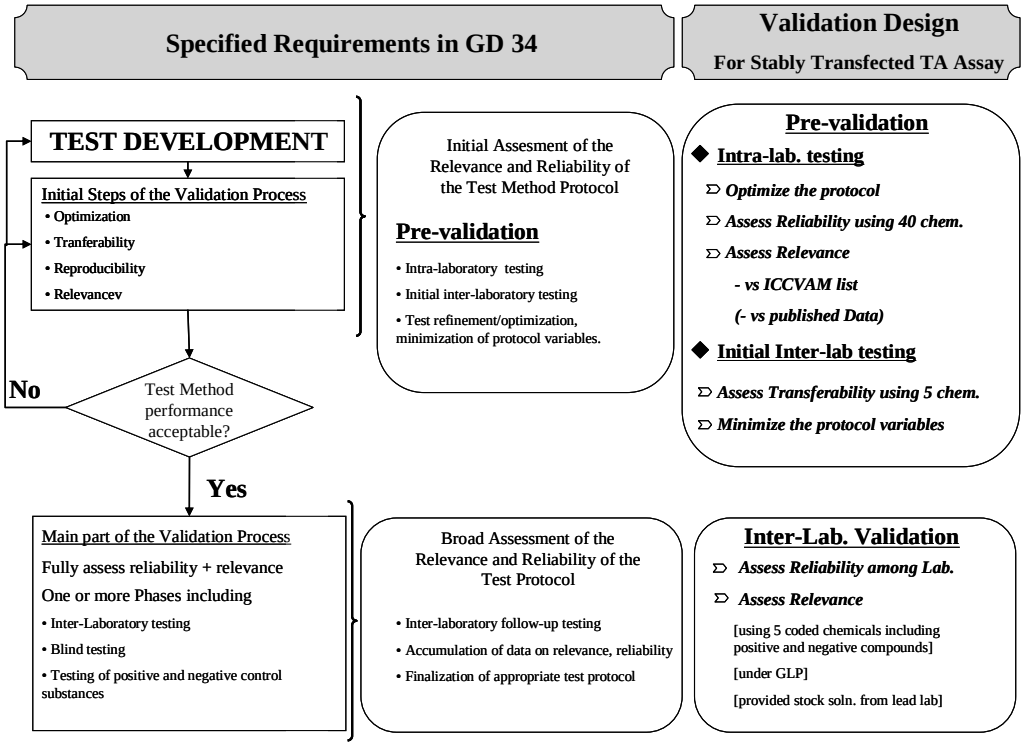


Fig. 1 Validation Design Scheme According to GD34 Specified Requirements.

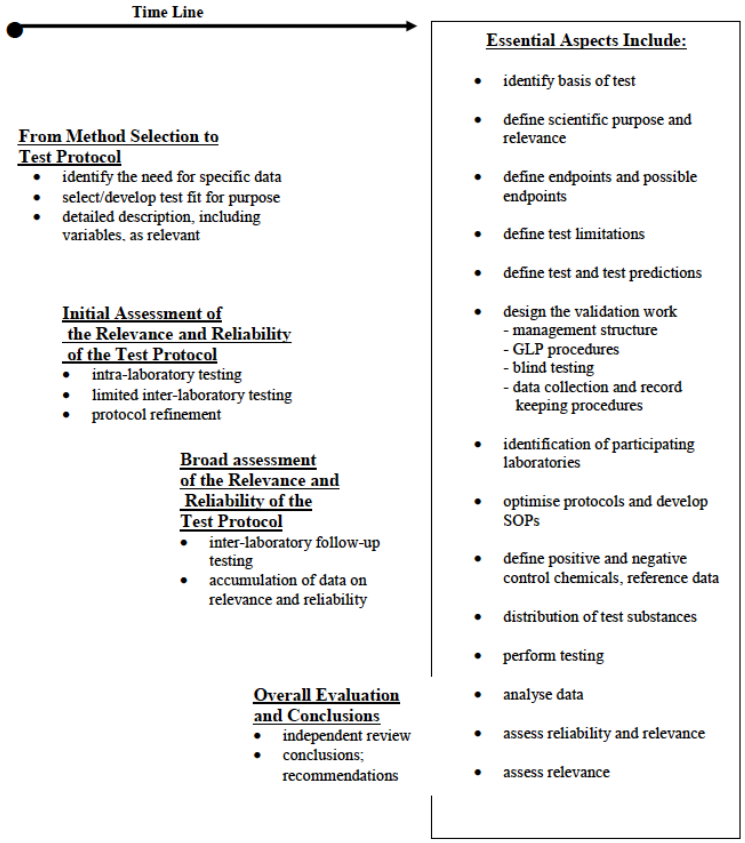


Fig. 2 Assessment Process of the Relevance and Reliability of New or Significantly Revised Testing Methods for Hazard Characterization Specified in GD 34

37 The pre-validation study of stably transfected TA assays using AR-EcoScreen™ cell line 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 in the ICCVAM list of Recommended Substances for Validation of *In Vitro* Androgen Receptor Transcriptional Activation Assays (ICCVAM, 2003).

38 The inter-laboratory validation study was planned by CERI and conducted at CERI's initiative with three other participating laboratories (APPENDIX2). 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.

39 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.

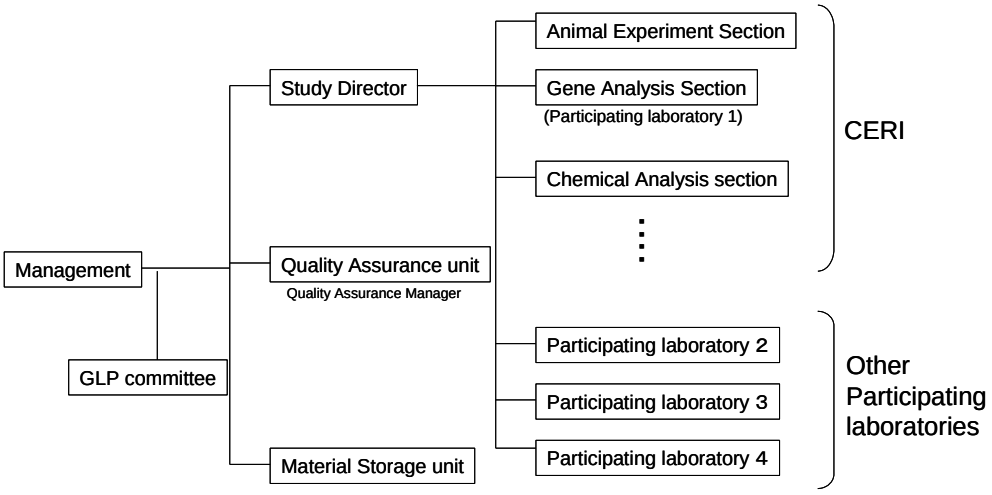


Fig. 3 Organization Schema of CERI GLP System Employed for the Inter-laboratory Validation Study.

40 The inter-laboratory validation study of the stably transfected TA assay using AR-EcoScreen™ 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 androgenic 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/IC50.

41 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 five coded test chemicals with or without androgenic activity in each laboratory. Assay data were gathered in CERI and were analysed with regard to reproducibility of the analytical parameters calculated as EC50, PC50, PC10 and IC50, IC30. 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.35)". Also, the details of IC30 are described in the same section (p.21).

4. TEST METHOD USED

4.1 TEST PROTOCOL

42 The standard operating procedure (SOP) used for the pre-validation study and the protocol used for inter-laboratory validation study are attached in Appendix 3. 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. In the antagonist assay, general cytotoxic compounds could apparently exhibit the reduction of transcriptional activation, thus cytotoxicity assay is additionally required to exclude the non-specific inhibition by test chemicals. In order to evaluate the cytotoxicity, cLuc-EcoScreen™ cell line which constitutively express luciferase gene was used in pre-validation study. In the inter-laboratory validation study, cytotoxicity was evaluated with renilla luciferase activity of AR-EcoScreen™ cell line, which originally established to expresses renilla luciferase constitutively instead of cLuc-EcoScreen™ cell used in the pre-validation. In this AR-EcoScreen™ system, AR-mediated transcriptional activation and cytotoxicity can be evaluated simultaneously in same assay plate, so it contributes to reduce the overall workload. The summary of the protocols is shown in Table 1.

Table 1 Summary of the protocol

Factors	For agonist assay	For antagonist assay
Cell line	AR-EcoScreen™ cell line	AR-EcoScreen™ cell line (cLuc-EcoScreen™ cell line for evaluating cytotoxicity was parallelly used only in the pre-validation phase.)
Cell medium	Dulbeco's modified Eagle's Minimum/Ham's F-12 nutrient mix (DMEM/F12) with 10% fetal bovine serum (FBS)	
Vehicle	*Pre-validation:* Dimethylsulfoxide (DMSO), Ethanol or Distilled Water *Inter-laboratory validation:* DMSO	
Vehicle control (VC)	0.1% of vehicle as final concentration. *Pre-validation:* Six-wells *Inter-laboratory validation:* 6wells	0.1% of vehicle as final concentration *Prev-validation:* 3-wells *Inter-laboratory validation:* 3-wells
Other controls	*Pre-validation:* 10 nM of 5α-dihydrotestosterone (DHT) and R1881 in three-wells *Inter-laboratory validation:* 10 nM of DHT in six-wells	*Pre-validation/Inter laboratory validation:* Spike-in controls (SPK); 12-well Agonist control (PCago; 10 nM of DHT); 3-wells Antagonist control (PCago; SPK +0.1 μM of hydroxyflutamide): 3-wells Cytotoxicity control (PC_{CT}; SPK + 10 μg/mL of cycloheximide); 3-wells
Concentrations tested for test chemicals	*Pre-validation:* Generally, 1 nM, 10 nM, 100 nM, 1 μM, 10 μM, 100 μM and 1mM* *Inter-laboratory validation:* Generally, 10 pM, 100 pM, 1 nM, 10 nM, 100 nM, 1 μM and 10 μM	*Pre-validation:* Generally, 10 nM, 100 nM, 1 μM, 10 μM, 100 μM and 1mM (10^{-3} – 10^{-8} M) *Inter-laboratory validation:* Generally, 100 pM, 1 nM, 10 nM, 100 nM, 1 μM and 10 μM
Cell density	10^4 cells/well	
Incubation time with test	18-20 hours	

Factors	For agonist assay	For antagonist assay
chemicals		
Number of test chemicals within pre-validation	40 chemicals for comparison with data listed as ICCVAM reference chemicals	
Number of test chemicals within inter-laboratory validations	5 coded test chemicals and one reference chemical (DHT)	
Number of assays per chemical	Three-runs of each chemical (in triplicate) on separate days for the pre-validation and inter-laboratory validation study	

* In the agonist assay in the pre-validation study, R1881, testosterone, DHT and methyl testosterone were tested at 1 pM, 10 pM, 100 pM, 1 nM, 10 nM, 100 nM and 1 µM to obtain full dose-response curve.

4.1.1 Cell line (stable clone: AR-EcoScreen™ cells)

43 AR-EcoScreen™ cell line is an androgen responsive stable transformant derived from a CHO-K1 cell line. This cell line was established by Otsuka Pharmaceutical Co. as follows: CHO-K1 cells (CHO-K1; ATCC No. CCL-61) purchased from ATCC through Dai-Nippon Pharmaceutical Company (Osaka, Japan) were stably transfected with both plasmids human AR expression vector and a firefly luciferase reporter vector bearing four tandem repeats of androgen responsive element (ARE) from prostate C3 gene-responsive element driven by a minimal heat shock protein promoter (Kojima et al. 2003).

Enhancer (ARE)	5'-AGTACGTGATGTTCT -3'

44 The human AR expression vector was generated by insertion of a RT-PCR amplified full-length of human AR cDNA, with an efficient Kozak's translation initiator sequence, from a commercial human prostate mRNA (Clontech, Palo Alto, CA), into the pZeoSV2 vector (Invitrogen, San Diego, CA).

45 Functional ERα, ERβ, AR, TRα and TRβ could not be detected in the host cell (CHO-K1; ATCC No. CCL-61), when tested by mock transfection assays with each hormone responsive reporter construct. Further, the established cell line, AR-EcoScreen™, was confirmed to be free of any mycoplasma infection.

46 It was confirmed that C3 promoter has no responsiveness to GR and that no functional GR was observed by applying GR ligand in the assay system.

47 The AR-EcoScreen™ cell line was obtained from Otsuka Pharmaceutical Co., Ltd. and then distributed to each participating laboratory by CERI for the validation study.

48 This cell line is currently has been deposited in the Health Science Research Resources Bank

(HSRRB) in Japan and it can be distributed worldwide as reference No. JCRB1328.

49 This cell can be used for commercial purpose, such as assays in CRO (Contact Research Organization) after independent contract with the cell depositor, Otsuka Pharmaceutical Ltd.

4.1.2 Medium

50 Dulbeco's modified Eagle medium/Ham's F-12 nutrient mix (DMEM/F12, Invitrogen), supplemented with a 10% dextran-coated-charcoal-treated fetal bovine serum (DCC-FBS) was used for the assay.

4.1.3 Chemical exposure to cells

4.1.3-.1) For pre-validations

51 Stock solutions (1000x) for each chemical were prepared in solvent. The maximum test concentration was set as 1 mM when 1M stock solution can be prepared. Solubility of test chemicals in the solvent (DMSO, water and ethanol) were determined before conducting the test. All chemicals except sodium azide and 17α-ethinylestradiol were dissolved in dimethysulfoxide (DMSO) at 10^{-2} to 1M depending on the solubility. Sodium azide and 17α-ethinyl estradiol was dissolved in distilled water and ethanol, respectively, following chart. The final concentration of DMSO or ethanol in the assay medium was 0.1%, which did not affect the cells.

52 In agonist assay, seven test concentrations were generally set at 1 mM, 100 µM, 10 µM, 1 µM, 100 nM, 10 nM, and 1 nM (10^{-9}-10^{-3}M). In the antagonist assay, six test concentrations were used (1 mM, 100 µM, 10 µM, 1 µM, 100 nM and 10 nM; 10^{-8}-10^{-3}M).

53 In order to prepare the desired concentrations of test chemicals for measuring agonistic activity, the stock solution was first serially diluted in common ratios of 10 with the solvent used. Then, these diluted samples in the solvent were further diluted with serum-free DMEM to prepare ten-fold concentrations of the desired test concentrations. Lastly, the desired test concentrations in triplicate were prepared by adding 10 µL of each sample solution to each well of the assay plates, containing $1x10^4$ cells/well/90 µL as illustrated in the assignment table (Table 2 and Table 3). For antagonist assay, test chemicals were diluted with medium containing DHT at 500 pM as final concentration.

54 In the agonist assay, positive control wells (n=3) treated with a natural ligand (10 nM of DHT) as agonist positive control-1 (PC_{AGO}-1), synthetic ligand (10 nM of R1881) as agonist positive control-2

(PC_{AGO}-2) and vehicle control wells (n=6) treated with DMSO (0.1%) alone were prepared on every assay plate. In the antagonist assay, , an antagonist (0.1 μM of hydroxyflutamide) as antagonist positive control (PC_{ATG}), a cytotoxic compound (10 μg/mL of cycloheximide) as cytotoxicity positive control (PC_{CT}), vehicle control wells treated with DMSO (0.1%) alone and vehicle control wells (n=6) treated with 500pM DHT were prepared on every assay plate.

55 The spike in concentration in the antagonist assay was set at 500pM, which was 80% of maximum induction of the full-dose response curve of DHT.

56 After adding the chemicals for both agonist and antagonist assays, the assay plates were incubated in a 5% CO_2 incubator at 37±1ºC for 20-24 hours to induce the reporter gene products.

Table 2 Plate Dose Assignment Table for Agonist Assay: Pre-validation Study

	Chemical 1			Chemical 2			Chemical 3			Chemical 4		
	1	2	3	4	5	6	7	8	9	10	11	12
A	1 mM*	→	→	→	→	→	→	→	→	→	→	→
B	100 μM	→	→	→	→	→	→	→	→	→	→	→
C	10 μM	→	→	→	→	→	→	→	→	→	→	→
D	1 μM	→	→	→	→	→	→	→	→	→	→	→
E	100 nM	→	→	→	→	→	→	→	→	→	→	→
F	10 nM	→	→	→	→	→	→	→	→	→	→	→
G	1 nM	→	→	→	→	→	→	→	→	→	→	→
H	VC	→	→	→	→	→	PC_{AGO}-1	→	→	PC_{AGO}-2	→	→

VC: Vehicle control (DMSO at 0.1%);
PC_{AGO}-1: Positive control (10 nM of DHT);
PC_{AGO}-2: Positive control (10 nM of R1881);
* Starting dose is from 1mM to 10 μM, depending on the maximum solubility of each compound.

Table 3 Plate Dose Assignment Table for Antagonist Assay: Pre-validation Study

	Chemical 1			Chemical 2			Chemical 3			Chemical 4		
	1	2	3	4	5	6	7	8	9	10	11	12
A	1 mM*	→	→	→	→	→	→	→	→	→	→	→
B	100 µM	→	→	→	→	→	→	→	→	→	→	→
C	10 µM	→	→	→	→	→	→	→	→	→	→	→
D	1 µM	→	→	→	→	→	→	→	→	→	→	→
E	100 nM	→	→	→	→	→	→	→	→	→	→	→
F	10 nM	→	→	→	→	→	→	→	→	→	→	→
G	SPK	→	→	→	→	→	→	→	→	→	→	→
H	VC	→	→	PC1	→	→	PC_{ATG}	→	→	PC_{CT}	→	→

VC: Vehicle control (DMSO at 0.1%);
PC1: Positive control (10 nM of R1881);
PC_{ATG}: Positive control (0.1 µM of hydroxyflutamide) ;
PC_{CT}: Positive control (10 µg/mL of cycloheximide);
SPK (DMSO at 0.1% spiked with 500p M DHT)
* Starting dose is from 1mM to 10 µM, depending on the maximum solubility of each compound.
** Gray colored wells spiked with $5x10^{-10}$M DHT

4.1.3-.2) For inter-laboratory validations

57 The stock solutions of test chemicals were prepared at 10 mM with DMSO at CERI, where they were coded and then provided to each participating laboratory.

58 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, 1 µM and 100 nM for agonist assay. 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 agonist assay

59 For antagonist assay, DMSO dilutions were generally set at 1 mM, 100 µM, 10 µM and 1 µM. The DMSO dilutions were further diluted with spike-in serum-free EMEM containing 500 pM DHT as final concentration to bring 10 µM, 1 µM, 100 nM, 10 nM, 1 nM and 100 pM (10^{-10}-10^{-5}M) for antagonist assay in each assay plate in triplicate.

60 On the basis of sensitivity of the assay system, the concentration range to be tested was generally set at 10^{-11}-10^{-5}M. The assay system can detect androgenic activity of well-known androgenic chemicals in this concentration range. 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.

61 A full dose response range of DHT was assigned in all assay plates to monitor the accuracy of

chemical dilution procedure in the inter-laboratory study.

62 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.

63 For agonist assay, positive control wells (n=6) treated with a natural ligand (10 nM of DHT) and vehicle control wells (n=6) treated with DMSO alone, were prepared on every assay plate. For antagonist assay, vehicle control (no spike-in, n=3), positive control for agonistic activity (PCago, 10 nM of DHT: n=3), positive control for antagonistic activity (PCatg, 0.1 μM of hydroxyflutamide, n=3), cytotoxicity control (CX, 10 μg/mL of cycloheximide, n=3) and spike-in control (500 pM of DHT) were set in each assay plate.

64 After adding the chemicals for both agonist and antagonist assays, the assay plates were incubated in a 5% CO_2 incubator at 37±1ºC for 20-24 hours to induce the reporter gene products.

65 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 for agonist, Table 5 for antagonist).

66 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 AR-EcoScreen™ cell line did not show any edge effects that would affect the assay results for practical use since no significant differences were observed between 36 edge wells and 60 center wells.

Table 4 Plate Dose Assignment Table for agonist assay:

Inter-laboratory Validation Study

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

VC: Vehicle control (DMSO);
BL: Blank;
PC: Positive control (10 nM of DHT)

Table 5 Plate Dose Assignment Table for antagonist assay:

Inter-laboratory Validation Study

	Chemical 1			Chemical 2			Chemical 3			DHT		
	1	2	3	4	5	6	7	8	9	10	11	12
A	10 µM	→	→	→	→	→	→	→	→	1µM	→	→
B	1 µM	→	→	→	→	→	→	→	→	100 nM	→	→
C	100 nM	→	→	→	→	→	→	→	→	10 nM	→	→
D	10 nM	→	→	→	→	→	→	→	→	1 nM	→	→
E	1 nM	→	→	→	→	→	→	→	→	100 pM	→	→
F	100 pM	→	→	→	→	→	→	→	→	10 pM	→	→
G	SPK	→	→	→	→	→	→	→	→	→	→	→
H	VC	→	→	PC_{AGO}	→	→	PC_{ATG}	→	→	PC_{CT}	→	→

VC: Vehicle control (DMSO);
PC_{AGO}: Positive control (10 nM of DHT);
PC_{ATG}-: Positive control (0.1 µM of hydroxyflutamide);
PC_{CT}: Positive control (10 µg/mL of cycloheximide);
SPK (DMSO at 0.1% spiked with 500pM DHT)
** Gray colored wells spiked with 500pM DHT

4.1.4 *Reagent for stably transfected TA assays and detection instrument*

67 Steady-Glo Luciferase Assay System (Promega, E2520) was used in the pre-validation study. Dual-Glo luciferase Assay System (Promega, E2920) or flush type Dual luciferase Assay System (Promega, E1910) was used in the antagonist assay in the inter-laboratory validation study to detect cytotoxicity by renilla luciferase activity.

4.1.5 *Test chemical*

4.1.5-.1) Dose selection for inter-laboratory validation study

68 The test concentration range employed in this assay was determined based upon the results from the pre-validation study, whilst also ensuring that there were no problems with solubility and cytotoxicity of test substances.

4.1.5-.2) Selection of test chemicals

(ア) For pre-validation

69 To demonstrate the relevance of the assay system in detecting androgenic and anti-androgenic activity, 40 chemicals 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 6 Chemicals Used for the Pre-validation of the AR-EcoScreen™ for both androgenic and anti-androgenic activities

Number	Chemical	CAS no.	Purity (%)	Supplier
1	Diethylstilbestrol	56-53-1	97	Sigma
2	Methyltrienolone (R1881)	965-93-5	97	Daiichi Chem
3	Cyproterone acetate	427-51-0	99.98	Sigma
4	Fluoxymestrone	76-43-7	97	Kanto
5	Dexamethasone	50-02-2	99	Wako
6	17β-Estradiol	50-28-2	97	Wako
7	Flutamide	13311-84-7	98	Sigma
8	Medroxyprogesterone acetate	71-58-9	98	Wako
9	Testosterone	58-22-0	97	Wako
10	4-Androstenedione	63-05-8	98	Sigma
11	Di - *n* - butyl phthalate	84-74-2	99.5	Wako
12	Diethylhexyl phthalate	117-81-7	99.5	Wako
13	5α-Dihydrotestosterone	521-18-6	95	Wako
14	Estrone	53-16-7	98	Wako
15	Linuron	330-55-2	99.5	Wako
16	*p,p'* - Methoxychlor	72-43-5	97	Wako
17	Spironolactone	52-01-7	97	Sigma
18	Sodium azide	26628-22-8	99	Wako
19	4 - *tert* - Octylphenol	140-66-9	98	Wako
20	Procymidone	32809-16-8	99.5	Wako
21	*p* - n -Nonylphenol	104-40-5	98.7	Wako
22	Bisphenol A	80-05-7	99	Wako
23	Progesterone	57-83-0	98	Wako
24	*p,p'* - DDE	72-55-9	99	Wako
25	Finasteride	98319-26-7	99	LKT labo
26	Hydroxyflutamide	52806-53-8	100	LKT labo
27	4-Hydroxytamoxifen	68047-06-3	98	Sigma
28	Actinomycin D	50-76-0	97	Wako
29	Vinclozolin	50471-44-8	99	Wako
30	Atrazine	1912-24-9	98	Wako
31	Mifepristone	84371-65-3	99.3	Wako
32	Fluoranthene	206-44-0	98	Wako
33	Kepone	143-50-0	NA	AccuStandard
34	*o,p'* - DDT	789-02-6	99	AccuStandard
35	Corticosterone	50-22-6	95	Wako
36	17α-Ethinyl estradiol	57-63-6	99	ICN
37	Ketoconazole	65277-42-1	99	Wako
38	Methyl testosterone	58-18-4	97	Wako
39	12 - *O* - Tetradecanoylphorbol-13-acetate	16561-29-8	99	Wako
40	2,4,5-Trichlorophenoxyacetic acid	93-76-5	98	Wako

Sigma : Sigma Chemical Co. (Sigma-Aldrich corp.)
Daiichi Chem: Daiichi Chemical Co., Ltd.
Kanto : Kanto Chemical Co., Inc.
Wako : Wako Pure Chemical Industries, Ltd.
AccuStandard : AccuStandard Inc.
ICN : ICN Chemicals, Inc.
LKT lab. : LKT Laboratories, Inc.

(イ) For inter-laboratory validations

70 For the inter-laboratory validation study in order to evaluate the protocol transferability among laboratories and to evaluate the relevance of the assay system, five chemicals shown in Table 7 that exhibit a wide range of strength of androgenic and anti-androgenic activity and one negative within the test concentration range from 10^{-11}-10^{-5}M were selected.

71 It should be noted that the number of known androgenic or anti-androgenic chemicals that were clearly demonstrated were very limited at the time of chemical selection for inter-laboratory validations. Therefore the appropriate number of chemicals and range of strength were hardly identifiable.

72 Under such conditions, test chemicals used for inter-laboratory validation were selected based on the following views;

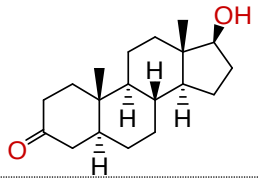
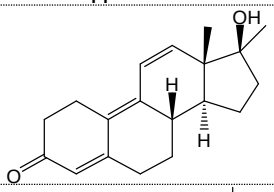
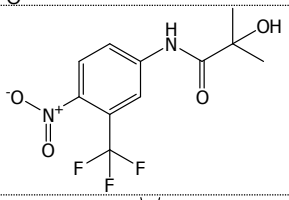
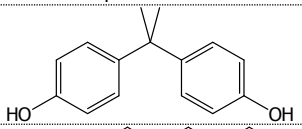
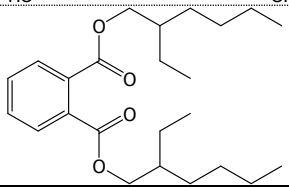
- A set of chemicals that can evaluate both AR agonistic and antagonistic activities for efficacy
- Test chemicals were desired to have no bi-functional activities with other nuclear receptors.
- The available knowledge on weak AR antagonists was limited and therefore bisphenol A that was known as AR antagonist in the internal study was selected as a negative for AR agonist and a weak positive for AR antagonist.

73 Moreover, the full dose response range of DHT 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.

4.1.5-.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. Platelayout of a series of concentrations of test chemicals were arbitrarily placed by each participating laboratory .

Table 7 Chemicals Used for Inter-laboratory Validation Study for androgenic and anti-androgenic activities

Name	CAS No.	Structure	Note
5α-Dihyrotestosterone DHT	521-18-6		Known as AR agonist
Methyltrienolone	965-93-5		Known as AR agonist
Hydroxyflutamide	52806-53-8		Known as AR antagonist
Bisphenol A	80-05-7		known as a weak AR antagonist
Di(2-ethylhexyl) phthalate	117-81-7		known as a negative for agonist and antagonist

4.1.6 *Method for Evaluation of Cytotoxicity*

75 In the antagonist assay, cytotoxic compounds could apparently reduce the AR mediated transcriptional activation due to its cytotoxic effects, thus additional assay is required to exclude the non-specific inhibition of the test chemical. To evaluate the cytotoxicity caused by test compounds, cLuc-EcoScreen™ cell, which was stably transfected luciferase gene (pc DNA luc) under the CMV promoter without induction into CHO-K1 cell (Satoh et al., 2004), was used during the pre-validation study. However, this approach requires an additional assay plate that was concurrently prepared for cytotoxicity evaluation. In order to make the assay procedure simple and easy, the AR-EcoScreen™ cells constitutively expresses the *Renilla* luciferase was developed (Araki et al, 2005a, 2005b). This cell line can conveniently evaluate both AR mediated effects and cytotoxicity effects by test chemicals in the same assay plate. The assay principle using cLuc-EcoScreen™ was substantially identical to that of the AR-EcoScreen™ cells to evaluate AR mediated effects, except for the use of

Renilla luciferase substrate to evaluate cytotoxicity effects. If a compound shows >20% reduction of relative cell viability, the compounds predicted to be cytotoxic at the tested concentration and data at the concentration where the renilla luciferase activity showed over 20% reduction is excluded from the calculation of linear IC30 and linearIC50 in anti-androgenic assay.

4.2 DATA RECORDING AND ANALYSES

76 The luminescence signal data as read by a luminometer were processed, and the average for the vehicle control (V.C.) wells was calculated. A fold induction of Positive control (10 nM DHT) was 8.88±0.52, as a historical data at the assay developer. 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.

77 In the multi-laboratory validation study, when PC10 for agonist assay or lin.IC30 for antagonist assay were derived, the chemical was regarded as positive, respectively, in each run. (Rationale of this criteria: See "0

78 Details about PC10 and lin.IC30 can be found in the following section. Also the justification for the use of the PC10 and lin.IC30 values is provided in paragraph 92 for agonist assay and paragraph 106 for antagonist assay.

4.2.1 *Calculation of EC50, PC values and lin. IC values*

79 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 analysed 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.

80 Furthermore, the PC values (e.g., PC50, PC10) were also calculated. For example, the PC50 and PC10 values were defined as the test chemical concentrations estimated to elicit either a 50% or a

10% transcription activity of the positive control (PC) response (10 nM of 5α-dihydrotestosterone (DHT)) 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).

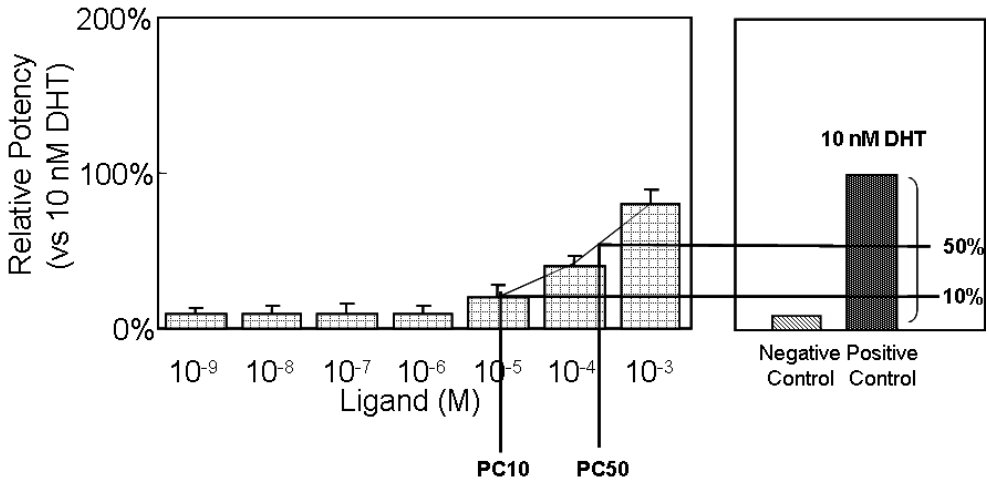


Fig. 4 Definition of PC50 and PC10 Values

81 Furthermore, the lin.IC50 and lin.IC30 values were also calculated. These lin.IC50 and lin.IC30 values were defined as the test chemical concentrations estimated to elicit either a 50% or a 30% inhibition of transcriptional activity induced by 500 pM DHT. Each IC value was calculated by a simple linear regression using two variable data points in the transcription activity (Fig. 5).

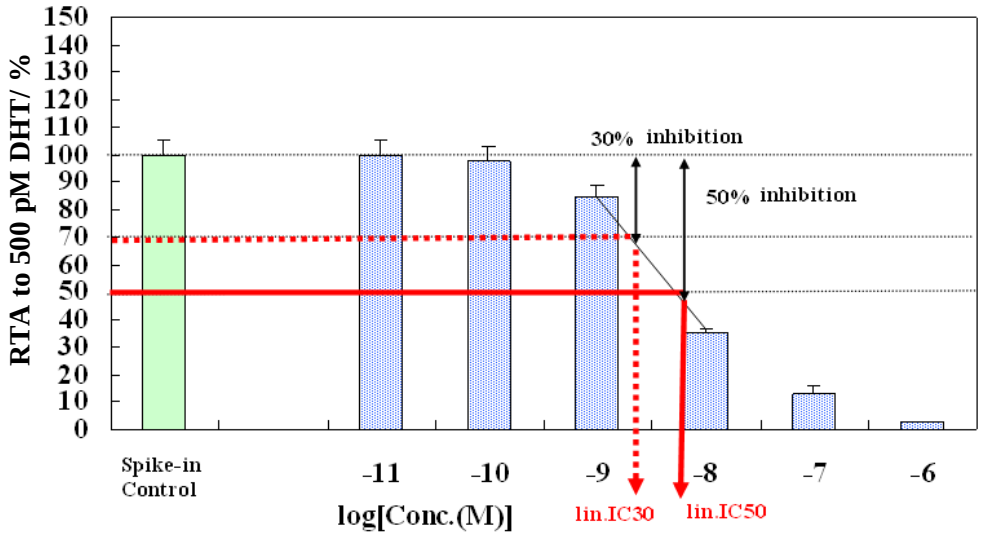
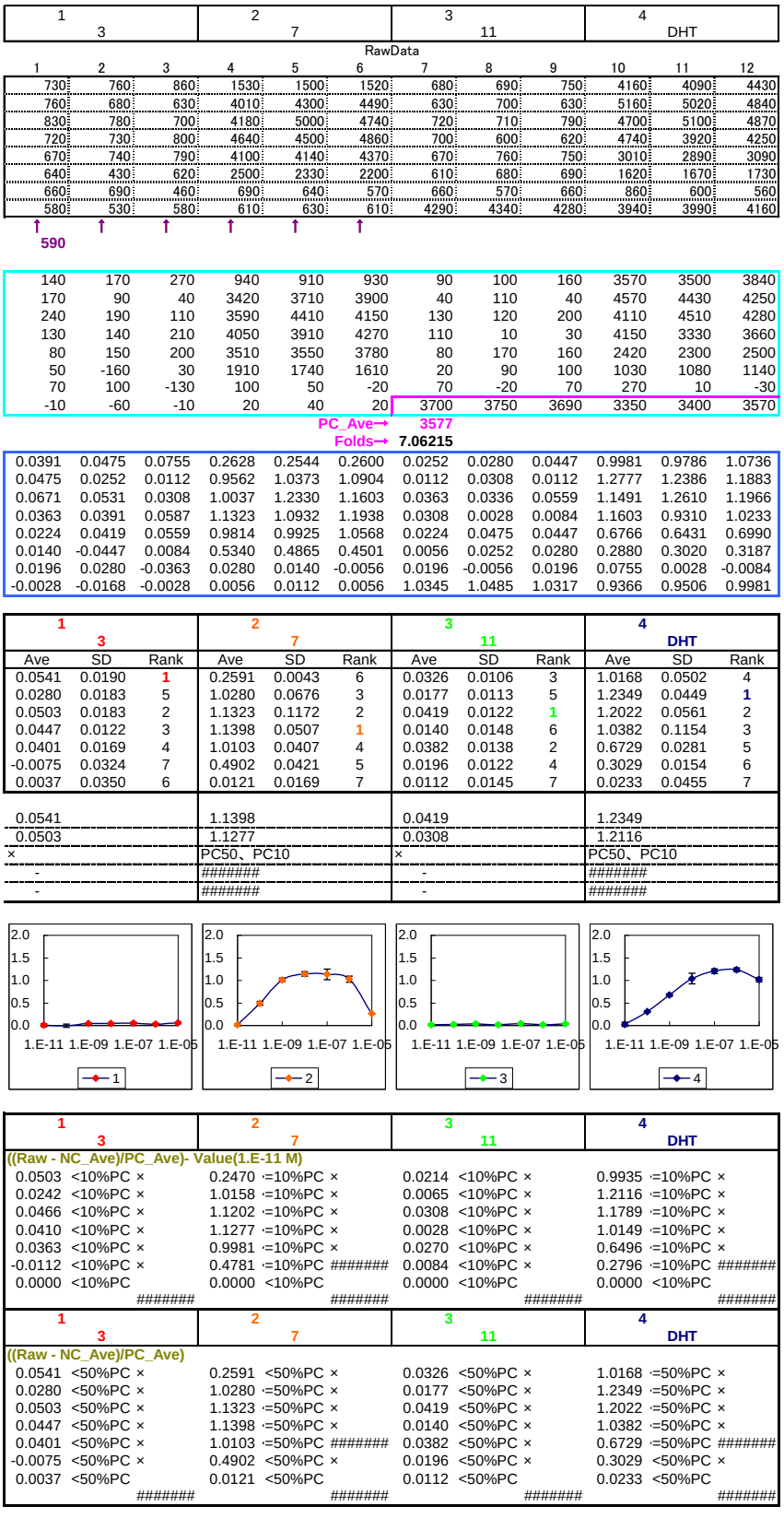


Fig. 5 Definition of lin.IC50 and lin.IC30

4.2.2 *Data Spreadsheet used in the Multi-laboratory Validation Study*

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

Fig. 6 Example of the excel spread sheet for processing raw data (Prepared by CERI)



1			2			3			4		
3			7			11			DHT		
RawData											
1	2	3	4	5	6	7	8	9	10	11	12
730	760	860	1530	1500	1520	680	690	750	4160	4090	4430
760	680	630	4010	4300	4490	630	700	630	5160	5020	4840
830	780	700	4180	5000	4740	720	710	790	4700	5100	4870
720	730	800	4640	4500	4860	700	600	620	4740	3920	4250
670	740	790	4100	4140	4370	670	760	750	3010	2890	3090
640	430	620	2500	2330	2200	610	680	690	1620	1670	1730
660	690	460	690	640	570	660	570	660	860	600	560
580	530	580	610	630	610	4290	4340	4280	3940	3990	4160
↑	↑	↑	↑	↑	↑						
590											
140	170	270	940	910	930	90	100	160	3570	3500	3840
170	90	40	3420	3710	3900	40	110	40	4570	4430	4250
240	190	110	3590	4410	4150	130	120	200	4110	4510	4280
130	140	210	4050	3910	4270	110	10	30	4150	3330	3660
80	150	200	3510	3550	3780	80	170	160	2420	2300	2500
50	-160	30	1910	1740	1610	20	90	100	1030	1080	1140
70	100	-130	100	50	-20	70	-20	70	270	10	-30
-10	-60	-10	20	40	20	3700	3750	3690	3350	3400	3570
				PC_Ave→		3577					
				Folds→		7.06215					
0.0391	0.0475	0.0755	0.2628	0.2544	0.2600	0.0252	0.0280	0.0447	0.9981	0.9786	1.0736
0.0475	0.0252	0.0112	0.9562	1.0373	1.0904	0.0112	0.0308	0.0112	1.2777	1.2386	1.1883
0.0671	0.0531	0.0308	1.0037	1.2330	1.1603	0.0363	0.0336	0.0559	1.1491	1.2610	1.1966
0.0363	0.0391	0.0587	1.1323	1.0932	1.1938	0.0308	0.0028	0.0084	1.1603	0.9310	1.0233
0.0224	0.0419	0.0559	0.9814	0.9925	1.0568	0.0224	0.0475	0.0447	0.6766	0.6431	0.6990
0.0140	-0.0447	0.0084	0.5340	0.4865	0.4501	0.0056	0.0252	0.0280	0.2880	0.3020	0.3187
0.0196	0.0280	-0.0363	0.0280	0.0140	-0.0056	0.0196	-0.0056	0.0196	0.0755	0.0028	-0.0084
-0.0028	-0.0168	-0.0028	0.0056	0.0112	0.0056	1.0345	1.0485	1.0317	0.9366	0.9506	0.9981

1			2			3			4		
3			7			11			DHT		
Ave	SD	Rank	Ave	SD	Rank	Ave	SD	Rank	Ave	SD	Rank
0.0541	0.0190	1	0.2591	0.0043	6	0.0326	0.0106	3	1.0168	0.0502	4
0.0280	0.0183	5	1.0280	0.0676	3	0.0177	0.0113	5	1.2349	0.0449	1
0.0503	0.0183	2	1.1323	0.1172	2	0.0419	0.0122	1	1.2022	0.0561	2
0.0447	0.0122	3	1.1398	0.0507	1	0.0140	0.0148	6	1.0382	0.1154	3
0.0401	0.0169	4	1.0103	0.0407	4	0.0382	0.0138	2	0.6729	0.0281	5
-0.0075	0.0324	7	0.4902	0.0421	5	0.0196	0.0122	4	0.3029	0.0154	6
0.0037	0.0350	6	0.0121	0.0169	7	0.0112	0.0145	7	0.0233	0.0455	7
0.0541			1.1398			0.0419			1.2349		
0.0503			1.1277			0.0308			1.2116		
×			PC50、PC10			×			PC50、PC10		
-			#######			-			#######		
-			#######			-			#######		

1				2				3				4			
3				7				11				DHT			
((Raw - NC_Ave)/PC_Ave)- Value(1.E-11 M)															
0.0503	<10%PC	×		0.2470	=10%PC	×		0.0214	<10%PC	×		0.9935	=10%PC	×	
0.0242	<10%PC	×		1.0158	=10%PC	×		0.0065	<10%PC	×		1.2116	=10%PC	×	
0.0466	<10%PC	×		1.1202	=10%PC	×		0.0308	<10%PC	×		1.1789	=10%PC	×	
0.0410	<10%PC	×		1.1277	=10%PC	×		0.0028	<10%PC	×		1.0149	=10%PC	×	
0.0363	<10%PC	×		0.9981	=10%PC	×		0.0270	<10%PC	×		0.6496	=10%PC	×	
-0.0112	<10%PC	×		0.4781	=10%PC	#######		0.0084	<10%PC	×		0.2796	=10%PC	#######	
0.0000	<10%PC			0.0000	<10%PC			0.0000	<10%PC			0.0000	<10%PC		
		#######				#######				#######				#######	
1				2				3				4			
3				7				11				DHT			
((Raw - NC_Ave)/PC_Ave)															
0.0541	<50%PC	×		0.2591	=50%PC	×		0.0326	<50%PC	×		1.0168	=50%PC	×	
0.0280	<50%PC	×		1.0280	=50%PC	×		0.0177	<50%PC	×		1.2349	=50%PC	×	
0.0503	<50%PC	×		1.1323	=50%PC	×		0.0419	<50%PC	×		1.2022	=50%PC	×	
0.0447	<50%PC	×		1.1398	=50%PC	×		0.0140	<50%PC	×		1.0382	=50%PC	×	
0.0401	<50%PC	×		1.0103	=50%PC	#######		0.0382	<50%PC	×		0.6729	=50%PC	#######	
-0.0075	<50%PC	×		0.4902	<50%PC	×		0.0196	<50%PC	×		0.3029	<50%PC	×	
0.0037	<50%PC			0.0121	<50%PC			0.0112	<50%PC			0.0233	<50%PC		
		#######				#######				#######				#######	

4.2.3 *Comparison with chemicals listed in ICCVAM Report (2003)*

83 In order to evaluate the assay relevance of the proposed stably transfected TA assay system (i.e., AR-EcoScreen™), following parameters for 40 chemicals were examined by two-by-two table analysis. The results listed in the ICCVAM Recommended Substances for Validation of In Vitro AR TA Agonism Assays were used as a reference assay data because there is no "gold standard" test method are available.

- accuracy (concordance): [a+d]/[a+b+c+d]
- Sensitivity: a/[a+c]
- Specificity: d/[b+d]

		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

84 For the two-by-two analysis to ensure the relevance of the assay, PC5, PC10, PC20, PC30, PC40 and PC50 were also calculated for chemicals listed in ICCVAM list. In this analysis, the best PC parameter to distinguish positive or negative as an AR agonist was also investigated.

85 For the two-by-two analysis to ensure the relevance of the assay, lin.IC30, lin.IC40 and lin.IC50 were also calculated for chemicals listed in ICCVAM list. The best lin.IC parameter was also investigated in this analysis.

4.2.4 *Comparison with AR Binding Data*

86 The results obtained by AR-EcoScreen™ were compared to the results obtained from a receptor binding assay using recombinant human AR (hAR) as supplemental information. The 31 chemicals selected from ICCVAM list were used for this comparison.

87 The hAR receptor binding assay was performed as follows: a solution (10 μL, final conc. 0.2 nM) of approximately 10 nM of recombinant human androgen receptor ligand binding domain fused with MBP expressed, 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 [1,2,4,5,6,7-^{3}H]

5α-Dihydrotestosterone (DHT) (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 DHT was calculated. Any chemicals possessing RBA values were defined as positive chemicals in the receptor binding assays.

5. RESULTS and DISCUSSIONS

5.1 Stability of response of AR-EcoScreen™ cell line

88 We monitored the response of AR-EcoScreen™ cell line on each plate using control wells containing 10 nM M methyltrienolone (R1881). The average response of each passage over 3 months of continuous culture was plotted in Fig. 7. Although the absolute light units of the response varied from 6,500 to 12,000 counts per second, and corresponding values for the solvent controls varied from 875 to 1,285 counts per second, the normalized fold-increase values remained within a range of 7.5 to 9.2. Thus, luciferase activity produced by the well known AR agonist (R1881) did not appear to decrease over prolonged periods, demonstrating stable integration of the reporter plasmid in the AR-EcoScreen™ cell line.

Outputs: The cell cultured at least less than 44 passages (within 3 month) can be used for testing.

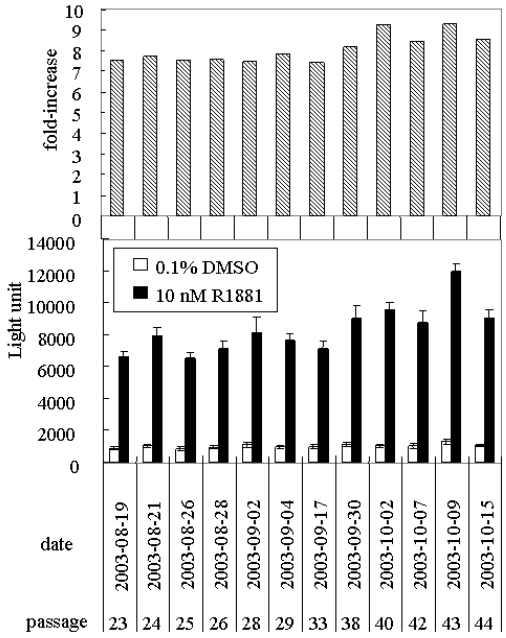


Fig. 7 Laboratory quality-control chart of AR-EcoScreen™.

AR-EcoScreen™ response to 10 nM methyltrienolone (R1881) and 0.1% DMSO.

Data are expressed as the light units or relative light units or relative fold increase (response/background).

5.2 RELEVANCE OF THE ASSAY SYSTEM

5.2.1 Comparison with chemicals listed in ICCVAM Report (2003)

89 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 androgenic and anti-androgenic activities is currently available. One possible approach to demonstrate the relevance of the proposed assay system (i.e., AR-EcoScreen™ assay) for detecting androgenic and anti-androgenic activities of chemicals is to compare the results with available data (ICCVAM list and AR binding data collected at CERI) collected from other assays that are designed to detect androgenic and anti-androgenic activities.

90 The 40 chemicals were selected from the list of ICCVAM (ICCVAM, 2003) and tested by AR-EcoScreen™ assay for evaluating both androgenic and anti-androgenic activities. The summary of the outcome based on positives/negatives judgment in section 5.2 (Data analysis and recording) is shown in **Table 8**. The detailed results and discussions are provided in the following sections. Also, this has been published by Araki et al (2005a).

Table 8 The Positive/negative Outcomes from the AR EcoScreen™ and the Data Reported in ICCVAM Report (2003)

No.	Chemical name	ICCVAM		AR EcoScreen	
		Agonist	Antagonist	PC10	lin.IC30
1	Diethylstilbestrol	N	P	N	P
2	Methyltrienolone (R1881)	P	N	P	N
3	Cyproterone acetate	P	P	P	P
4	Fluoxymestrone	P	N	P	N
5	Dexamethasone	P	N'	P	N
6	17b-Estradiol	P	P	P	P
7	Flutamide	N	P	N	P
8	Medroxyprogesterone acetate	P	N	P	N
9	Testosterone	P	N	P	N
10	4-Androstenedione	P	N'	P	N
11	Di - n -butyl phthalate	N	N'	N	N
12	Diethylhexyl phthalate	N	N'	N	N
13	5a-Dihydrotestosterone	P	N'	P	N
14	Estrone	P	N'	P	P
15	Linuron	P	P	P	P
16	p,p'- Methoxychlor	N	P	N	N
17	Spironolactone	P	P	P	P
18	Sodium Azide	N'	N'	N	N
19	4- tert -Octylphenol	N	P	N	P
20	Procymidone	N	P	N	P
21	p - n -Nonylphenol	N	N'	N	P
22	Bisphenol A	N	P	N	P
23	Progesterone	P	P	P	P
24	p,p'-DDE	P	P	N	P
25	Finasteride	N'	N'	N	P
26	Hydroxyflutamide	P	P	N	P
27	4-Hydroxytamoxifen	N	N'	N	P
28	Actinomycin D	N'	N'	P	N
29	Vinclozolin	N	P	N	P
30	Atrazine	N	N	N	N
31	Mifepristone	P	P	P	P
32	Fluoranthene	P	P	P	P
33	Kepone	N	P	N	N
34	o,p' -DDT	N	P	N	N
35	Corticosterone	N	N'	P	N
36	17a-Ethinyl estradiol	N	N'	N	P
37	Ketoconazole	N'	N'	N	P
38	Methyl testosterone	P	N'	P	N
39	12 - O -Tetradecanoylphorbol-13-acetate	N'	N'	N	N
40	2,4,5-Trichlorophenoxyacetic acid	N'	N'	N	P

P: positives, N: negatives, N': anticipated negatives

***5.2.1-1**) For detection of androgenic activity*

91 The positive/negative result outcomes based on the PC, PC10, PC20, PC30, PC40 or PC50 obtained from AR-EcoScreen™ assay were compared with 34 chemicals of which positives or negatives were clearly demonstrated in ICCVAM list, (ICCVAM, 2003) by two-by-two table analyses (Table 9).

92 The highest concordance, highest sensitivity and lowest false negative rate were obtained when PC5

and PC10 was used to judge positives or negatives. However, PC5 values were not always exceed the values of vehicle control plus 2 SD (mean +2SD of VC). This means the response correspond to PC5 is not always considered as statistically significant. Therefore, PC10 was considered as the best parameter to judge AR agonistic activity and used for the further discussion.

93 In ICCVAM report (ICCVAM, 2003), 18 positives, 16 negatives and 6 presumed negatives are listed.

94 Eighteen chemicals were considered to be androgenic chemicals based on PC10 value obtained from AR-EcoScreen™ assay as shown in Table 10 by the criteria (5.2 Data analysis and recording) and its response curves of those 18 chemicals are shown in Fig. 10-1 and Fig. 10-2. Actinomycin D in these 18 chemicals was an anticipated negative in ICCVAM list.

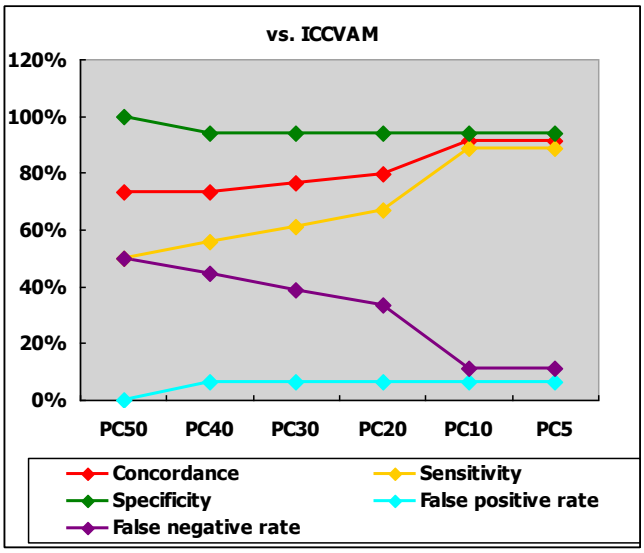
95 The concordance between the results obtained from the stably transfected TA assay using AR-EcoScreen™ cell line and the reference data in the ICCVAM report was 91%. Further, sensitivity and specificity rates were 89% and 82%, respectively.

96 According to the comparison with the positive/negative judgment in ICCVAM list (2003), 3 chemicals were classified incorrectly in the androgenic assay. Two chemicals were misclassified as negatives (hydroxyflutamide (HF) and *p,p'*-DDE) and positives (corticosterone), respectively.

97 Schrader and Cooke (2000) has previously reported that *p,p*'-DDE enhance luciferase activity 1.5- to 2-fold in PC-3 LUCAR+ cells at 5×10^{-5} and 10^{-4} M. However, the response in PC-3 LUCAR+ cells is highly cross-reactive with the glucocorticoid receptor (GR) agonist, dexamethasone. Therefore the agonist activity of *p,p'*-DDE may be due, in part, to cross-reactivity with the GR in PC-3 LUCAR+ cells.

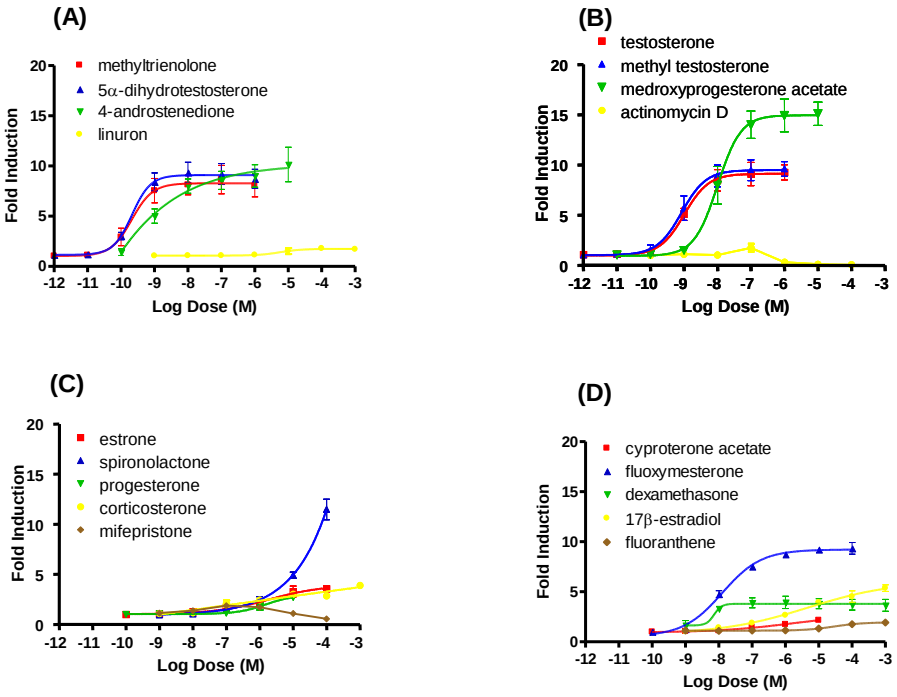
98 At high concentrations (1.0×10^{-5} and 5.0×10^{-5} M) of HF, HF induced AR-mediated transcriptional activation (Wong et al., 1995; Kemppainen and Wilson, 1996). However, HF showed cytotoxicity greater than 10^{-7}M in the antagonist assay and no AR agonistic activity was observed.

Table 9 Two-by-two Table Analysis of 34 Selected Chemicals Listed in the ICCVAM Report (2003) as Recommended Chemicals for AR/TA agonist assay



	PC50	PC40	PC30	PC20	PC10	PC5
Concordance	73.5%	73.5%	76.5%	79.4%	91.2%	91.2%
Sensitivity	50.0%	55.6%	61.1%	66.7%	88.9%	88.9%
Specificity	100.0%	93.8%	93.8%	93.8%	93.8%	93.8%
False positive rate	0.0%	6.3%	6.3%	6.3%	6.3%	6.3%
False negative rate	50.0%	44.4%	38.9%	33.3%	11.1%	11.1%

Fig. 8 Dose-response curves of 18 positive chemicals in the AR agonist detection assay



99 Actinomycin D and Mifepristone showed slight downward response in high concentration range which seemed to be caused by cytotoxicity effects. Therefore PC50 values for these chemicals had not been calculated.

100 Medroxyprogesterone acetate showed higher fold induction (approx 15 fold) in higher concentration range than that of other positive chemicals (approx 10 fold). The reason for this reinforced response of Medroxyprogesterone is unclear.

101 Dose-response for the partial antagonist, such as Linuron showed maximum fold induction tend to be repressed. It means the practical agonist activity would be less than that expressed as EC50 values. So RPCMax which is the maximum level of luciferase activity induced by a test chemical, expressed as a percentage of the response induced by 10 nM DHT on the same plate, as well as the PCMax (concentration associated with the RPCMax) would be better parameters for express practical strength of agonist activity as defined in the STTA assay (OECD TG455).

102 Furthermore, the available 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) were plotted with the logEC50s obtained from the proposed assay (Fig. 9).Log_{10}[EC50 (M)] values obtained in AR EcoScreen™ agonist assay for 6 chemicals listed in ICCVAM report (2003) correlate well with the values reported by ICCVAM (2003). As shown in Fig. 9, the correlation coefficient between the Log_{10}[EC50 (M)] of AR EcoScreen™ and that of ICCVAM was successful (R^2=0.780, n=6).

Table 10 EC50 Values Obtained from the Stably Transfected TA Assay using AR-EcoScreen™ and the Median EC50 Values Reported in the Other Assays for Detection of Androgenic Activity

Chemical	EC_{50} (M)	PC_{50} (M)	EC_{50} (M)
	AR-EcoScreen™	AR-EcoScreen™	ICCVAM
Methyltrienolone	2.65×10^{-10}	2.3×10^{-10}	N.A.
Cyproterone acetate	2.41×10^{-7}	-	N.A.
Fluoxymesterone	1.00×10^{-8}	7.6×10^{-9}	N.A.
Dexamethasone	2.37×10^{-9}	-	N.A.
17β- Estradiol	4.99×10^{-6}	2.83×10^{-6}	N.A.
Hydroxymethylprogesterone acetate	1.08×10^{-8}	2.88×10^{-9}	N.A.
Testosterone	1.06×10^{-9}	6.13×10^{-10}	2.00×10^{-10}
4-Androstenedione	1.02×10^{-9}	6.12×10^{-10}	1.50×10^{-9}
5α-Dihydrotestosterone	2.22×10^{-10}	1.65×10^{-10}	1.50×10^{-10}
Estrone	2.18×10^{-6}	-	5.50×10^{-8}
Linuron	1.17×10^{-5}	-	N.A.
Spironolactone	2.45×10^{-5}	5.35×10^{-6}	N.A.
Progesterone	2.19×10^{-6}	-	N.A.
Actinomycin D	1.96×10^{-5}	-	N.A.
Mifepristone	3.23×10^{-5}	-	1.40×10^{-8}
Fluoranthene	1.29×10^{-8}	-	N.A.
Corticosterone	4.48×10^{-7}	-	N.A.
Methyl testosterone	7.05×10^{-10}	4.21×10^{-10}	8.10×10^{-10}

Actinomycin D was an anticipated negative in ICCVAM list (2003).

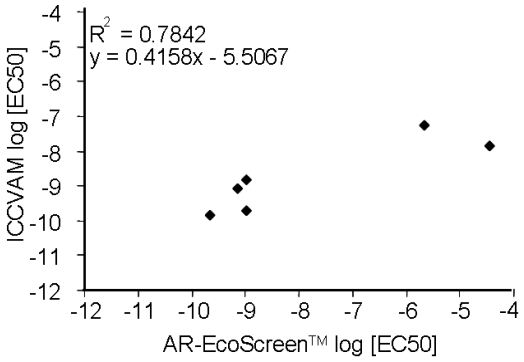


Fig. 9 The Relationship between LogEC50s and Median Log EC50s in the ICCVAM Report (2003)

103 As for the corticosterone misclassified as a positive in AR-EcoScreen™, it was because corticosterone was tested at concentrations very high concentration (10^{-3} M) in this study to meet the ICCVAM testing guidelines (ICCVAM, 2003).

Outputs:

- ***The relevance of the proposed assay system was well demonstrated*** *with comparison with ICCVAM reference data .*

- *AR EcoScreen™ system can detect known AR agonist satisfactory based on PC10 as discrimination parameters.*

5.2.1-2) *For detection of anti-androgenic activity*

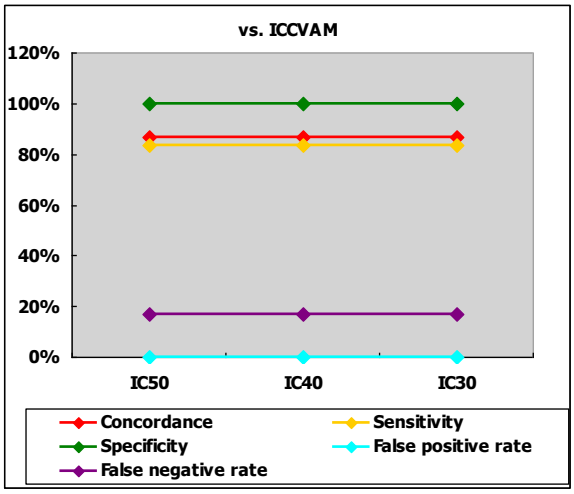
104 The two-by-two table analysis based on lin. IC30, IC40 and IC50 were summarized in Table 11.

105 In the selected 40 chemicals from ICCVAM list (ICCVAM, 2003), 17 chemicals were listed as "anticipated negatives". 23 chemicals were worth to analyze to ensure the relevance of AR EcoScreen™ system. 15 chemicals were considered to be anti-androgenic chemicals in AR EcoScreen™ system based on any of lin. IC30, IC40 and IC50 judgments (5.2 Data analysis and recording) (Table 12).

106 Any of the parameters (lin. IC30, IC40 and IC50) provided the same performance. However, the screening assay for 253 chemicals conducted by Araki et al. showed spline-based IC30 had the higher detection sensitivity than spline-based IC50. Whereas, the comparison between Lin.IC30 and lin.IC50 was further analyzed using the same data and it was confirmed that lin.IC30 was also promising parameter than lin.IC50.

107 The concordance between the results obtained from the stably transfected TA assay using AR-EcoScreen™ cell line and the reference data in the ICCVAM report for anti-androgenic activities was 87%. Further, sensitivity and specificity rates were 83% and 100%, respectively.

Table 11 Two-by-two Table Analysis of 23 Selected Chemicals Listed in the ICCVAM Report (2003) as Recommended Chemicals for AR/TA antagonist assay



	Lin. IC50	Lin. IC40	Lin. IC30
Concordance	87.0%	87.0%	87.0%
Sensitivity	83.3%	83.3%	83.3%
Specificity	100.0%	100.0%	100.0%
False positive rate	0.0%	0.0%	0.0%
False negative rate	16.7%	16.7%	16.7%

108 The response curves of 25 chemicals that can provide lin.IC30 including anticipated negatives are shown in Fig. 10-1 and Fig. 10-2 with cytotoxicity data.

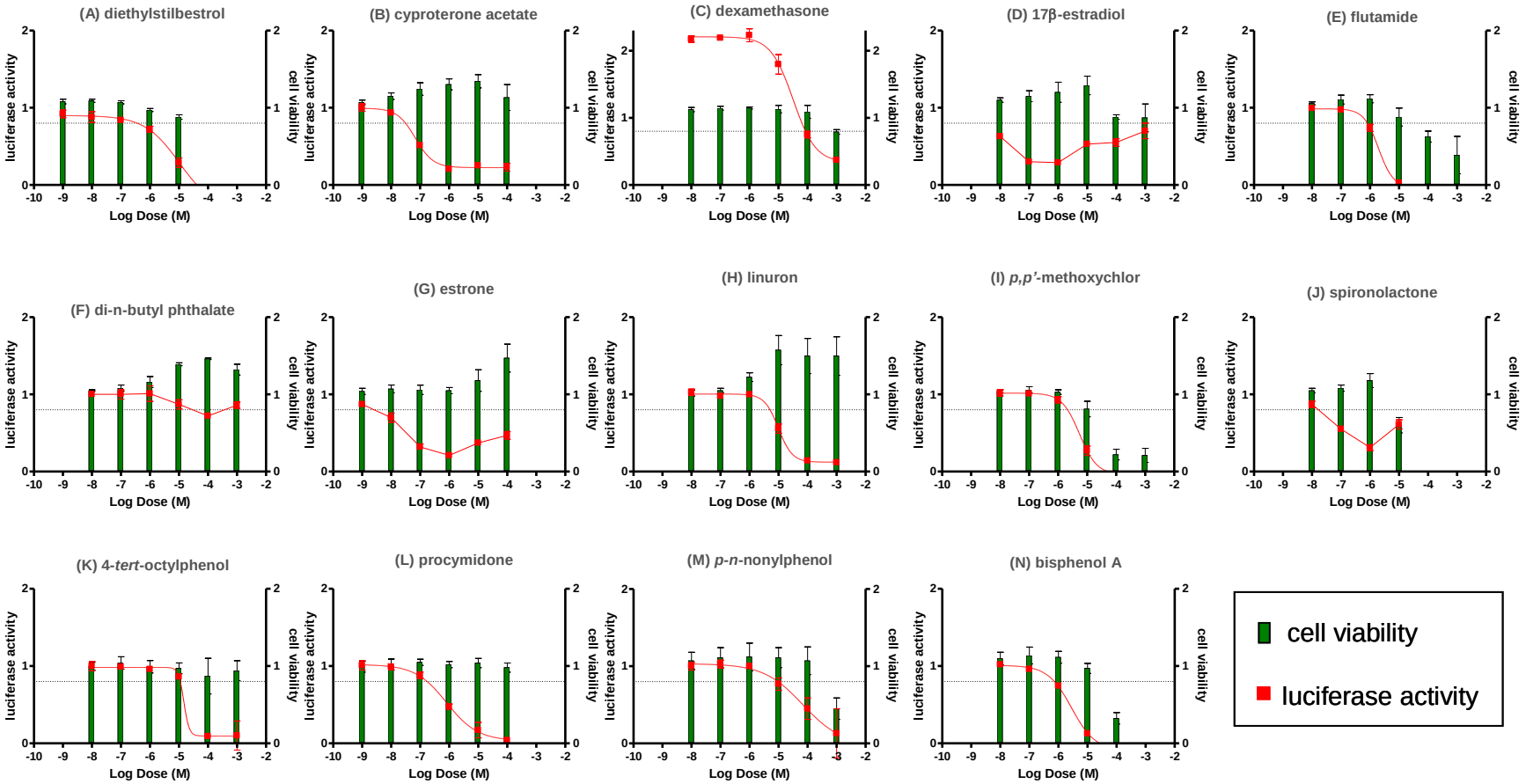


Fig. 10-1 Dose-response curves of test chemicals in the AR antagonist assay.

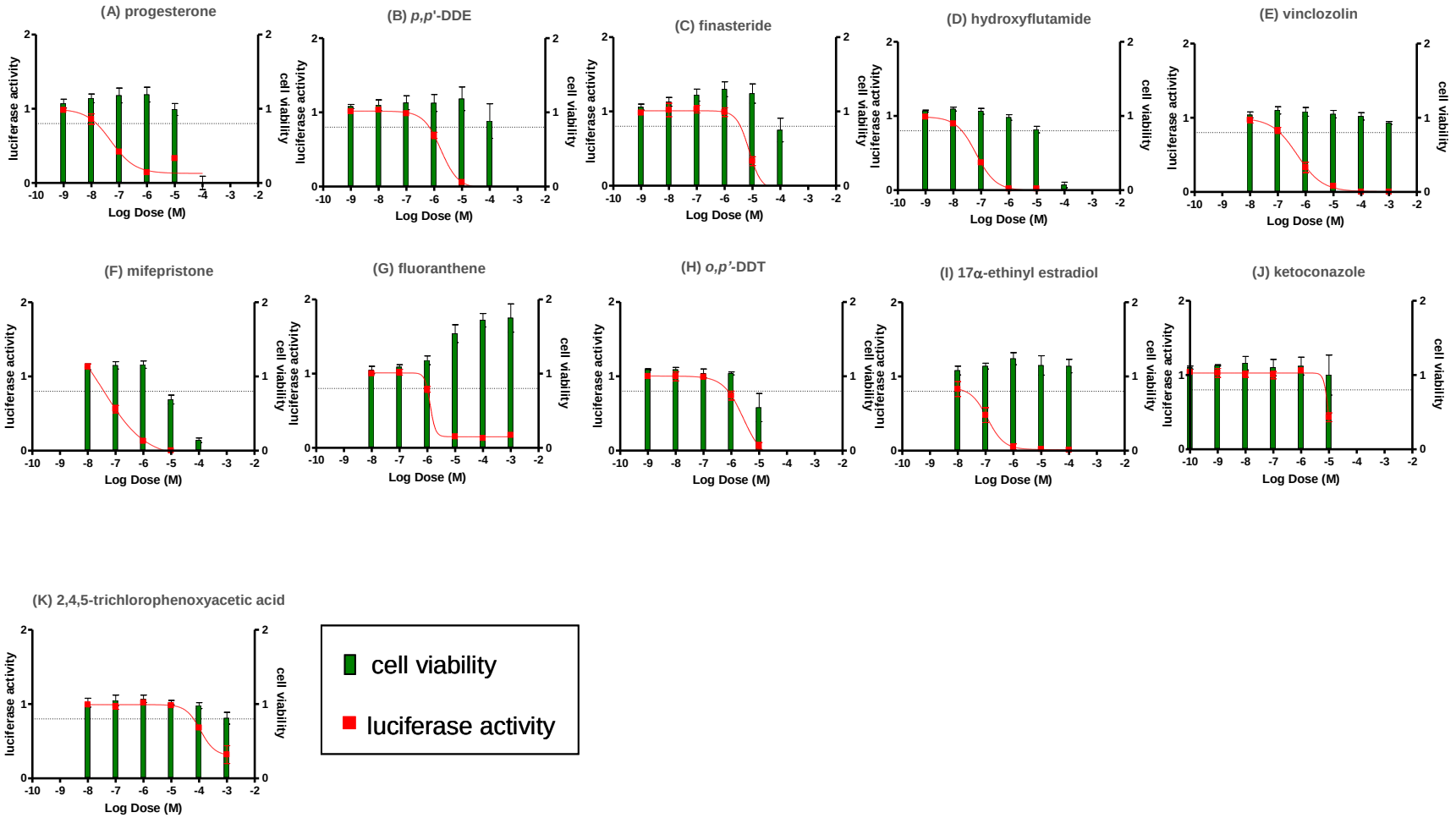


Fig. 10-2 Dose-response curves of test chemicals in the AR antagonist assay.

109 Log_{10}[IC50 (M)] values in AR EcoScreen™ assay was compared to that of ICCVAM report. However, the correlation coefficient between the Log_{10}[IC50 (M)] of AR EcoScreen™ and that of ICCVAM was relatively low (R^2=0.435, n=14) (Fig. 11). In order to evaluate the anti-androgenic activity, certain concentration of a spiked reference chemical (e.g., DHT, R1881) is needed to be added in the test system. However, the reported methods in ICCVAM list were different not only the test systems (including cell line, methods) but also spike-in reference chemical and/or its concentration. Due to limitation of available data, IC50 values of AR EcoScreenTM and that in ICCVAM list was considered not correlate well.

Table 12 IC50 Values Obtained from the Stably Transfected TA Assay using AR-EcoScreen™ and the Median IC50 Values Reported in the Other Assays for Detection of Anti-androgenic Activity

Chemical Name	IC_{50} (M)	IC_{50} (M)
	AR-EcoScreen™	ICCVAM
Diethylstilbestrol	4.98×10^{-6}	3.60×10^{-7}
Cyproterone acetate	7.68×10^{-8}	1.00×10^{-7}
Dexamethasone	4.45×10^{-5}	N.A.
17β-Estradiol	7.69×10^{-9}	5.00×10^{-7}
Flutamide	1.66×10^{-6}	N.A.
di - n -butyl phthalate	5.47×10^{-4}	N.A.
Estrone	3.98×10^{-8}	N.A.
Linuron	1.08×10^{-5}	5.00×10^{-6}
p,p' - Methoxychlor	4.01×10^{-6}	N.A.
Spironolactone	1.69×10^{-7}	3.00×10^{-7}
4 - tert -Octylphenol	2.69×10^{-5}	3.00×10^{-6}
Procymidone	7.12×10^{-7}	7.50×10^{-6}
p - n -Nonylphenol	3.59×10^{-5}	N.A.
Bisphenol A	2.06×10^{-6}	1.00×10^{-6}
Progesterone	5.74×10^{-7}	3.00×10^{-7}
p,p' -DDE	1.63×10^{-6}	3.00×10^{-6}
Finasteride	5.43×10^{-6}	N.A.
Hydroxyflutamide	4.91×10^{-8}	1.00×10^{-7}
Vinclozolin	4.04×10^{-7}	2.80×10^{-7}
Mifepristone	7.07×10^{-8}	5.00×10^{-8}
Fluoranthene	2.04×10^{-6}	4.60×10^{-6}
o,p' -DDT	2.13×10^{-6}	N.A.
17α-Ethinyl estradiol	7.80×10^{-8}	N.A.
Ketoconazole	6.98×10^{-6}	N.A.
2,4,5-Trichlorophenoxyacetic acid	2.63×10^{-4}	N.A.

Estrone, p-n-nonylphenol and finasteride were anticipated negatives in ICCVAM list.

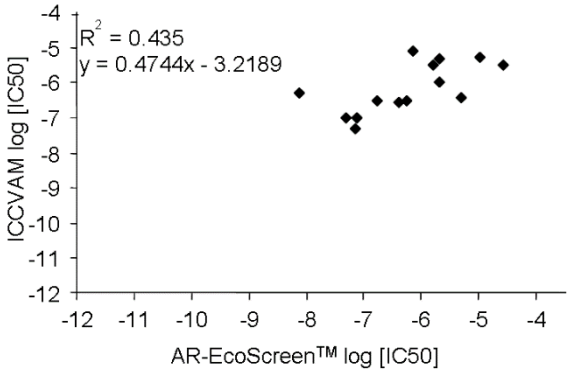


Fig. 11 The Relationship between LogIC50s and Median Log IC50s in the ICCVAM Report (2003)

110 Three chemicals (Kepone, *o,p'*-DDT and *p,p'*-methoxychlor) were misclassified as negative in the AR EcoScreen™.

111 The anti-androgenic effect of kepone was accompanied by a significant reduction in cell viability (Schrader and Cooke, 2000), and the ability of kepone to displace androgen from the AR was only observed at concentrations higher than 5.0×10^{-5} M (Kelce et al., 1995). In this study, the reduction of DHT-induced luciferase activity by kepone was observed above 10^{-5} M, and was accompanied by an apparent parallel reduction in cell viability (cytotoxicity was defined as the viability was less than 80%). Similarly, *o,p'*-DDT and *p,p'*-methoxychor showed cytotoxic effects at 10^{-9} or 10^{-8} M .

Outputs:

- ***The relevance of the proposed assay system was well demonstrated*** *with comparison with ICCVAM reference data.*
- *AR EcoScreen™ system can detect known AR antagonist satisfactory based on lin.IC30 as discrimination parameters.*

5.2.2 *Comparison with AR Binding Data*

112 AR binding is one of the representative assay that has relevant mode of action with AR transcriptional activation assays since AR transcriptional activation is triggered by chemical bindings to ARs. In this analysis, the results from the proposed AR TA assay were compared with AR binding assay using human recombinant AR ligand binding domain It should be noted that an AR binding assay cannot distinguish AR agonist and antagonist and this AR binding assay has not been validated yet.

113 Chemicals that have been selected in ICCVAM list were used for this comparison and the AR binding data from METI program were used. Therefore, not all chemicals in ICCVAM list were tested and a set of data on 31 chemicals in ICCVAM list was available on this comparison. AR binding data used for this analysis is shown in Table 14.

114 Before comparing the AR binding data with AR-EcoScreen™ assay, the AR binding data was compared with the results in ICCVAM list. The concordance between positive/negative outcomes from AR binding and ICCVAM results were 83.9%.

115 In comparison with the results from AR-EcoScreen™ assay, the concordance with AR binding assay results was 77.4% ((8+7+7+2)/31). This value is comparable with that of outcomes from ICCVAM List (83.9%). Although 5 chemicals (dibutyl phthalate, procymidon, *p*-*n*-nonylphenol, vinclozolin and 2,4,5,-trichlorophenoxyacetic acid) were detected as AR antagonist in AR-EcoScreen assay, the AR binding assay did not show binding affinities to AR for these chemicals. Possible reason could be the range of test concentration. In the AR-EcoScreen assay, chemicals were tested up to the available solubility, setting the maximum concentration as 1 mM (see the maximum concentration in Table 14). Chemicals were tested only up to 0.1 mM under the AR binding assay. At least, dibutyl phthalate and vinclozolin could displace hot ligand (DHT) greater than 20% at the highest test concentration. The difference of assay system that AR binding assay is a non-cell based assay (i.e., no metabolism is present in the assay system) could be the other possibility.

116 Two chemicals (4-hydroxytamoxifen and kepone) that have AR binding affinities were not detected as neither AR agonist nor AR antagonist in AR-EcoScreen assay.

117 As the conclusion from this analysis, the effects detected by AR-EcoScreen assay are relevant with respect to the chemical binding to AR.

Outputs:

- ***The relevance of the proposed assay system was well demonstrated*** *with comparison with AR binding assay data as the effects detected by AR-EcoScreen assay is relevant with the chemical binding to AR.*

Table 13 Comparison between AR-EcoScreen ™ and AR binding data

		AR-EcoScreen ™				Sum
		P/P	P/N	N/P	N/N	
AR Binding Assay	P	8 Cyproterone acetate, Dexamethasone, 17β-estradiol, Estrone, Linuron (= Lorox), Spironolactone, Progesterone, RU-486	7 Fluoxymesterone, Hydroxymethylprogesterone acetate, Testosterone, Androstenedione, 5α-Dehydrotestosterone, Corticosterone, 17α-Methyltestosterone	7 Diethylstilbestrol, Flutamide, Methoxychlor, 4-*tert*-Octylphenol, Bisphenol A, *p,p'*-DDE, Ethynyl estradiol	2 4-Hydroxytamoxifen, Kepone	24
	N	0	0	5 Dibutyl phthalate, Procymidon, p-n-Nonylphenol, Vinclozolin, 2,4,5-Trichlorophenoxyacetic acid	2 DEHP, Atrazine	7
		8	7	12	4	31

Concordance:	[(8+7+7) + 2] / 31 =	77.4%
Sensitivity:	(8+7+7) / 24 =	91.7%
Specificity:	2 / 7 =	28.6%

Table 14 AR Binding Data

Chemical Name	CAS No	AR_RBA%	AR_RBA P/N	AR Eco Screen		ICCVAM		Max conc. (mM)
				Ago	Atg	Ago	Atg	
Diethylstilbestrol (=DES)	56-53-1	0.0136	P	N	P	N	P	0.1
Cyproterone acetate	427-51-0	12.1	P	P	P	P	P	0.1
Fluoxymesterone	76-43-7	6.04	P	P	N	P	N	0.1
Dexamethasone)	50-02-2	0.0393	P	P	P	P	N'	1
17β-Estradiol	50-28-2	6.6	P	P	P	P	P	1
Flutamide	13311-84-7	0.0812	P	N	P	N	P	1
Hydroxymethylprogesterone acetate	71-58-9	51	P	P	N	P	N	0.01
Testosterone	58-22-0	68.5	P	P	N	P	N	1
Androstenedione	63-05-8	0.644	P	P	N	P	N'	0.1
Dibutyl phthalate	84-74-2	N.D.	N	N	P	N	N'	1
Di-sec-octyl phthalate	117-81-7	N.B.	N	N	N	N	N'	1
5α-Dehydrotestosterone	521-18-6	105	P	P	N	P	N'	0.1
Estrone	53-16-7	0.113	P	P	P	P	N'	0.1
Linuron	330-55-2	0.0259	P	P	P	P	P	1
Methoxychlor	72-43-5	0.0159	P	N	P	N	P	1
Spironolactone	52-01-7	3.08	P	P	P	P	P	1
4-tert-Octylphenol	140-66-9	0.0125	P	N	P	N	P	1
Procymidon	32809-16-8	N.B.	N	N	P	N	P	0.1
p-*n*-Nonylphenol	104-40-5	N.B.	N	N	P	N	N'	1
Bisphenol A	80-05-7	0.0301	P	N	P	N	P	1
Progesterone	57-83-0	3.59	P	P	P	P	P	0.1
p,p'-DDE	72-55-9	0.0497	P	N	P	P	P	0.1
4-Hydroxytamoxifen	68047-06-3	0.0543	P	N	N	N	N'	0.1
Vinclozolin	50471-44-8	N.D.	N	N	P	N	P	1
Atrazine	1912-24-9	N.B.	N	N	N	N	N	1
RU-486	84371-65-3	9.08	P	P	P	P	P	1
Kepone	143-50-0	0.0186	P	N	N	N	P	0.1
Corticosterone	50-22-6	0.299	P	P	N	N	N'	1
Ethynyl estradiol	57-63-6	0.482	P	N	P	N	N'	1b
17α-Methyltestosterone	58-18-4	78.8	P	P	N	P	N'	0.1
2,4,5-Trichlorophenoxyacetic acid	93-76-5	N.B.	N	N	P	N'	N'	1

N.D. Hot ligand was replaced only 20-50% and IC50 was not calculated.

N.B.: Hot ligand was not replaced greater than 20%.

5.3 INTER-LABORATORY REPRODUCIBILITY (RELIABILITY) AND PROTOCOL TRANSFERABILITY.

118 For the inter-laboratory validation study, assays for androgenic and anti-androgenic effects were both performed three runs on separate days (triplicate/run) in the four different

laboratories.

5.3.1 For detection of androgenic activity

119 One known androgenic (methyltrienolone (R1881)) and three androgenic negative chemicals (bisphenol A, di(2-ethylhexyl) phthalate (DEHP) and hydroxyflutamide (HF)) were tested in a coded manner and a standard chemical (DHT) was tested as a standard chemical in a un-coded manner 3 times on different days at 4 participating laboratories.

120 For evaluating the androgenic activity, logEC50 that has long been used as general parameters, logPC50 and logPC10 (both have been newly introduced in OECD TG 455 for detection of ER agonistic chemcial) were used as trial parameters.

121 The values logEC50, logPC50 and logPC10 induced by the DHT among 4 laboratories are shown in, Table 15.

122 The summary results for logEC50, logPC50 and logPC10 are shown in Table 16, Table 17 and Table 18, respectively.

123 Based on the criteria for judging positive or negative for evaluating androgenic effect potency, all known androgenic chemicals (R1881 and DHT) were determined as androgenic positive. Also, all know androgenic negative chemicals (bisphenol A, DEHP and HF) were determined as androgenic negative in all four laboratories, though the logPC10 for HF and DEHP were calculated one time at one laboratory, respectively,

124 Thus, the assay system can detect known androgenic positive and negative chemicals correctly among participating laboratories.

125 The coefficient variation (CV) values of $\log_{10}$[PC10 (M)], $\log_{10}$[PC50 (M)], and $\log_{10}$[EC50 (M)] for standard chemical (DHT) measured in three different days at each participating laboratory ranged from 0.4 to 8.0%, from 1.2 to 7.1% and from 0.0 to 7.4%, respectively. These results demonstrated the high reproducibility and reliability within each participating laboratories.

126 The overall CVs amongst 4 participating laboratiries were 3.6%, 4.5%, 4.4% for $\log_{10}$[EC50 (M)], $\log_{10}$[PC50 (M)] and $\log_{10}$[PC10 (M)] for DHT, respectively. These low CV values exert that the protocol can provide highly reproducible and reliable results for the standard chemical (DHT).

127 In addition to this finding, the coded agonistic positive chemical (R1881) showed also lower CV values (1.9%, 3.7%, 0.7% for $\log_{10}$[EC50 (M)], $\log_{10}$[PC50 (M)] and $\log_{10}$[PC10 (M)], respectively). This result also ensures the high reproducibility and reliability of the assay protocol/system.

128 As for the acceptability criteria for the androgenic activity using this AR-STTA assay, following criteria was recommended;

✓ Fold induction [PC (10 nM of DHT)] / V.C.		> =6.4	Lowest valid fold-induction from Multi-lab study			
✓ Fold-induction of 10% of PC (10 nM of DHT) (Fold-induction of V.C. = "1")		1+2SD of vehicle control	Make PC10 significant			
✓ Reference chemicals		logPC10	logPC50	logEC50	Hill Slope	Test range
	5α-Dehydrotestosterone (DHT)	-9.87 ~- 12.08	-9.00 ~ - 11.03	-9.13 ~ - 11.02	0.577 ~ 4.358	10^{-6} ~ 10^{-12}M
	R1881	-10.57 ~ - 11.07	-9.10 ~ - 10.86	-9.37 ~ - 10.83	3.996 ~ 0.599	10^{-5} ~ 10^{-11}M

V.C.: Vehicle Control

The range of logPc10, logPC50, logEC50 and Hill Slope was established based on Average ± 2SD from the multi-laboratory validation study, which is used in the ER-STTA approach.

Outputs:

- *Androgenic positive and negative chemicals were correctly detected by AR-EcoScreen™ assay system among all participating laboratories. Therefore* ***the relevance of the assay was verified in the inter-laboratory study.***
- *The positive indication parameters (logEC50, logPC50 and logPC10) have low CV values (below 3.6%, 4.5 and 4.4, respectively) and low variability (varied less than 2 order of magnitude). Thus,* ***the high reproducibility of the protocol and reliability of the assay system was demonstrated****.*
- *The recommended acceptability criteria were established for androgenic activity.*

Table 15 The Reproducibility of the Agonist Assay with a Positive Control Substance, 5α-Dihydrotestosterone

Test Substance	Test vial No.	Laboratory	Trial	Log_{10} [PC10(M)]		Log_{10} [PC50(M)]		HILLSLOPE	R2	Log_{10} [EC50(M)]	
				Data	Mean	Data	Mean			Data	Mean
5α-Dihydrotestosterone	DHT	ceri	1-1	-10.97	-10.91	-9.90	-9.97	0.9032	0.9740	-10.09	-10.09
			1-2	-10.84		-10.04		1.3180	0.9640	-10.08	
			2-1	-10.81	-10.81	-10.11	-10.11	2.4190	0.9746	-10.13	-10.13
			3-1	-10.66	-10.70	-9.60	-9.68	1.3040	0.9872	-9.74	-9.81
			3-2	-10.74		-9.75		1.3490	0.9839	-9.87	
			4-1	-10.66	-10.66	-9.65	-9.65	1.6290	0.9947	-9.79	-9.79
	DHT	sumitomo	1-1	-9.95	-9.92	-9.37	-9.27	1.6460	0.9909	-9.28	-9.22
			1-2	-9.88		-9.16		1.6220	0.9880	-9.16	
			2-1	-11.12	-11.37	-10.33	-10.47	1.2650	0.9354	-10.33	-10.45
			2-2	-11.62		-10.60		1.2130	0.9979	-10.58	
			3-1	-11.36	-11.50	-10.50	-10.54	1.4610	0.9960	-10.49	-10.56
			3-2	-11.64		-10.58		1.3160	0.9935	-10.64	
	DHT	otsuka	1-1	-11.73	-11.73	-10.47	-10.47	0.7300	0.9615	-10.26	-10.26
			2-1	-11.72	-11.72	-10.48	-10.48	0.8727	0.9788	-10.29	-10.29
			3-1	-11.72	-11.72	-10.77	-10.77	7.6160	0.9202	-10.98	-10.98
			4-1	-11.82	-11.82	-11.05	-11.05	4.5990	0.9453	-11.02	-11.02
	DHT	kaneka	1-1	-10.68	-10.67	-9.63	-9.62	1.8400	0.9935	-9.83	-9.82
			1-2	-10.66		-9.60		1.6260	0.9879	-9.80	
			2-1	-10.71	-10.71	-9.69	-9.67	1.6430	0.9834	-9.83	-9.82
			2-2	-10.70		-9.66		1.4900	0.9829	-9.81	
			3-1	-10.70	-10.70	-9.68	-9.65	1.7120	0.9832	-9.85	-9.81
			3-2	-10.69		-9.62		1.4060	0.9895	-9.77	
Total	MAX			-9.88	-9.92	-9.16	-9.27	7.6160	0.9979	-9.16	-9.22
	MIN			-11.82	-11.82	-11.05	-11.05	0.7300	0.9202	-11.02	-11.02
	Ave.			-10.97	-11.07	-10.01	-10.10	1.8627	0.9776	-10.07	-10.15

Table 16 The Reproducibility of Log_{10}[EC50 (M)] in the Agonist Assay

Test Substance	Test vial No.	Laboratory	Trial	HILLSLOPE	R2	Log_{10} [EC50 (M)]							
						Data	intra-Lab				inter-Lab		
							Mean	SE	SD	CV	Mean	SD	CV
Methyltrienolone (R1881)	5	ceri	1	1.8910	0.9978	-10.14	-10.02	0.09	0.16	-1.6	-10.03	0.10	0.19	-1.9
			2	1.9650	0.9857	-10.08							
			3	1.0690	0.9793	-9.84							
	6	sumitomo	1	n.c.	n.c.	n.c	-10.30	0.01	0.02	-0.2			
			2	1.3540	0.9722	-10.29							
			3	1.3730	0.9537	-10.31							
	7	otsuka	1	1.3270	0.9755	-9.92	-9.97	0.02	0.04	-0.4			
			2	5.6580	0.9828	-9.99							
			3	1.6910	0.9857	-10.00							
	8	kaneka	1	1.5250	0.9865	-9.91	-9.84	0.05	0.08	-0.8			
			2	1.2110	0.9808	-9.75							
			3	1.3360	0.9855	-9.87							
5α-Dihydrotestosterone	DHT	ceri	1-1	0.9032	0.9740	-10.09	-9.95	0.09	0.18	-1.8	-10.12	0.18	0.36	-3.6
			1-2	1.3180	0.9640									
			2-1	2.4190	0.9746	-10.13								
			3-1	1.3040	0.9872	-9.81								
			3-2	1.3490	0.9839									
			4-1	1.6290	0.9947	-9.79								
	DHT	sumitomo	1-1	1.6460	0.9909	-9.22	-10.08	0.43	0.75	-7.4				
			1-2	1.6220	0.9880									
			2-1	1.2650	0.9354	-10.45								
			2-2	1.2130	0.9979									
			3-1	1.4610	0.9960	-10.56								
			3-2	1.3160	0.9935									
	DHT	otsuka	1-1	0.7300	0.9615	-10.26	-10.64	0.21	0.42	-3.9				
			2-1	0.8727	0.9788	-10.29								
			3-1	7.6160	0.9202	-10.98								
			4-1	4.5990	0.9453	-11.02								
	DHT	kaneka	1-1	1.8400	0.9935	-9.82	-9.82	0.00	0.00	0.0				
			1-2	1.6260	0.9879									
			2-1	1.6430	0.9834	-9.82								
			2-2	1.4900	0.9829									
			3-1	1.7120	0.9832	-9.81								
			3-2	1.4060	0.9895									
MAX								0.43	0.75	0.0		0.18	0.36	-1.9
MIN								0.00	0.00	-7.4		0.10	0.19	-3.6
Ave.								0.11	0.21	-2.0		0.14	0.28	-2.7

n.c. : not caluculated

Table 17 The Reproducibility of Log_{10}[PC50 (M)] in the Agonist Assay

Test Substance	Test vial No.	Laboratory	Trial	Log_{10} [PC50 (M)]								
				Data	intra-Lab				inter-Lab			
					Mean	SE	SD	CV	Mean	SE	SD	CV
Hydroxyflutamide	1	ceri	1	-	-	-	-	-	-	-	-	-
			2	-								
			3	-								
	2	sumitomo	1	-								
			2	-								
			3	-								
	3	otsuka	1	-								
			2	-								
			3	-								
	4	kaneka	1	-								
			2	-								
			3	-								
Methyltrienolone (R1881)	5	ceri	1	-9.87	-9.83	0.06	0.11	-1.1	-9.93	0.18	0.36	-3.7
			2	-9.92								
			3	-9.71								
	6	sumitomo	1	<-11.00	-10.39	0.02	0.02	-0.2				
			2	-10.41								
			3	-10.38								
	7	otsuka	1	-9.98	-9.98	0.01	0.02	-0.2				
			2	-9.99								
			3	-9.95								
	8	kaneka	1	-9.53	-9.52	0.02	0.04	-0.4				
			2	-9.47								
			3	-9.55								
Diethylhexyl phthalate	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	-	-	-	-	-	-	-	-	-
			2	-								
			3	-								
	14	sumitomo	1	-								
			2	-								
			3	-								
	15	otsuka	1	-								
			2	-								
			3	-								
	16	kaneka	1	-								
			2	-								
			3	-								
5α-Dihydrotestosterone	DHT	ceri	1	-9.97	-9.85	0.11	0.23	-2.3	-10.07	0.23	0.45	-4.5
			2	-10.11								
			3	-9.68								
			4	-9.65								
	DHT	sumitomo	1	-9.27	-10.09	0.41	0.72	-7.1				
			2	-10.47								
			3	-10.54								
	DHT	otsuka	1	-10.47	-10.69	0.14	0.27	-2.6				
			2	-10.48								
			3	-10.77								
			4	-11.05								
	DHT	kaneka	1	-9.62	-9.65	0.06	0.11	-1.2				
			2	-9.67								
			3	-9.65								
MAX						0.41	0.72	-0.2		0.23	0.45	-3.7
MIN						0.01	0.02	-7.1		0.18	0.36	-4.5
Ave.						0.11	0.19	-1.9		0.20	0.41	-4.1

Table 18 The Reproducibility of Log_{10}[PC10 (M)] in the Agonist Assay

Test Substance	Test vial No.	Laboratory	Trial	Log_{10} [PC10 (M)]							
				Data	intra-Lab				inter-Lab			
					Mean	SE	SD	CV	Mean	SE	SD	CV
Hydroxyflutamide	1	ceri	1	-	-	-	-	-	-5.06	-	-	-
			2	-								
			3	-								
	2	sumitomo	1	-5.06	-5.06	-	-	-				
			2	-								
			3	-								
	3	otsuka	1	-	-	-	-	-				
			2	-								
			3	-								
	4	kaneka	1	-	-	-	-	-				
			2	-								
			3	-								
Methyltrienolone (R1881)	5	ceri	1	-10.82	-10.79	0.02	0.03	-0.3	-10.76	0.04	0.08	-0.7
			2	-10.81								
			3	-10.76								
	6	sumitomo	1	<-11.00	<-11.00	-	-	-				
			2	<-11.00								
			3	<-11.00								
	7	otsuka	1	-10.82	-10.81	0.00	0.01	-0.1				
			2	-10.81								
			3	-10.81								
	8	kaneka	1	-10.69	-10.67	0.02	0.03	-0.3				
			2	-10.63								
			3	-10.68								
Diethylhexyl phthalate	9	ceri	1	-	-	-	-	-	-5.33	-	-	-
			2	-								
			3	-								
	10	sumitomo	1	-	-	-	-	-				
			2	-								
			3	-								
	11	otsuka	1	-	-	-	-	-				
			2	-								
			3	-								
	12	kaneka	1	-5.33	-5.33	-	-	-				
			2	-								
			3	-								
Bisphenol A	13	ceri	1	-	-	-	-	-	-	-	-	-
			2	-								
			3	-								
	14	sumitomo	1	-	-	-	-	-				
			2	-								
			3	-								
	15	otsuka	1	-	-	-	-	-				
			2	-								
			3	-								
	16	kaneka	1	-	-	-	-	-				
			2	-								
			3	-								
5α-Dihydrotestosterone	DHT	ceri	1	-10.91	-10.77	0.06	0.11	-1.0	-11.03	0.24	0.48	-4.4
			2	-10.81								
			3	-10.70								
			4	-10.66								
	DHT	sumitomo	1	-9.92	-10.93	0.51	0.88	-8.0				
			2	-11.37								
			3	-11.50								
	DHT	otsuka	1	-11.73	-11.75	0.03	0.05	-0.4				
			2	-11.72								
			3	-11.72								
			4	-11.82								
	DHT	kaneka	1	-10.67	-10.69	0.06	0.11	-1.1				
			2	-10.71								
			3	-10.70								
MAX						0.51	0.88	-0.1		0.24	0.48	-0.7
MIN						0.00	0.01	-8.0		0.04	0.08	-4.4
Ave.						0.10	0.18	-1.6		0.14	0.28	-2.6

5.3.2 For detection of anti-androgenic activity

129 Ideally, the ratio of Spike-in/PC DHT should have been 80/100 (80% correspond to EC80 of DHT). However, these ratio obtained in "Kaneka-lab." were all around 20% (Corresponding to EC20 of DHT). Therefore, the spike-in concentration in Kaneka-lab. was definitely lower than the expected concentration. Accordingly, these data were excluded from the data analysis for anti-androgenic activity of multi-lab validation study.

130 In the assay for detecting anti-androgenic activity, 3 possible parameters (log[IC50 (M)], log[linearIC50 (M)] and log[linearIC30 (M)] were analyzed in this inter-laboratory validation study.

131 The summarized results of $\log_{10}$[IC50(M)], $\log_{10}$[linearIC50(M)] and $\log_{10}$[linearIC30(M)] were shown in Table 19, Table 20 and Table 21, respectively

132 In the anti-androgenic assay, data at the concentration where the renilla luciferase activity showed over 20% reduction (i.e., < 80% cell viability) were excluded from the calculation of linear IC50 and linearIC30.

133 Hydroxyflutamide (HF) and bisphenol A were both judged as anti-androgenic positives based on the criteria (see section 5.2). Methyltrienolone (R1881), diethylhexyl phthalate and 5a-dihydrotestosterone (DHT) were correctly judged as anti-androgenic negatives in this assay system.

134 These judgments were all agreed with the known information (ICCVAM, 2003). Therefore, the relevance of this assay system demonstrated in the pre-validation study was also supported in the inter-laboratory validation study.

135 For the antagonist assay, the CVs of $\log_{10}$[IC50] of HF and bisphenol A in each laboratory were from 1.2 to 3.5% and from 0.9 to 3.0%, respectively. These values were considered low. Therefore, high intra-laboratory reproducibility was demonstrated for $\log_{10}$[IC50]. In a similar way, the CVs of $\log_{10}$[linearIC50(M)] of HF and bisphenol A in each laboratory ranged from 0.9 to 3.6 and from 0.6 to 2.8, respectively. The CVs of $\log_{10}$[linearIC30 (M)] of HF and bisphenol A in each laboratory ranged from 1.2 to 5.9 and from 0.46 to 1.6, respectively. These data also demonstrated the high reproducibility of concerning to $\log_{10}$[linearIC50(M)] and $\log_{10}$[linearIC30(M)] determined by the assay system within 4 participating laboratories. Furthermore, it can be said that the results obtained by this

assay/protocol were highly reliable.

136 For the antagonist assay, the CV of log_{10}[IC50], log_{10}[linearIC50(M)] log_{10}[linearIC30(M)] among laboratories (as parameters for inter-laboratory variability) ranged from 7.8 to 8.3, from 6.4 to 8.6 and from 7.1 to 9.0, respectively. Thus, high reproducibility and reliability of the test system/protocol among laboratories were again demonstrated.

137 In conclusion, AR-EcoScreen™ assay showed high inter-laboratory reproducibility and the protocols were optimized well to transfer the other laboratories. Therefore AR-EcoScreen™ assay meet the criteria that is required for the OECD testing guideline.

138 As for the acceptability criteria for the androgenic activity using this AR-STTA assay, following criteria was recommended;

✓ Fold induction of spike-in [Spike-in of 500 pM DHT] /[Vehicle Control]		> =5.0	Lowest valid fold-induction from Multi-lab study			
✓ PC_{ATG} inhibitory ratio		= <0.46	Highest valid inhibitory ratio from Multi-lab study			
✓ Reference chemicals		log linearIC30	log linearIC50	logIC50	Hill Slope	Test range
	Hydroxyflutamide	-6.41 ~ -8.37	-6.17 ~ -7.80	-6.26 ~ -7.71	-2.503 ~ -0.652	10^{-5} ~ 10^{-10}M
	Bisphenol A	-4.48 ~ -7.52	-4.29 ~ -7.05	-4.38 ~ -6.89	-2.973 ~ -0.598	10^{-5} ~ 10^{-10}M

The range of logPc10, logPC50, logEC50 and Hill Slope was established based on Average ± 3SD from the multi-laboratory validation study taking into considerations the following reasons;

- ✓ In order to make the acceptable range "2 order of magnitude"
- ✓ An antagonism assay has more additional variation factors such as "Spike-in" than agonism assay.
- ✓ The number of laboratory that data employed for analysis were reduced from 4 lab. to 3 lab. because the spike-in concentration at a laboratory was lower than the required concentration.

Outputs:

- *Anti-androgenic positive and negative chemicals were correctly detected by AR-EcoScreen™ assay system among all participating laboratories. Therefore* ***the***

relevance of the assay was verified in the inter-laboratory study.

- *The positive indication parameters (logIC50, log[linearIC50] and log[linearIC30]) have low CV values (below 8.3, 8.6 and 9.0%, respectively) and low variability (varied less than 2 order of magunitude). Thus,* ***the high reproducibility and technical transferability of the protocol and reliability of the assay system was demonstrated****.*
- *The recommended acceptability criteria were established from the multi-laboratory validation study.*

Table 19 The Reproducibility of the Assay System with a Positive Control Substance in Antagonist Assay

Test Substance	Test vial No.	Laboratory	Trial	HILLSLOPE	R2	Log_{10} [IC50 (M)]							
						Data	intra-Lab				inter-Lab			
							Mean	SE	SD	CV	Mean	SE	SD	CV
Hydroxyflutamide	1	ceri	1	-0.9761	0.9900	-7.27	-7.11	0.08	0.15	-2.1	-7.27	0.35	0.61	-8.3
			2	-1.3750	0.9945	-7.07								
			3	-1.6860	0.9892	-6.99								
	2	sumitomo	1	-0.8839	0.9524	-7.00	-6.73	0.14	0.24	-3.5				
			2	-1.5020	0.9882	-6.56								
			3	-1.4530	0.9913	-6.63								
	3	otsuka	1	-1.2090	0.9818	-7.19	-7.11	0.05	0.09	-1.2				
			2	-1.4830	0.9920	-7.13								
			3	-1.2430	0.9920	-7.02								
	4	kaneka	1	-0.8388	0.9381	-8.09	-8.14	0.10	0.18	-2.2				
			2	-0.8218	0.9139	-7.99								
			3	-0.5689	0.9006	-8.34								
Bisphenol A	13	ceri	1	-3.7610	0.9221	n.e.	-5.47	0.12	0.16	-3.0	-5.62	0.25	0.44	-7.8
			2	-1.7280	0.9612	-5.59								
			3	-1.0790	0.8644	-5.36								
	14	sumitomo	1	-2.4200	0.9455	-5.29	-5.24	0.03	0.05	-0.9				
			2	-1.1760	0.9518	-5.20								
			3	-1.2440	0.9737	-5.24								
	15	otsuka	1	-1.4630	0.9731	-5.49	-5.52	0.02	0.04	-0.6				
			2	-1.4710	0.9772	-5.56								
			3	-1.3450	0.9515	-5.50								
	16	kaneka	1	-0.9544	0.9301	-6.32	-6.26	0.04	0.07	-1.2				
			2	-1.3300	0.9242	-6.18								
			3	-0.9781	0.9788	-6.27								
MAX								0.14	0.24	-0.6		0.35	0.61	-7.8
MIN								0.02	0.04	-3.5		0.25	0.44	-8.3
Ave.								0.07	0.12	-1.8		0.30	0.52	-8.1

n.e. : not evaluated for cytotoxicity

Table 20 The Reproducibility of Log_{10}[linearIC50 (M)] in the Antagonist Assay

Test Substance	Test vial No.	Laboratory	Trial	Log_{10} [lin.IC50 (M)]								
				Data	intra-Lab				inter-Lab			
					Mean	SE	SD	CV	Mean	SE	SD	CV
Hydroxyflutamide	1	ceri	1	-7.27	-7.13	0.08	0.14	-1.9	-7.19	0.23	0.46	-6.4
			2	-7.12								
			3	-7.00								
	2	sumitomo	1	-6.96	-6.69	0.14	0.24	-3.6				
			2	-6.51								
			3	-6.59								
	3	otsuka	1	-7.26	-7.15	0.07	0.12	-1.7				
			2	-7.16								
			3	-7.01								
	4	kaneka	1	-7.87	-7.81	0.04	0.07	-0.9				
			2	-7.82								
			3	-7.73								
Methyltrienolone (R1881)	5	ceri	1	n.e.	-	-	-	-	-5.22	-	-	-
			2	n.e.								
			3	n.e.								
	6	sumitomo	1	n.e.	-	-	-	-				
			2	n.e.								
			3	n.e.								
	7	otsuka	1	n.e.	-5.22	-	-	-				
			2	n.e.								
			3	-5.22								
	8	kaneka	1	n.e.	-	-	-	-				
			2	-								
			3	-								
Diethylhexyl phthalate	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	n.e.	-5.44	0.11	0.15	-2.8	-5.65	0.24	0.49	-8.6
			2	-5.54								
			3	-5.33								
	14	sumitomo	1	-5.38	-5.31	0.04	0.06	-1.2				
			2	-5.25								
			3	-5.30								
	15	otsuka	1	-5.45	-5.48	0.02	0.03	-0.6				
			2	-5.51								
			3	-5.48								
	16	kaneka	1	-6.38	-6.37	0.02	0.03	-0.5				
			2	-6.34								
			3	-6.40								
5α-Dihydrotestosterone	DHT	ceri	1	-	-	-	-	-	-	-	-	-
			2	-								
			3	-								
	DHT	sumitomo	1	-	-	-	-	-				
			2	-								
			3	-								
	DHT	otsuka	1	-	-	-	-	-				
			2	-								
			3	-								
			4	-								
	DHT	kaneka	1	-	-	-	-	-				
			2	-								
			3	-								
MAX					0.14	0.24	-0.5		0.24	0.49	-6.4	
MIN					0.02	0.03	-3.6		0.23	0.46	-8.6	
Ave.					0.06	0.11	-1.7		0.24	0.47	-7.5	

n.e. : not evaluated for cytotoxicity

Table 21 The Reproducibility of Log_{10}[linearIC30 (M)] in the Antagonist Assay

Test Substance	Test vial No.	Laboratory	Trial	Log_{10} [lin.IC30 (M)]								
				Data	intra-Lab				inter-Lab			
					Mean	SE	SD	CV	Mean	SE	SD	CV
Hydroxyflutamide	1	ceri	1	-7.71	-7.57	0.07	0.13	-1.7	-7.64	0.27	0.54	-7.1
			2	-7.54								
			3	-7.46								
	2	sumitomo	1	-7.55	-7.07	0.24	0.42	-5.9				
			2	-6.78								
			3	-6.89								
	3	otsuka	1	-7.64	-7.53	0.05	0.09	-1.2				
			2	-7.51								
			3	-7.46								
	4	kaneka	1	-8.43	-8.38	0.09	0.16	-1.9				
			2	-8.52								
			3	-8.20								
Methyltrienolone (R1881)	5	ceri	1	n.e.	-	-	-	-	-5.46	-	-	-
			2	n.e.								
			3	n.e.								
	6	sumitomo	1	n.e.	-	-	-	-				
			2	n.e.								
			3	n.e.								
	7	otsuka	1	n.e.	-5.46	-	-	-				
			2	n.e.								
			3	-5.46								
	8	kaneka	1	n.e.	-	-	-	-				
			2	-								
			3	-								
Diethylhexyl phthalate	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	n.e.	-5.74	0.06	0.09	-1.6	-5.98	0.27	0.54	-9.0
			2	-5.81								
			3	-5.68								
	14	sumitomo	1	-5.61	-5.63	0.01	0.02	-0.4				
			2	-5.63								
			3	-5.66								
	15	otsuka	1	-5.72	-5.77	0.02	0.04	-0.7				
			2	-5.80								
			3	-5.79								
	16	kaneka	1	-6.80	-6.78	0.03	0.06	-0.9				
			2	-6.72								
			3	-6.84								
5α-Dihydrotestosterone	DHT	ceri	1	-	-	-	-	-	-	-	-	-
			2	-								
			3	-								
	DHT	sumitomo	1	-	-	-	-	-				
			2	-								
			3	-								
	DHT	otsuka	1	-	-	-	-	-				
			2	-								
			3	-								
			4	-								
	DHT	kaneka	1	-	-	-	-	-				
			2	-								
			3	-								
MAX						0.24	0.42	-0.4		0.27	0.54	-7.1
MIN						0.01	0.02	-5.9		0.27	0.54	-9.0
Ave.						0.07	0.13	-1.8		0.27	0.54	-8.1

n.e. : not evaluated for cytotoxicity

6. DISCUSSION

139 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 a global concern regarding endocrine disruption effects resulting from chemical exposure, particularly those mediated by the ER and AR. 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.

140 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.

141 In order to develop and validate a test protocol to support the development of test guidelines for the detection of chemicals possessing the potential androgenic and anti-androgenic activity through human androgen receptor (hAR), we conducted a series of validation tests for the AR 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.

142 Validation work on the hAR 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 Otsuka Pharmaceutical Co., Ltd., Japan and the inter-laboratory validation study was conducted within four Japanese domestic laboratories upon the initiative of CERI.

143 In the pre-validation study, the method of the AR transcriptional activation assay using the AR-EcoScreen™ cell line was evaluated. The overall sensitivity and specificity of the method were examined by comparing the result of the chemical compounds with their ICCVAM classification (ICCVAM, 2003). In the AR agonist assay, 16 of the 18 positive

agonists and 15 of the 16 negative chemicals were identified correctly.

144 The results obtained by the stably transfected TA assay and the information given in the ICCVAM report (2003) were compared with regard to 40 chemicals. The information in ICCVAM report (2003) was collected based on several different *in vitro* assay systems to detect androgenic activities, and the assay performance parameters for the stably transfected TA assay, concordance, sensitivity and specificity, were 91%, 89% and 94%, respectively. The concordance, sensitivity and specificity for antagonistic activity were 87%, 83% and 100%, respectively.

145 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.

146 In order to establish the test guideline, the proficiency chemicals that is used prior to testing unknown chemicals in the STTA assay, the responsiveness of the test system should be confirmed by each laboratory. The recommended list of proficiency chemicals were selected from the list of pre-validation study in Table 22 for androgenic assay and Table 23 for anti-androgenic assay, respectively.

Table 22 Recommended proficiency chemicals for androgenic assay

No.	Chemical name	CAS No.	PC50	PC10	ICCVAM
1	Flutamide	13311-84-7	N	N	N
2	4-*tert*-Octylphenol	140-66-9	N	N	N
3	Bisphenol A	80-05-7	N	N	N
4	Dexamethasone	50-02-2	N	P	P
5	Medroxyprogesterone acetate	71-58-9	P	P	P
6	Testosterone	58-22-0	P	P	P
7	4-Androstenedione	63-05-8	P	P	P
8	Spironolactone	52-01-7	P	P	P
9	Progesterone	57-83-0	N	P	P
10	17a-Methyltestosterone	58-18-4	P	P	P

Table 23 Recommended proficiency chemicals for anti-androgenic assay

No.	Chemical name	CAS No.	Lin.IC50	Lin.IC30	ICCVAM
1	Methyltrienolone (R1881)	965-93-5	N	N	N
2	Fluoxymestrone	76-43-7	N	N	N
3	Medroxyprogesterone acetate	71-58-9	N	N	N
4	Cyproterone acetate	427-51-0	P	P	P
5	Flutamide	13311-84-7	P	P	P
6	Spironolactone	52-01-7	P	P	P
7	4- tert -Octylphenol	140-66-9	P	P	P
8	Procymidone	32809-16-8	P	P	P
9	Progesterone	57-83-0	P	P	P
10	Vinclozolin	50471-44-8	P	P	P

147 Accordingly, the overall assay performance of the stably transfected TA assay system using the AR-EcoScreen™ cell line was deemed satisfactory for practical use, and in accordance with GD 34 (See Table 24).

Table 24 Checklist for GD34 requirements

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 androgenic/anti-androgenic activities mediated androgen receptor.
– 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 AR agonists or spike-in DHT, is thought to be mediated by direct binding where AR-liganded AR binds directly to androgen responsive element (ARE) 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 appendices in the validation report. Further statistical discussions on data analysis and decision criteria are

		provided in the validation report.
– 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	Five chemicals 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 assay developer laboratory for further comparison with 40 chemicals selected from the ICCVAM list, and these data give a strong indication of relevance of the proposed test method. Under limited budget constraint and to achieve efficient study design, common set of chemicals were selected and serve for both agonist and antagonist assay system evaluations. Test chemicals used in this validation study were selected based on best available information at that time. Although this study was conducted under the limited budget constraint, the assay results showed clear inter-lab reproducibility, and the performance standard for this assay could be established based on the results of this study. Moreover, the relevance of this assay can be confirmed with 40 chemicals tested in pre-validation phase. Such an approach is intended to improve the efficiency, reduce costs and speed up the validation process t.
– 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 laboratory of assay developer. – Additionally a data comparison was conducted with the proposed test method and the hAR Binding assay with good concordance.
– g) Ideally, all data supporting the validity of a test method should have been obtained in accordance with the	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

principles of GLP.		conducted to GLP. – Under the ER-STTA validation study, there was consensus from the preliminary validation assessment panel (PVAP) 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. Therefore, the AR-STTA validation study is considered acceptable since the same approach as the ER-STTA was applied.
– 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 ready 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.

6.1 LIMITATIONS OF THE ASSAY, AND FURTHER VALIDATION CONSIDERATIONS

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

148 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 AR activation only. It has not been directly extrapolated to the complex *in vivo* androgenic or anti-androgenic 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* AR screening assays, by utilizing the test method to produce data for different purposes, including the development of a prediction model.

149 Although the proposed stably transfected TA assay system shows good concordance with other *in vitro* and *in vivo* AR 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).

6.1.2 Metabolic capability and TA assays

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

7. CONCLUSIONS

151 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.

152 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).

153 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 AR mediated endocrine disruption.

154 A Japanese human AR mediated stably transfected TA assay system using AR-EcoScreen™ 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 hARα. The assay is a therefore a promising method to use in the prescreening process of an endocrine disruptor screening strategy.

8. RECOMMENDATIONS

155 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 DHT, R1881 etc.

156 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 androgens 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 androgens, PC10 and PC50 values calculated using linear regression can be obtained with accuracy and reproducibility. PC50 values can also provide the relative androgenic potency and this parameter reflects AR mediated biological effects from the results of comparative studies with AR binding. 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.

9. ACKNOWLEDGMENTS

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10. REFERENCES

Araki, N., Ohno, K., Takeyoshi, M. and Iida, M. (2005a). Evaluation of a rapid in vitro androgen receptor transcriptional activation assay using AR-EcoScreen™ cells. Toxicology In Vitro **19**, 335-352.

Araki N, Ohno K, Nakai M, Takeyoshi M, Iida M. (2005b) Screening for androgen receptor activities in 253 industrial chemicals by in vitro reporter gene assays using AR-EcoScreen cells. Toxicol In Vitro, 19, 831-42.

Blair, R.M., Fang, H., Branham, W.S., Hass, B.S., Dial, S.L., Moland, C.L., Tong, W., Shi, L., Perkins, R. and Sheehan, D.M. (2000) The Estrogen Receptor Relative Binding Affinities of 188 Natural and Xenochemicals: Structural Diversity of Ligands. Toxicol. Sci. **54**, 138-153.

Blankvoort, B.M., de Groene, E.M., van Meeteren-Kreikamp, A.P., Witkamp, R.F., Rodenburg, R.J. and Aarts, J.M. (2001) Development of an androgen reporter gene assay (AR-LUX) utilizing a human cell line with an endogenously regulated androgen receptor. Analytical Biochemistry **298**, 93-102.

Coecke, S., Ahr, H., Blaauboer, B.J., Bremer, S., Casati, S., Castell, J., Combes, R., Corvi, R., Crespi, C.L., Cunningham, M.L., Elaut, G., Eletti, B., Freidig, A., Gennari, A., Ghersi-Egea, J.F., Guillouzo, A., Hartung, T., Hoet, P., Ingelman-Sundberg, M., Munn, S., Janssens, W., Ladstetter, B., Leahy, D., Long, A., Meneguz, A., Monshouwer, M., Morath, S., Nagelkerke, F., Pelkonen, O., Ponti, J., Prieto, P., Richert, L., Sabbioni, E., Schaack, B., Steiling, W., Testai, E., Vericat, J.A. and Worth, A. (2006) Metabolism: a bottleneck in in vitro toxicological test development. The report and recommendations of ECVAM workshop 54. Altern. La.b Anim. **34**, 49-84

ECB (European Chemical Bureau) [URL: http://ecb.jrc.it/ accessed 20.09.06.]

EDMVS (2002) Endocrine Disruptor Methods Validation Subcommittee (EDMVS) Third Plenary Meeting March 25-27,
[URL: http://www.epa.gov/scipoly/oscpendo/meetings/mtg_032502.htm accessed 20.09.06.]

EDSTAC (1998) Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) Final report. [URL: http://www.epa.gov/scipoly/oscpendo/pubs/edspoverview/finalrpt.htm accessed 20.09.06.]

Gray, L.E. Jr. (1998) Tiered screening and testing strategy for xenoestrogens and antiandrogens. Toxicol. Lett. **102-103**, 677-680.

Hartung, T., Bremer, S., Casati, S., Coecke, S., Corvi, R., Fortaner, S., Gribaldo, L., Halder, M., Hoffmann, S., Roi, A.J., Prieto, P., Sabbioni, E., Scott, L., Worth, A., and Zuang, V. (2004) A modular approach to the ECVAM principles on test validity. ATLA **32**, 467-472.

ICCVAM (2003) ICCVAM Evaluation of In Vitro Test Methods for Detecting Potential Endocrine Disruptors: Estrogen Receptor and Androgen Receptor Binding and Transcriptional Activation Assays. [URL: http://iccvam.niehs.nih.gov/methods/endocrine.htm#fineval accessed 22.09.06.]

Kemppainen, J.A. and Wilson, E.M. (1996) Agonist and antagonist activities of hydroxyflutamide and Casodex relate to androgen receptor stabilization. Urology 48, 157-163.

Kenakin, T. (1993) Competitive antagonism. In Pharmacological Analysis of Drug-Receptor Interaction. Second Edition, pp. 278-322. Raven Press, New York.

Kojima, H., Iida, M., Katsura, E., Kanetoshi, A., Hori, Y. and Kobayashi, K. (2003) Effects of a diphenyl ether-type herbicide, chlornitrofen, and its amino derivative on androgen and estrogen receptor activities. Environmental Health Perspectives **111**, 497-502.

Kojima, H., Katsura, E., Takeuchi, S., Niiyama, K. and Kobayashi, K. (2004) Screening for estrogen and androgen receptor activities in 200 pesticides by in vitro reporter gene assays using chinese hamster ovary cells. Environmental Health Perspectives **112**, 524-531.

Miyamoto, H., Yeh, S., Wilding, G. and Chang, C. (1998) Promotion of agonist activity of antiandrogens by the androgen receptor coactivator, ARA70, in human prostate cancer DU145 cells. Proceedings of the National Academy of Sciences of the United States of America **95**, 7379-7384.

Miyamoto, H., Rahman, M., Takatera, H., Kang, H.Y., Yeh, S., Chang, H.C., Nishimura, K., Fujimoto, N. and Chang, C. (2002) A dominant-negative mutant of androgen receptor coregulator ARA54 inhibits androgen receptor-mediated prostate cancer growth. Journal of Biological Chemistry **277**, 4609-4617.

OECD (2001) OECD 3rd meeting of the validation management group for the screening and testing of endocrine disrupters (mammalian effects). ENV/JM/TG/EDTA (2001). Paris: Joint meeting of the chemicals committee and the working party on chemicals, pesticides and biotechnology .

OECD (2002) OECD conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals [URL: http://www.oecd.org/dataoecd/17/33/23652447.doc accessed 22.09.06.]

OECD (2006) OECD draft Detailed Review Paper, "The use of metabolising systems for in vitro testing of endocrine disruptors"

Satoh, K., Ohyama, K., Aoki, N., Iida, M. and Nagai, F. (2004) Study on anti-androgenic effects of Bisphenol A Diglycidyl Ether (BADGE), Bisphenol F Digly-cidyl Ether (BFDGE) and their derivatives using cells stably transfected with human androgen receptor, AR-EcoScreen. Food and Chemical Toxicology **42**, 983-993.

Schrader, T. J. and Cooke, G. M. (2000) Examination of selected food additives and organochlorine food contaminants for androgenic activity in vitro. Toxicological Sciences **53**, 278-288.

Sonneveld, E., Riteco, J.A., Jansen, H.J., Pieterse, B., Brouwer, A., Schoonen, W.G. and van der Burg, B. (2006) Comparison of in vitro and in vivo screening models for androgenic and estrogenic activities. Toxicol. Sci. **89**, 173-187.

Suzuki, T., Kitamura, S., Khota, R., Sugihara, K., Fujimoto, N. and Ohta, S. (2005) Estrogenic and antiandrogenic activities of 17 benzophenone derivatives used as UV stabilizers and sunscreens. Toxicol. Appl. Pharmacol. **203**, 9-17.

Takeyoshi, M., Yamasaki, K., Sawaki, M., Nakai, M., Noda, S. and Takatsuki, M. (2002) The efficacy of endocrine disruptor screening tests in detecting anti-estrogenic effects downstream of receptor-ligand interactions. Toxicol. Lett. **126**, 91-98.

Tamura, H., Maness, S.C., Reischmann, K., Dorman, D.C., Gray, L.E. and Gaido, K.W. (2001) Androgen receptor antagonism by the organophosphate insecticide fenitrothion. Toxicological Sciences **60**, 56-62.

Wong, C., Kelce, W.R., Sar, M. and Wilson, E.M. (1995) Androgen receptor antagonist versus agonist activities of the fungicide vinclozolin relative to hydroxyflutamide. Journal of Biological Chemistry **270**, 19998-20003.

APPENDIX-1 PRE-VALIDATION STUDY PLAN

AR-EcoScreen™

STUDY PLAN

Prevalidation study for the AR mediated reporter gene assay using AR-EcoScreen™ cell line

Study No. CR05-PV0001

Study Dates:	Initiation of Study: 20 Jun 2003 Completion of Study: 15 Aug 2003
Testing Facility:	Otsuka Pharmaceutical Co, Ltd. 224-18 Ebisuno, Hiraishi, Kawauchi-cho, Tokushima-shi, Tokushima, JAPAN
Study Director:	Mitsuru Iida, PhD
Investigator(s):	Naohiro Araki, Ken Ohno, Emiko Nishikawa, Katsue Yamada

Signature Page

We, the undersigned, certify that this report accurately reflects all relevant data collected in this study.

Prepared by:
Study Director

Naohiro Araki Date
Researcher
EcoScreen R&D Section, Diagnostic Division

Reviewed and approved by:
Director

Mitsuru Iida Date
Director of Section
EcoScreen R&D Section, Diagnostic Division

1. Materials and Methods

1.1 Test and Reference Compounds

1.1.1 Test Compounds

Table 1 Test Compounds

Number	Chemical Name	Lot no.	Purity (%)	Supplier
1	Diethylstilbestrol	PKQ7182	97	Sigma
2	Methyltrienolone (R1881)	3147-179	97	Daiichi Chem
3	Cyproterone acetate	0010315-X	99.98	Sigma
4	Fluoxymestrone	A004112291	97	Kanto
5	Dexamethasone	WAR7210	99	Wako
6	17β-Estradiol	ASR2564	97	Wako
7	Flutamide	G28585	98	Sigma
8	Medroxyprogesterone acetate	TPE2317	98	Wako
9	Testosterone	TCE7702	97	Wako
10	4-Androstenedione	49H0634	98	Sigma
11	Di - *n* -butyl phthalate	JSE9760	99.5	Wako
12	Diethylhexyl phthalate	RWQ9074	99.5	Wako
13	5α-Dihydrotestosterone	ASQ7976	95	Wako
14	Estrone	DWF5588	98	Wako
15	Linuron	MLP9891	99.5	Wako
16	*p,p'*- Methoxychlor	RWR9082	97	Wako
17	Spironolactone	023K1300	97	Sigma
18	Sodium azide	TCM6820	99	Wako
19	4 - *tert* -Octylphenol	JSL9944	98	Wako
20	Procymidone	RWK9785	99.5	Wako
21	*p* - *n* -Nonylphenol	JSJ9459	98.7	Wako
22	Bisphenol A	MLL9662	99	Wako
23	Progesterone	ASQ7977	98	Wako
24	*p,p'* -DDE	JSF9541	99	Wako
25	Finasteride	23920302	99	LKT labo
26	Hydroxyflutamide	1FRA-91-1	100	LKT labo
27	4-Hydroxytamoxifen	4636C	98	Sigma
28	Actinomycin D	DWH6818	97	Wako
29	Vinclozolin	RWR9057	99	Wako
30	Atrazine	YWM9872	98	Wako
31	Mifepristone	2399105	99.3	Wako
32	Fluoranthene	J2561A	98	Wako
33	Kepone	121800MT	NA	AccuStandard
34	*o,p'* -DDT	10955	99	AccuStandard
35	Corticosterone	370-007	95	Wako
36	17α-Ethinyl estradiol	3713F	99	ICN
37	Ketoconazole	4671F	99	Wako
38	Methyl testosterone	TCM7092	97	Wako
39	12 - *O* -Tetradecanoylphorbol-13-acetate	L10617	99	Wako
40	2,4,5-Trichlorophenoxyacetic acid	MLG9061	98	Wako

Storage conditions: -80°C under protection from light.

1.1.2 Reference Compound

Table 2 Reference Compound

Compound Name	Lot Number	Supplier
5α-Dihydrotestosterone (DHT)	ASQ7976	Wako
Hydroxyflutamide (HF)	1FRA-91-1	LKT labo
Methyltrienolone (R1881)	3147-179	Daiichi Chem
Cycloheximide (CHX)	WK036-18371	Wako

1.1.3 Solvent

Table 2 Solvent

Name	Lot Number	Supplier
Dimethyl sulfoxide (DMSO)	45-24511	Wako Pure Chemical
Ethanol	KSM 8342	Wako Pure Chemical
distilled water	NA	NA

1.2. Reagents

Table 4 Reargents

Reagent Name	Lot Number	Supplier
Dulbeco's modified eagle medium (DMEM)/F12	1166524	Gibco
Charcol dextran-treated fetal bovine serum (c-FCS)	AML7637	Hyclone
Trypsin EDTA 10 ×	1156364	Gibco
Phosphate-buffered saline (PBS)	AE1G111	Nikken
Steady Glo Luciferase Assay System	14969	Promega
Zeocin	116752Z	Invitrogen
Hygromicin	22-03-HGL	Invitrogen

1.3. Prepared Reagents

- Cell culture medium (DMEM/F12, 10% fetal bovine serum (FBS), penicillin-streptomycin; FBS was inactivated by incubation at 56°C for 30 min. To prepare the media, 50 mL FBS and 5 mL PS were added to a 500-mL bottle of DMEM and stored at 4°C.
- Assay medium (DMEM/F12, 10% charcol dextran-treated fetal bovine serum (c-FCS), penicillin-streptomycin); c-FCS was inactivated by incubation at 56°C for 30 min. To prepare the media, 50 mL cFCS and 5 mL PSG were added to a 500-mL bottle of DMEM and stored at 4°C.
- 1 × trypsin EDTA solution; Ten mL of 10 × trypsin EDTA solution was diluted with 90 mL phosphate-buffered saline (PBS) and stored at 4°C.

1.4. Equipment

Table 3 Equipment

Name	Supplier	Model	Place
Centrifuge	Eppendorf	5804R	EcoScreen laboratory Room 10
Luminometer	Wallac	ARVO-SX	EcoScreen laboratory Room 12
Top loading balance	Metler	AT201	Shared storage room

1.5 Preparation of Test and Reference Compounds

Test and reference compounds were dissolved in DMSO at a concentration of 10^{-2} to 1M. .Sodium azide and 17α ethinyl estradiol will be dissolved in distilled water and ethanol, respectively. For the agonist detection assay, the six substance concentrations (e.g. 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} M) spaced at log intervals were prepared by diluting with DMSO. Then the test and reference compounds were diluted with serum-free DMEM 100 fold and 10 μL of these were added to the cell culture plates. Thus, the final concentration of DMSO in the cell growth medium was 0.1%. For the antagonist detection assay, the five substance concentrations (e.g. 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} M) spaced at log intervals were prepared by diluting with DMSO. Then the test and reference compounds were diluted with 5.6×10^{-8} M

DHT and serum-free DMEM 100 fold and 10 μL of these were added to the cell culture plates. Final DHT concentration of culture medium will be 5.6×10^{-10} M, which will produce approximately 80% of the maximal AR-mediated signal.

1.7 Test System

1.7.1 Cell Culture

1.7.1.1. Cell line for AR agonism and antagonism detection

1) Name of cell line: AR-EcoScreen™
2) Source: Ovary, Chinese Hamster
3) Supplier: Otsuka Pharmaceutical Co, Ltd.
4) Pre-culture conditions: DMEM/F12, 1 × PS, 10% FBS, 25 μg/mL hygromycin, 50 μg/mL zeocin.
    a) Medium: DMEM/F12
        i) Supplier: Gibco
        ii) Lot number: 1166524
    b) Serum: heat inactivated FBS
        i) Supplier: JRH
        ii) Lot number: 3J0482
        iii) FBS was inactivated by incubation at 56°C for 30 min
        iv) After heat inactivation, FBS was stored at -20°C
    c) Penicillin-Streptomycin , liquid
        i) Supplier: ICN
        ii) Lot number: 16700024
    d) Preparation of medium

    Fifty mL FBS and 5 mL PS were added to a 500-mL bottle of DMEM/F12.

1.7.1.2. Cell line for Cytotoxicity evaluation

1) Name of cell line: cLuc-EcoScreen™
2) Source: Ovary, Chinese Hamster
3) Supplier: Otsuka Pharmaceutical Co, Ltd.
4) Pre-culture conditions: DMEM/F12, 1 × PS, 10% FBS, 50 μg/mL zeocin.
    e) Medium: DMEM/F12
        i) Supplier: Gibco

ii) Lot number: 1166524

f) Serum: heat inactivated FBS

i) Supplier: JRH

ii) Lot number: 3J0482

iii) FBS was inactivated by incubation at 56°C for 30 min

iv) After heat inactivation, FBS was stored at -20°C

g) Penicillin-Streptomycin , liquid

i) Supplier: ICN

ii) Lot number: 16700024

h) Preparation of medium

Fifty mL FBS and 5 mL PS were added to a 500-mL bottle of DMEM/F12.

1.7.2. Experimental Design

Table 4 Experimental Design for Agonist Assay

	Group Name	Dose or Concentration	N or # trialsa	Incubation time
1	DMSO	0.1 %	6	18 h
2	Test Compound	10^{-9} M	3	18 h
3	Test Compound	10^{-8} M	3	18 h
4	Test Compound	10^{-7} M	3	18 h
5	Test Compound	10^{-6} M	3	18 h
6	Test Compound	10^{-5} M	3	18 h
7	Test Compound	10^{-4} M	3	18 h
8	Test Compound	10^{-3} M	3	18 h
9	Methyltrienolone	10^{-8} M	3	18 h
10	5α-Dihydrotestosterone	10^{-8} M	3	18 h

Table 5 Experimental Design for Antagonist Assay

	Group Name	Dose or Concentration	N or trials	Incubation time
1	DMSO	0.1 %	3	18 h
2	Test Compound	10^{-8} M	3	18 h
3	Test Compound	10^{-7} M	3	18 h
4	Test Compound	10^{-6} M	3	18 h
5	Test Compound	10^{-5} M	3	18 h
6	Test Compound	10^{-4} M	3	18 h
7	Test Compound	10^{-3} M	3	18 h
8	5α-Dihydrotestosterone (DHT)	5×10^{-10} M	12	18 h

Table 5 Experimental Design for Antagonist Assay				
	Group Name	**Dose or Concentration**	**N or trials**	**Incubation time**
9	Methyltrienolone (R1881)	10^{-8} M	3	18 h
10	Hydroxyflutamide (HF)	10^{-7} M	3	18 h
11	Cycloheximide (CHX)	1 μg/mL	3	18 h

A rationale for dose (or concentration) determinations: Limit concentration will be 1mM based on the ICCVAM recommendation. Some test chemicals will be tested from the lower concentration, depend on their solubility. Each trial was tested using three replicates for each concentration. aEach trial was carried out with three independent plates that were prepared from the different bathes of the cell culture.

1.8 Experimental Procedures

1.8.1 Thawing the Cells

The cell lines stored in liquid nitrogen was carefully thawed in a 37°C water bath. As soon as the ice melted, the cells were transferred into 50 mL pre-warmed culture medium. The cell pellet was formed by centrifugation at 1000 rpm for 5 min, the supernatant was discarded, and dissolved in 20 mL fresh culture medium. Then the resuspended cells were transferred to a 75-cm^2 plastic flask and incubated at 37°C in the CO_2 incubator.

1.8.2. Cell Culture Maintenance

The cell lines was maintained in the 175 cm^2 flask at 37°C and at 100% humidity in the CO_2 incubator. The flask was trypsinized 1 time/3 − 4 days to divide into a new flask.

1.8.3 Preparation for Testing

The medium was removed from the sub-confluent cell culture in a 175-cm^2 flask and washed with 20 mL PBS. The flask was trypsinized with 1.4 mL of 1 × trypsin EDTA solution for 5 min at 37°C and 20 mL culture medium was added to the flask. The cell solution was placed in a sterile 50-mL centrifuge tube and underwent spinning at 1000 rpm for 5 min. The cell pellet was suspended in 10 mL assay medium. The resuspended cells were diluted with the assay medium to prepare 1.0×10^5-cells/mL cell suspension. Ninety μL of 1.0×10^5-cells/mL cell suspension was transferred into a 96-well plate and incubated over night in the CO_2 incubator.

1.8.4. Sample Addition

Dosing medium was applied to the 96-well plates after 16 hours post-plating (Figure 1) and the plates were incubated for 16-24 hours following addition of the sample.

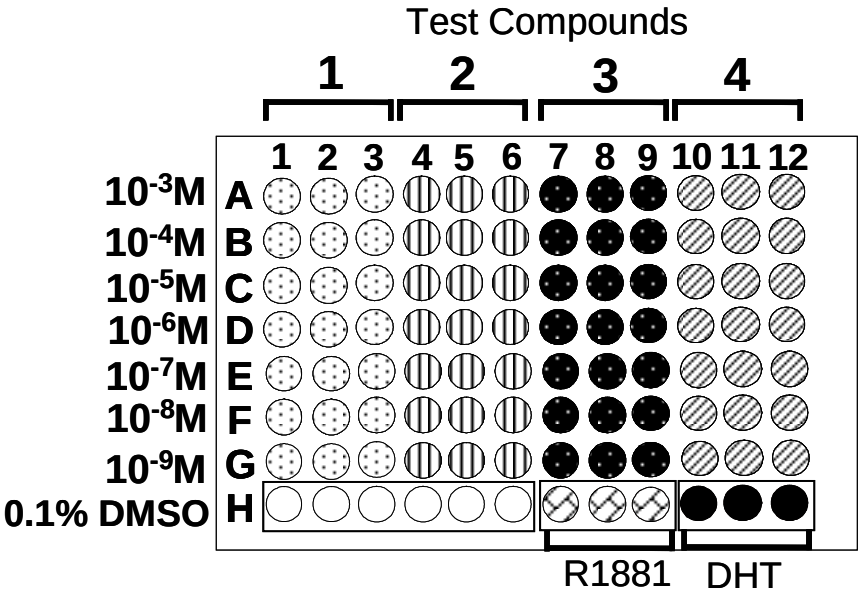


Figure 1 Plate Format for Agonist Assay

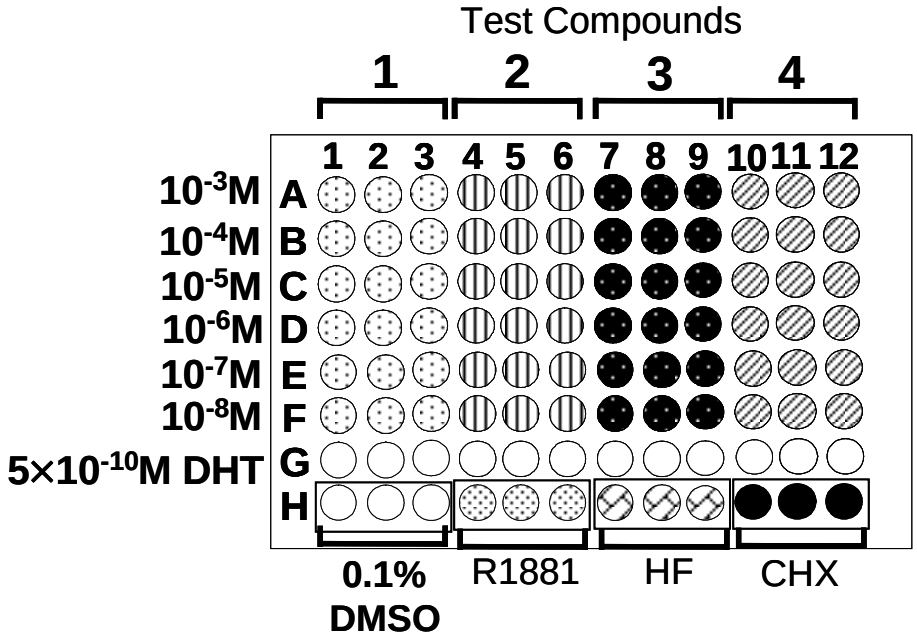


Figure 2 Plate Format for Antagonist Assay

1.8.5. Measurement

Luciferase activity was determined by adding 40 μL of Steady-Glo buffer per well, followed by incubation for 5 min at room temperature. Relative light units (RLU) were recorded using a luminometer.

1.9. Parameters Determined

The luminescence value (RLU) of each dose was converted to the relative value as follow.

- Agonist assay

  Fold Induction (FI) = mean RLU/mean blank response (0.1% DMSO).

- Antagonist assay:

  Relative value = (mean RLU - mean blank response (0.1% DMSO))/(mean 5×10^{-10} M DHT response - mean blank response (0.1% DMSO)).

- Cytotoxicity

  Relative value = (mean RLU- mean CHX RLU)/(mean 5×10^{-10} M DHT response - mean CHX RLU)

1.10 Statistical Analysis

Data will be collected with Microsoft Excel 2000 format. The compounds were evaluated using the FI and relative value. The level of significance was set as 5% for all statistical analyses. The EC_{50} and IC_{50} with a 95% confidence interval was calculated by four-parameter logistic equation of Prims 4 software (GraphPad, USA).

APPENDIX-2 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)

EDC Analysis Center, Otsuka Life Science Initiative,

Otsuka Pharmaceutical Co., Ltd.

224-18, Ebisuno Hiraishi, Kawauchi-cho, Tokushima

7710195, Japan

Testing facility 3 (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 4 (Enforcement of the study)

KANEKA Techno-Research Co., Ltd.

1-8, Miyamae-cho, Takasago-cho, Takasago-shi,

Hyogo 6768688, Japan

APPENDIX-3 STANDARD OPERATING PROCEDURE (SOP) FOR DETECTION OF ANDROGENIC AND ANTI-ANDROGENIC ACTIVITY USING THE REPORTER GENE ASSAY

STANDARD OPERATING PROCEDURE (SOP)

for detection of androgenic and anti-androgenic activity using the reporter gene assay

Description: This document provides a methodology for detecting the androgenic and anti-androgenic activity of chemicals by the reporter gene assay technique using the AR-EcoScreen^{TM} 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

5a-dihydrotestosterone (DHT)

3. Vehicle for chemical stock solutions

Dimethylsulfoxide (DMSO) should be used for the vehicle.

4. Materials

4.1 Cell lines

The AR-EcoScreen™ stable cell line (Otsuka Pharmaceutical Co., Ltd.) will be used for the assay.

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

Cells should be maintained in Dulbeco's modified Eagle medium/Ham's F-12 nutrient mix (DMEM/F-12) supplemented with a 10% fetal bovine serum (FBS), penicillin/streptomycin (100 U/mL), hygromycin (25 μg/ml) and zeocin (50 μg/ml) 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 (DHT), 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. 4.

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 for the agonism detection and Dual-Glo (Promega, E2920 and its equivalents) are used for the antagonism detection. A bottle of Luciferase Assay Substrate is

dissolved with the Luciferase Assay Buffer. A Stop & Glo substrate should be diluted with Stop & Glo buffer 100 fold before use. The dissolved substrate should either be used immediately or stored below -20ºC. 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 1mM, 100 µM, 10 µM, 1 µM, 100 nM and 10 nM working solutions. Exactly 10 µL of 10 mM chemical stock and 6 working solutions will dilute in serum-free DMEM/F12 (90 µL).

Then 10µL of the diluted test samples will dilute in DMEM/F12 (90 µL) again and added to each well of the assay plate according to the assignment table shown in Fig.1.

Positive control wells (n=6) treated with 10 nM of DHT 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 (10 nM of DHT)

4.8 Luciferase assay (See support protocol No. 5)

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.

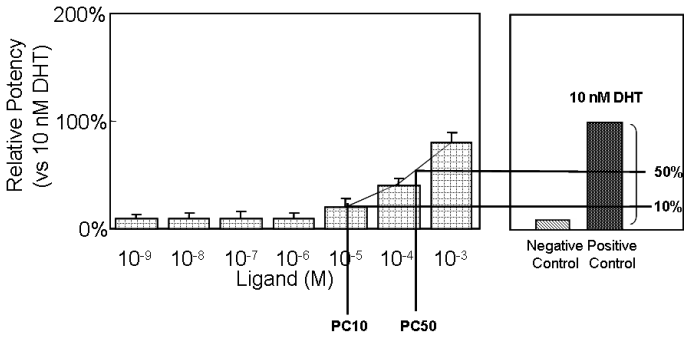


Fig. 2 Description of PC10 and PC50

SUPPORT PROTOCOLS

① No. 1. 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 10 mL pre-warmed 10%FBS-DMEM/F-12 in a 15 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 DMEM/F-12 and place it in a 75 cm^2 flask dish.
6. Incubate the cells in a 5% CO_2 incubator at 37°C.

SUPPORT PROTOCOLS

② No. 2. Propagation

2. Remove the medium from the culture dish with a sterile pipette or sucker.
3. Rinse the cell with 10 mL of PBS.
4. Remove the PBS with a sterile pipette or sucker.
5. Add 600 μL 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.
6. Allow the Trypsin treated cells to stand for about 5-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.)

7. Tap the dish gently.
8. Wash with 10 mL of 10%FBS-DMEM/F-12 to remove the adherent cells.
9. Count the number of cells.
10. Dilute the cell suspension with 10%FBS-DMEM/F-12 to 3.0-6.0 x 10^5 cells/20mL.
11. Place 20 mL of cell suspension in a 75 cm^2 flask.
12. Incubate the cells in a 5% CO_2 incubator at 37°C.

SUPPORT PROTOCOLS

① No. 3. Preparation of frozen stock

1. Remove the medium from the culture dish with a sterile pipette or sucker.
2. Rinse the cell with 10 mL of PBS.
3. Remove the PBS with a sterile pipette or sucker.
4. Add 600 μL 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-DMEM/F-12 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 10^4-10^6 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

② No. 4 Preparation of the assay plate

Prepare a dish of cultured AR-EcoScreen™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 600 µL 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 10 mL of 10% Charcoal Dextran treated Fetal Bovine Serum (c-FCS) DMEM/F-12 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% c-FCS DMEM/F-12 to obtain a final cell density of 1 x 10^5 cells/mL.
11. Add 90µL 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

③ No. 5-1. 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-4 PROTOCOL USED FOR THE INTER-LABORATORY VALIDATION STUDY

Study Code: R10-0014

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

February, 2005

Chemicals Evaluation and Research Institute
CERI-Japan

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

Participating laboratories

Testing facility 1 (coordination and enforcement of the study)
Chemical Evaluation and Research Institute
Hita Laboratory
3-822, Ishii-machi,Hita-shi, Oita, 877-0061, Japan

Testing facility 2 (enforcement of the study)
Otsuka Pharmaceutical Co., Ltd.
EDC analysis center
224-18, Ebisuno, Hiraishi, Kawauchi-cho, Tokushima, 771-0195, Japan.

Testing facility 3 (enforcement of the study)
Sumitomo Chemical Co., Ltd.
Environmental Health Science Laboratory
3-1-98, Kasuga denaka, Konohana-ku, Osaka, 544-8558, Japan

Testing facility 4 (enforcement of the study)
Kaneka Techno Research Co., Ltd.
Environmental Analysis Center
1-8, Miyamaemachi, Takasago-cho, Takasago, 676-8688, Japan

Aim of the study: To appraise the reliability and reproducibility of the AR 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 February 7, 2005
Completion Date March 31, 2005

Persons concerned in the study

Study director

Masahiro Takeyoshi
Chemical Evaluation and Research Institute, Hita Laboratory

Person in charge of individual studies conducted by each laboratory

Testing facility 1:

Hideki MIyaura
Chemical Evaluation and Research Institute, Hita Laboratory

Testing facility 2:

Mitsuru Iida
Otsuka Pharmaceutical Co., Ltd., EDC analysis center

Testing facility 3:

Kouichi Saito
Sumitomo Chemical Co., Ltd.
Environmental Health Science Laboratory

Testing facility 4:

Yoshio Hato
Kaneka Techno Research Co., Ltd.
Environmental Analysis Center

Quality Assurance Supervisor

Kouichiro Mizuguchi
Chemical Evaluation and Research Institute, Hita Laboratory

Peer reviewers

(To be announced)

Materials and Methods

Materials

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 – AR agonist

2.1 Chemical name

5α-Dihyrotestosterone (DHT)

2.2 Lot No. (To be announced)

2.3 Manufacturer

Wako Pure Chemicals, Japan

2.4 Storage

To be stored at room temperature in a shading bottle.

3. Positive control substance – AR antagonist

3.1 Chemical name

Hydroxyflutamide (HF)

3.2 Lot No.

1-FRA-91-1

3.3 Manufacturer

Wako Pure Chemicals, Japan

3.4 Storage

-20°C

4. Positive control substance – Cytotoxicity

4.1 Chemical name

Cycloheximide (Cx)

4.2 Lot No.

B45646

4.3 Manufacturer

Wako Pure Chemicals, Japan

4.4 Storage

To be stored at room temperature.

5. Vehicle control (vehicle for chemical stock solutions)

5.1 Chemical name

Dimethylsulfoxide (DMSO)

5.2 Lot No.

KLK5473

5.3 Manufacturer

Wako Pure Chemicals, Japan

5.4 Storage

To be stored at room temperature in a shading bottle.

6. Materials

6.1 Test systems

The AR-EcoScreen stable cell line (Otsuka Pharmaceutical Co., Ltd.) will be used for the assay. Each laboratory should conduct three series of assays with the four test chemicals listed in Table 1 on independent days.

6.2 Cell lines

AR-EcoScreen stable cell line will be provided by the CERI as a frozen vial.

6.3 Preparation of the medium

Three kinds of mediums should be used for the propagation of cell, preparation of the assay plate and dilution of the chemicals as below.

6.3.1 Medium No.1: for diluting the sample

D-MEM/F-12 Phenol Red Free (Gibco, #21041-025). Stored at 4°C.

6.3.2 Medium No.2 for cell propagation

At first, fetal bovine serum (FBS) (JRH, #12103-78P, Lot No.9B2018) is heat-inactivated at 56°C for 30 min. Twenty-five mL of heat-inactivated FBS and 5mL Penicillin/Streptocycin (Dainippon Pharmaceutical, #DNS1670049) are added to the 500 mL bottle of D-MEM/F-12 (Gibco, #DNS1670049) and stored at 4°C. Zeocin (100 mg/mL Zeocin™, Invitrogen, #R250-1) and Hygromycin (100 mg/mL HygroGold™, Invivigen, #ant-hg-1) should be added to the culture medium at 200 μg/mL and 100 μg/mL, respectively.

6.3.3 Medium No.3 for preparation of the assay plate

At first, charcoal dextran treated fetal bovine serum (C-FCS) (Hyclone, #SH30068.03) is heat-inactivated at 56°C for 30 min. Twenty-five mL of heat-inactivated C-FCS and 5mL Penicillin/Streptocycin (Dainippon Pharmaceutical, #DNS1670049) are added to the 500 mL bottle of D-MEM/F-12 (Gibco, #DNS1670049) and stored at 4°C.

Methods

1 Cell Culture

Cells should be incubated in the incubator set at 37°C and 5% CO_2.

0.05% Trypsin solution

Ten mL of 0.5% trypsin solution (Gibco, #15400-054) is diluted with 90 mL PBS (-) and store at 4°C.

1.2 Thawing the cells

Remove the vial from the liquid nitrogen or freezer and immediately transfer it to a 37°C water bath. While holding the tip of the vial, gently agitate the vial. When completely thawed, transfer the cell stock into 10mL pre-warmed Medium No.2 in a 15 mL conical tube (Falcon, #36-2096, or equivalent). Centrifuge the tube at 1000 rpm (200-300×g) for five minutes; then remove the supernatant carefully. Resuspend the cell with 10 mL of Medium No.2 and place an adequate number of the cells ($3\text{-}6\times10^5$ cells/20mL) in a 75 cm^2 culture flask. Incubate the cells in a 5% CO_2 incubator at 37°C.

1.3 Propagation

Remove the medium from the culture flask with a sterile pipette or sucker. Rinse the cell with 10 mL of PBS. Remove the PBS with a sterile pipette or sucker. Add 600 μL of Trypsin-EDTA solution, enough to coat the bottom of the culture flask. Allow the Trypsin-treated cell to stand for about five 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. Tap the flask gently and wash with 5 mL of Medium No.2 to remove the adherent cells. Count the number of cells and dilute the cell suspension with medium

containing 200 μg/mL zeocin and 100 μg/mL hygromycin to 3-6×10^5 cells/20mL in a 75 cm^2 culture flask. Incubate the cells in a 5% CO_2 incubator at 37°C.

1.4 Preparation of frozen stock of the cells

Remove the medium from the culture flask with a sterile pipette or sucker. Rinse the cell with 10 mL of PBS. Remove the PBS with a sterile pipette or sucker. Add 600 μL of Trypsin-EDTA solution, enough to coat the bottom of the culture flask. Allow the Trypsin-treated cell to stand for about five 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. Tap the flask gently and wash with 5 mL of Medium No.2 to remove the adherent cells. Count the number of cells. Centrifuge the tube at 1000 rpm (200-300×g) for five minutes, and remove the supernatant carefully. Add Cell-Banker* (Juji Field Inc.) and resuspend the cell at a density of ca. 1 × 10^4 -10^6 cells/mL. Make 1 mL aliquots of cell stock. 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).

1.5 Preparation of the assay plate

Remove the medium from the culture dish with a sterile pipette or sucker. Rinse the cells with 10 mL of PBS. Remove the PBS with a sterile pipette or sucker. Add 2 mL of Trypsin-EDTA solution, enough to coat the bottom of the culture dish, and then remove the excess. 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. Tap the dish gently. Wash with 10 mL of Medium No.3 to remove the adherent cells. Count the number of cells. Dilute the cell suspension with Medium No.3 to obtain a final cell density of 1 × 10^5 cells/mL. Add 90μL of cell suspension into each well of a 96-well assay plate (Nunc #136101 or equivalent). Incubate the cell in a 5% CO_2 incubator at 37°C for over night (24 hours).

2. Preparation of chemicals

Positrive control and test chemicals are dissolved in DMSO at 10 mM by CERI and shipped to each laboratory before test. The chemicals are stored at -20°C.

3. Preparation of positive control for cytotoxicity

Cycloheximide, postitive control for cytotoxicity, is dissolved at 10 mg/mL in DMSO and stored at -20°C.

4. Preparation of dosing medium for agonist assay

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 positive control in triplicate.

To achieve the above-described test conditions, 25 μL of each chemical stock solution provided by CERI are added to A1, A4, A7 well of 96 well plate, and 90 μL of DMSO added to the gray colored wells (B1 to H1, B4 to H4, B7 to G7, B10 to G10) and 90 μL of medium No.1 is added to the shaded column (No.2, 3, 5, 6, 8, 9, 11, 12) as Fig.2. Ten μL of 10mM stock solution in A1, A4 and A7 are transferred to the row B and then repeat this dilution to the row G of the plate to prepare 1mM to 10 nM serial dilution in DMSO. For DHT, 1mM diluted stock solution should be added to the A10 well and serial dilution in DMSO should be prepared as the test sample. For the quality control of the plate, 15 μL of 10 μM DHT are added to the H7 and H10 well, respectively. Using 8-channel pipette, 10μL of column 1 should be transferred to column 2 and then column 2 to column 3. Column 4-6, column 7-9, column 10-12 should be prepared in a same way. Column 3, 6, 9 and 12 are dosed to the cell plate.

Fig.2 Plate assignment of diluting the test chemicals for the agonist assay

	Test Chemical 1			Test Chemical 2			Test Chemical 3			PC (DHT)		
	1	2	3	4	5	6	7	8	9	10	11	12
A	10 mM			10 mM			10 mM			1 mM		
B	1 mM			1 mM			1 mM			100 µM		
C	100 µM			100 µM			100 µM			10 µM		
D	10 µM			10 µM			10 µM			1 µM		
E	1 µM			1 µM			1 µM			100 nM		
F	100 nM			100 nM			100 nM			10 nM		
G	10 nM			10 nM			10 nM			1 nM		
H	DMSO			DMSO			PC (DHT)			PC (DHT)		

Gray:DMSO, Shaded: Medium No.1,

Preparation of dosing medium for antagonist assay

In the 96 well plate, 25 µL of each chemical stock solution are added to A1, A4, A7 well and 90 µL of DMSO added to the gray colored wells (B1 to H1, B4 to G4, B7 to G7, B10 to G10) and 90 µL of medium No.1 is added to the shaded column (No.3, 6, 9, 12, H2 and H5 well), and 56 nM DHT 0.1% DMSO solution is added to the backed column (No. 2, 5, 8, 11) as Fig.3. Ten µL of 10mM stock solution in A1, A4 and A7 are transferred to the row B and then repeat this dilution to the row F of the plate to prepare 1mM to 100 nM serial dilution in DMSO. For HF, 1mM diluted stock solution should be added to the A10 well and serial dilution in DMSO should be prepared as the test sample. For the quality control of the plate, 15 µL of 10 µM DHT is added to the H4, 15 µL of 1mM HF is to the H7 well, and then 15 µL of 10mg/mL Cx is to the H7 well, respectively. Using 8-channel pipette, 10µL of column 1 should be transferred to column 2 and then column 2 to column 3. Column 4-6, column 7-9, column 10-12 should be prepared in a same way. Column 3, 6, 9 and 12 are dosed to the cell plate.

Fig.3 Plate assignment of diluting the test chemicals for the antagonist assay

	Test Chemical 1			Test Chemical 2			Test Chemical 3			PC (HF)		
	1	2	3	4	5	6	7	8	9	10	11	12
A	10 mM			10 mM			10 mM			1 mM		
B	1 mM			1 mM			1 mM			100 µM		
C	100 µM			100 µM			100 µM			10 µM		
D	10 µM			10 µM			10 µM			1 µM		
E	1 µM			1 µM			1 µM			100 nM		
F	100 nM			100 nM			100 nM			10 nM		
G	DMSO			DMSO			DMSO			DMSO		
H	DMSO			DHT			HF			Cx		

Gray :DMSO, Shaded: Medium No.1, Black: 56nM DHT 0.1% DMSO.

5 Chemical Exposure

5.1 Chemical Exposure for agonist assay

From the column no.3, 6, 9 and 12 of the plate prepared for dilution (Fig.2), 10 µL of serially diluted sample will be added to each well of the assay plate according to the assignment as Fig.4. After adding the chemicals, the assay plate will be incubated in a CO_2 incubator for 20-24 hours to induce the reporter products.

Fig. 4 Plate assignment for the agonist assay

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

NC: Negative Control (0.1% DMSO), PC: Positive Control (10nM DHT)

5.2 Chemical Exposure for antagonist assay

From the column no.3, 6, 9 and 12 of the plate prepared for dilution (Fig.2), 10 µL of serially diluted sample will be added to each well of the assay plate according to the assignment as Fig.4. Row H will be used as solvent control wells. After adding the chemicals, the assay plate will be incubated in a CO_2 incubator for 20-24 hours to induce the reporter products.

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

Gray: 500 pM DHT spiked, VC: Vehicle Control (0.1% DMSO), NC: Negative Control (0.1% DMSO), PC (DHT) Positive Agonist Control (10nM DHT), PC (HF) :Postive Antagonist Control (100nM HF), Cx: Positive Toxicity Control: 10 µg/mL Cx

6 Reagent

6.1 Reagent for agonist assay

Glo-type luciferase assay reagent: A vial of 100 mL of luciferase assay buffer (Promega, #E4550, or equivalent) is added into a vial containing luciferase assay substrate (Promega, #E4550, or equivalent), and dissolve the substrate thoroughly. Store the substrate below -20°C before use.

Flash-type luciferase assay reagent: A vial of luciferase assay buffer (Promega, #E1500, or equivalent) is added into a vial containing luciferase assay substrate (Promega, #E1500, or equivalent), and dissolve the substrate thoroughly. Store the substrate below -20°C if necessary. Dilute 5 mL of 5×Cell Culture Lysis Reagent (Promega, #E1531, or equivalent) with 45 mL of distilled water and store at 4°C before use.

6.2 Reagent for antagonist assay

Dual Glo-type luciferase assay reagent: A vial of 100 mL of Dual-Glo luciferase assay buffer (Promega, #E2920, or equivalent) is added into a vial containing Dual-Glo luciferase assay substrate (Promega, #E2920, or equivalent), and dissolve the substrate thoroughly. Store the substrate below -20°C before use.

Dilute stop&Glo substrate with 100× volume of Stop&Glo buffer stored at 4°C before use.

7. Chemiluminescence detection

7.1 Chemiluminescence detection of agonist assay

Add 40 µL of Steady-Glo luciferase buffer to the assay plate incubated over night at 37°C in CO_2 incubator. Shake the plate for 5 min at room temperature. Luminescence intensity will be recorded with a luminometer.

7.2 Chemiluminescence detection of antagonist assay

Remove 60 µL of culture supernatant from the assay plate incubated over night at 37°C in CO_2 incubator and add 40 µL of Dual-Glo luciferase buffer. Shake the plate for 10 min at room temperature. Luminescence intensity will be recorded with a luminometer. Furthermore, 40µL of Stop-Glo luciferase buffer which is prepared by 1/100 dilution of Stop&Glo substrate will be added and shake the plate for additional 10 min at room temperature. Then, again luminescence intensity will be recorded with a luminometer.

8. Analysis of data

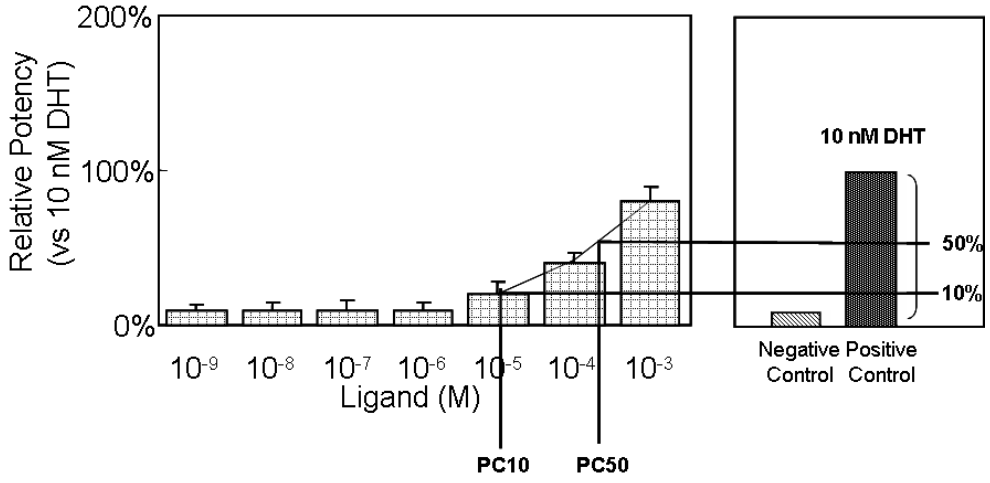
8.1 Analysis of data for agonist assay

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 can be calculated with the following equation by the commercial software (GraphPad® PRIZM GraphPad software Inc.):

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.



8.2 Analysis of data for antagonist assay

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 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, IC50 can be calculated with the following equation by the commercial software (GraphPad® PRIZM GraphPad software Inc.):

9. 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.

10. 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.

11. 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.

12. 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.

13. 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

HR code No.	Name	CAS No.	Lot.	Storage Condition
HR5971	Methyltrienolone	965-93-5	3411-228	-20°C
HR5972	Hydroxyflutamide	52806-53-8	1-FRA-19-1	-20°C
HR5561	Bisphenol A	80-05-7	GF01	RT
HR5565	Diethylhexyl phthalate	117-81-7	ELE1799	RT

APPENDIX-5 CONSIDERATION OF THE EDGE EFFECTS ON THE ASSAY SYSTEM

1) Experiment

The distribution of luminescent intensity in a assay plate were examined by measuring the chemiluminescence signal of half the wells on a assay plate stimulated with the positive control substance, 100pM of DHT or the vehicle (dimethylsulphoxide, DMSO, final concentration at 0.1%). This experiment was conducted according to the SOP.

Data obtained in this experiment were shown in Table 1. To ensure the edge effect, we analyzed the results by t-test: Two-Sample Assuming Equal Variances, and no significant differences were observed between edge and center in the vehicle control plate treated with DMSO and in the positive control plate treated with 100pM of DHT(Table 2 and Table 3).

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

Table 1 The raw data of the luminesecence intensity of each well in 100pM of DHT treated the left half of plate and in DMSO treated the right half of plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	76500	72828	74396	74092	72580	75676	16428	15128	15544	15876	15900	15800
B	73340	74880	72496	72232	68268	70088	16220	15180	14924	15336	14904	15468
C	71820	69604	68648	69412	68472	67596	15244	14416	14420	14680	20388	15816
D	73128	70164	70056	67176	65724	67880	15476	14416	14648	14616	14656	15388
E	74628	71940	71716	69776	66008	67160	14876	14336	14140	14020	14164	14100
F	75092	73120	74012	69768	67780	70452	15216	14232	14656	14460	15052	14248
G	76864	74172	74040	73108	69216	69540	14784	14740	14504	14336	14400	14472
H	78364	75368	77736	75188	71732	70472	15468	14460	14908	15044	14836	14652

Screened wells mean the edge.

Table 2 t-test of chemiluminescent signals in the assay plate with 100pM of DHT

	center	edge
mean	71222	72647
variance	8289809	12151713
Observations	30	18
degree of freedom	46	
t Stat	-1.53	
P-value two-tailed	0.13	

Table 3 t-test of chemiluminescent signals in the assay plate with DMSO

	center	edge
mean	14915	15196
variance	1286998	425470
Observations	30	18
degree of freedom	46	
t Stat	-0.96	
P-value two-tailed	0.34	

Report of the Second Validation Study For Androgen Receptor (AR)-Mediated Stably Transfected Transcriptional Activation (AR-STTA) Assay to Detect Androgenic and Anti-androgenic Activities of Chemicals: AR EcoScreen™

Prepared by

Study management team of the 2nd validation study of AR STTA
2015

Index

1. SUMMARY

1. The AR STTA assay (AR EcoScreen^{TM}) is a trans-activation assay based on Chinese Hamster Ovary cells stably transfected with human AR and an AR response element fused to a luciferase reporter gene. This assay is designed to screen for substances that may induce (agonist) or inhibit (antagonist) AR-mediated transcription. The cell line employs androgen responsive element (ARE) from prostate C3 gene-responsive element driven by a minimal heat shock protein promoter. This construct is confirmed to have minimal induction of glucocorticoid receptor (GR) mediated responses. AR Ecoscreen has the great advantage to provide AR specific response with minimal GR crosstalk.

2. The validation report of this assay system has been submitted to OECD in 2010. However the Peer review panel report stated that a dedicated inter-laboratory study should be carried out, using the final test protocol to test substances covering a broad range of activity, especially including non-active substances and weak agonists and antagonists as a major recommendation. This report describes the results of an additional inter-laboratory validation study corresponding to the first major recommendation of Peer review comment for the first validation report. As for the other major recommendations, responses are stated in Appendix 2.

3. The additional validation study consisted of Phase-1 and Phase-2 studies. The Phase-1 study was to confirm the overall laboratory proficiency by testing the same lot number of reference chemicals and to collect data to set an acceptability criteria for mestanolone which was the newly added reference chemical for the agonist study. The Phase-2 study was to provide the supplemental data according to previous peer review comments of this assay and to evaluate the assay performance (within/between-laboratory reproducibility and predictive capacity) by blind testing of 5 coded chemicals for agonistic and 5 for antagonistic activities.

4. In the Phase-1 study, all laboratories passed the acceptability criteria within the minimum three runs. The inexperienced Korean laboratory yielded successful results for the additional reference chemical for the agonist assay, mestanolone that met the tentative acceptability criteria decided based on the results obtained with three Japanese laboratories. In the Phase-2 agonist study, all laboratories yielded correct positive/negative outcomes corresponding to the candidate effects. Consequently, the Accuracy, Sensitivity and Specificity of the agonist assay were all calculated to be 100% in all laboratories. In addition, the %CV of LogPC10(M) and LogPC50(M) for positive chemicals were less than 5% and high reproducibility of this assay was confirmed.

5. In the Phase-2 antagonist study, the Accuracy, Sensitivity and Specificity for all four laboratories were calculated to be 95%, 92% and 100%, respectively, due to the false negative response of one chemical in one laboratory. The cause of the false negative response for the chemical was confirmed to be a dose-selection issue rather than a specificity issue. In addition, the %CV of LogIC30(M) and LogIC50(M) for positive chemicals in the additional trial were less than 4%, and high reproducibility of this assay was confirmed. Therefore, the concordance of positive/negative outcomes of coded test chemicals were more than 80% for each of agonist and antagonist assay, and the high performance of this assay was confirmed.

6. The results of the additional validation study show that the original protocol is well established and robust, however the maximum dose selected by the solubility test described in the original protocol may occasionally affect the sensitivity of the assay. Therefore the following sentence should be including in the section of solubility test in the guideline:

   "A solubility test is a very important step to determine the maximum concentration for the assay and it

may affect the sensitivity of the assay. Maximum concentration should be selected based on the avoidance of precipitation at highest concentration ranges in culture media. Precipitation observed at any concentration should be noted, but these data should not be included in the dose-response analysis."

2. INTRODUCTION

7. Numerous natural compounds in the environment, as well as many synthetic compounds, may disrupt the endocrine functions of wildlife and humans. At the present time, there is a global concern regarding endocrine disruption effects resulting from chemical exposure, particularly those mediated by the estrogen receptor (ER) and androgen receptor (AR). 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. The Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) convened by the EPA recommended a tiered testing approach for the evaluation of endocrine, androgen and thyroid related effects of commercial chemicals and environmental contaminants (EDSTAC, 1998). Under this testing paradigm, Tier I screening would identify chemicals with a potential to affect the estrogen, androgen and thyroid systems. Since EDSTAC recommended that in vitro assays, such as receptor binding and reporter gene assays, be used as part of a tier 1 screening battery, many efforts have been taken to develop reporter gene assay systems for evaluating ER and AR mediated effects of chemicals.

8. Several reporter gene assay systems 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. Currently no *in vitro* assays screening (anti)androgenic activity of chemicals have been peer reviewed for potential test guideline development and subsequent use for OECD regulatory purposes, however the need is recognized and understood to be urgent.

9. We have developed the reporter gene assay system using the AR EcoScreen cell and compiled a validation report based on results from the pre-validation study with 40 chemicals and the inter-laboratory validation study performed with the four participating laboratories using the same 5 chemicals for both androgenic and anti-androgenic activities.

10. The validation report was submitted to OECD in 2010. However the peer review panel report stated that a dedicated inter-laboratory study should be carried out, using the final test protocol to test substances with various activities, including non-active substances and weak agonists and antagonists.

11. According to the major peer review recommendation, we made a plan for the additional inter-laboratory validation study. This additional validation study was conducted with four participating laboratories in 2013-2014.

3. OBJECTIVES

12. The aim of this study was to evaluate intra-laboratory repeatability and intra- and inter-laboratory reproducibility of Androgen Receptor (AR) EcoScreen protocol using additional chemicals according to the major recommendation in the OECD peer review comments for the previously conducted 1st validation study.

4. VALIDATION DESIGN

13. The validation study for the stably transfected TA assay using AR-EcoScreen™ cell line to detect androgenic/anti-androgenic activities consisted of the Phase-1 and Phase-2 studies. Prior to starting the validation study, each laboratory conducted the proficiency test following the technical training.

4.1 ORGANIZATION

Schematic drawing of the organization for the additional validation is shown in Fig. 1.

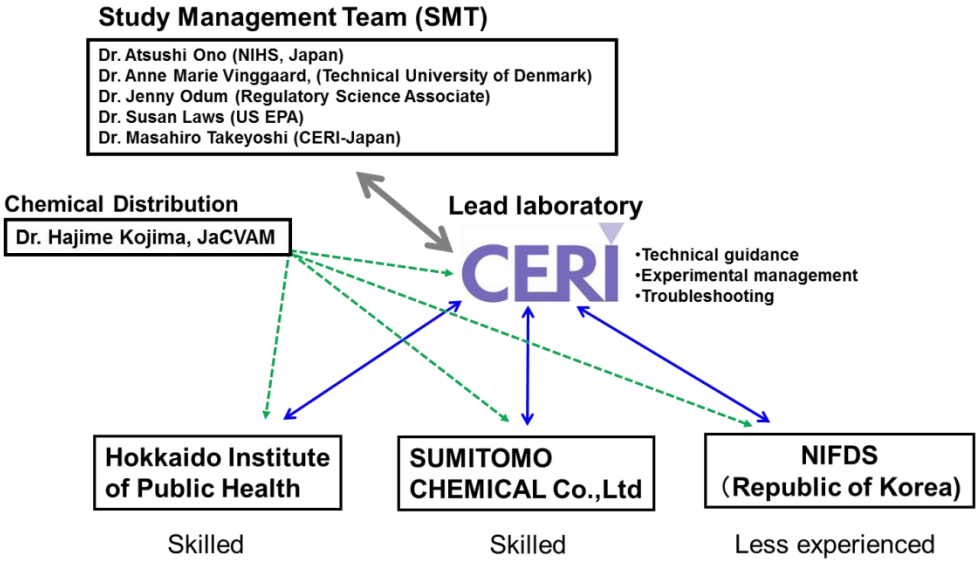


Fig. 1 Schematic drawing of the study organization

4.1.1 *Sponsor*[*]

Ministry of Economy, Trade and Industry, Japan.

4.1.2 *Supporters*

Ministry of Health, Labour and Welfare, Japan.
National Institute of Health Sciences (NIHS)
Japanese Center for the Validation of Alternative Methods (JaCVAM)

4.1.3 *Participating laboratories*[*]

The validation study is conducted by four participating laboratories as follows;

- Chemicals Evaluation and Research Institute (CERI, Lead Laboratory)
- Environmental Health Science Laboratory of Sumitomo Chemical Co. Ltd,
- Hokkaido Institute of Public Health
- National Institute of Food and Drug Safety Evaluation, Republic of Korea (NIFDS)

14. The lead laboratory representing the test method was responsible for providing the test method

[*] See Appendix 1 for detailed contact address

protocol and the necessary assay datasheets (MSExcel format) and worksheets (MSWord format), etc. The lead laboratory was also responsible for providing, if necessary, new versions of the protocols during the entire validation trail. The lead laboratory and the other participating laboratory were contacted by the Project Coordinator for technical issues.

4.1.4 *The Study management team (SMT)*

SMT was organized with following members to support the validation process;

Dr. Atsushi Ono (NIHS, Japan)	Project Coordinator Quality assurance
Dr. Masahiro Takeyoshi (CERI-Japan)	Expertise of this assay Quality assurance
Dr. Anne Marie Vinggaard (Technical University of Denmark) Dr. Jenny Odum (Regulatory Science Associate) Dr. Susan Laws (US EPA)	Validation study expertise

4.1.5 *Chemical Distribution Management*[*]

Dr. Hajime Kojima (JaCVAM, Japan)

4.1.6 *Chemicals and other materials*

15. Reference chemicals and test chemicals were shipped according to proper regulatory procedures. Each participating laboratory was notified by Chemical Distribution Management when any reference chemicals, and test chemicals were shipped. Upon receipt, chemicals were stored under appropriate storage conditions as per recommendations provided by Chemical Distribution Management. Each participating laboratory notified the SMT Project Coordinator upon receipt.

16. The information with regard to the lot number of serum and the list of the other materials used in the validation were announced by CERI prior to the start of validation study, and all laboratories obtained the same products that were to be used for the study with a very few exceptions, namely the dimethyl sulfoxide used as a vehicle by CERI in Phase-2 study.

4.1.6-.1) Reference chemicals and vehicle

17. Reference chemicals and vehicle used in the validation study (Table 1) were distributed from Distribution Management (JaCVAM) prior to the start of Phase-1 study to Japanese participant laboratory and prior to the start of Phase-2 to NIFDS. Japanese participant laboratory conducted Phase-1 and 2 studies using distributed chemicals. NIFDS conducted Phase-1 study using chemicals obtained locally and conducted Phase-2 study using distributed chemicals. Solvent (DMSO, CAS: 67-68-5) was obtained from Sigma as product code of D8418, and the lot number of DMSO used in the study was SHBB3758V except in the Phase-2 study of CERI in which the lot number was SHBC3313V.

Table 1-1 List of reference chemicals used in the validation study

Chemical Name	CASRN	MW (g/mol)	Supplier	Product Code	Lot No.
5α-Dihydrotestosterone	521-18-6	290.44	TCI	A0462	JN01
Mestanolone	521-11-9	304.47	APIN	27879m	212259
Di(2-ethylhexyl)phthalate	117-81-7	390.56	sigma	67261	BCBG7259V
Hydroxyflutamide	52806-53-8	292.21	LKT lab	H9718	26801402
Bisphenol A	80-05-7	228.29	sigma	239658	MKBF3852V

Table 1-2 Vehicle used in the validation study

Chemical name	CASRN	MW (g/mol)	Supplier	Product Code	Lot No.
Dimethyl sulfoxide	67-68-5	78.13	sigma	D8418	SHBB3758V SHBC3313V *

* Product used for Antagonist assay in CERI Phase-2.

4.1.6-.2) *Test chemicals*

18. Test chemicals have been selected based on the suggestion from the voluntary chemical selection team of OECD validation management group of non-animal (VMG-NA) by considering the following points:

+ ICCVAM recommendations: ICCVAM Evaluation of In Vitro Test Methods for Detecting Potential Endocrine Disruptors: Estrogen and Androgen Receptor Binding and Transcriptional Activation Assays[1]
+ appropriate negative and positive effects on published AR Ecoscreen™ assay results
+ historical data of lead laboratory
+ availability
+ costs

19. Coded test chemicals (Table 2) were packaged so as to conceal their identities and shipped prior to starting Phase-2. Coded test chemicals, along with a sealed health and safety information package were shipped to the designated Safety Officer. The Safety Officer retained the safety information package and passed the coded test chemicals to the Study Director. The safety information package contained necessary information about the substance hazards and provided instructions for emergency actions. A disclosure key for identifying the test chemicals by code was also included in the package. Consequently, there was no occasion to open the safety information package in any participant laboratories.

[1] ICCVAM Evaluation of In Vitro Test Methods for Detecting Potential Endocrine Disruptors:

Estrogen Receptor and Androgen Receptor Binding and Transcriptional Activation Assays

URL: http://iccvam.niehs.nih.gov/methods/endodocs/edfinrpt/edfinrpt.pdf

Table 2 List of chemicals used in Phase-2 study

Chemical Name	CASRN	MW	Supplier	Product Code	Lot No.	Expected result
Testosterone	58-22-0	288.42	sigma	46923	SZBA235XV	Ago /P
17β-estradiol	50-28-2	272.39	sigma	E8875	SLBC5955V	Ago /P
Medroxyprogesterone 17-acetate	71-58-9	386.53	sigma	46412	SZB8248XV	Ago /P
17α-ethinyl estradiol	57-63-6	296.41	sigma	E4876	071M1429V	Ago /N
Butylbenzyl phthalate	85-68-7	312.36	aldrich	308501	MKBH8959V	Ago /N
Flutamide	13311-84-7	276.21	sigma	F9397	SLBC6159V	Ant/P
Prochloraz	67747-09-5	376.67	sigma	45631	SZBA112XV	Ant/P
Vinclozolin	50471-44-8	286.11	sigma	45705	SZB7292XV	Ant/P
Atrazine	1912-24-9	215.69	sigma	45330	SZB8175XV	Ant/N
6-Propyl-2-thiouracil	51-52-5	170.23	sigma	P3755	BCBG1817V	Ant/N

Ago /P: Positive in agonist assay, Ago /N: Negative in agonist assay, Ant/P: Positive in antagonist assay, Ant/N: Negative in antagonist assay

4.1.6-.3) *Test chemical supply and allocation*

20. Chemicals used in Phase-2 study were assigned according to the following Table 3;

Table 3 Chemical code and allocation of chemicals used in Phase-2 study

Chemical name	CERI	Sumitomo	Hokkaido	NIFDS
17β-estradiol	ARA31	ARA01	ARA16	ARA46
17α-ethinyl estradiol	ARA32	ARA02	ARA17	ARA47
Testosterone	ARA33	ARA03	ARA18	ARA48
Medroxyprogesterone 17-acetate	ARA34	ARA04	ARA19	ARA49
Butylbenzyl phthalate	ARA35	ARA05	ARA20	ARA50
Flutamide	ART36	ART06	ART21	ART51
Atrazine	ART37	ART07	ART22	ART52
Vinclozolin	ART38	ART08	ART23	ART53
Prochloraz	ART39	ART09	ART24	ART54
6-Propyl-2-thiouracil	ART40	ART10	ART25	ART55

5. PROTOCOL

21. In this validation study, the identical protocol was used (ANNEX 1) in all laboratories. The draft protocol was written by the lead laboratory and finalized by SMT. Positive/Negative judgment was made using the preferable criteria decided in the 1st validation report (see paragraph 92 for agonist assay and paragraph 106 for antagonist assay in the 1st validation report).

22. The summary of the protocol is shown in Table 4.

Table 4 Summary of the AR STTA agonist and antagonist protocol

<table>
<tr><th>Study phase</th><th>Purpose</th><th>Procedures in brief</th></tr>
<tr><td rowspan="2">Proficiency test</td><td>a) Edge effects confirmation at each participating laboratory</td><td>a) Edge effects
(1) Expose 10nM 5α-Dihydrotestosterone (DHT) to all wells in a 96-well plate
(2) Check if the value of coefficient of variation (CV) value among all wells of luminescence intensity is less than 10%.
If yes, no edge effects are expected and all wells of 96-well plate can be used.
If no and if the larger %CV are due to data from wells at the edges, the wells on the edge should not be used for further evaluation.</td></tr>
<tr><td>b) Confirmation of the technical transfer status at each participating laboratory by testing the same stock solution* of minimal reference chemicals.</td><td>b) the technical transfer status
Test the same aliquots of stock solution* of minimal reference chemicals (5α-Dihydrotestosterone and Di(2-ethylhexyl)phthalate (DEHP) for agonist assay, Hydroxyflutamide and DEHP for antagonist assay) at their own laboratory sites.

*The same aliquots of stock solutions were also used in technical training at the lead laboratory, CERI.</td></tr>
<tr><td>Phase-1</td><td>Confirm the overall laboratory proficiency by testing the reference chemicals with the same lot numbers and collecting data to set a reference criteria for mestanolone.</td><td>Test “AR agonist, antagonist and negative chemicals”
<table>
<tr><th>Assay</th><th>Chemical Name</th><th>Expected effect</th></tr>
<tr><td rowspan="3">Agonist</td><td>5α-Dihydrotestosterone</td><td>Positive</td></tr>
<tr><td>Mestanolone</td><td>Positive</td></tr>
<tr><td>Di(2-ethylhexyl)phthalate</td><td>Negative</td></tr>
<tr><td rowspan="3">Antagonist</td><td>Hydroxyflutamide</td><td>Positive</td></tr>
<tr><td>Bisphenol A</td><td>Positive</td></tr>
<tr><td>Di(2-ethylhexyl)phthalate</td><td>Negative</td></tr>
</table>
In addition, data from the NIFDS will be used to confirm the validity of acceptability criteria.</td></tr>
<tr><td>Phase-2</td><td>Test coded chemicals</td><td>Test the agonist and antagonist activities of the 10 coded chemicals</td></tr>
</table>

6. VALIDATION STUDY PROCESS

6.1 Technical transfer meeting

23. Before the validation study started, a technical transfer meeting was held for the two domestic laboratories at CERI from 9 to 11 October, 2013.

24. The NIFDS staff had been technically trained during the technical transfer meeting held in CERI from October 16 to October 18, 2012.

6.2 Edge effect check

25. Edge effect check was conducted by all participating laboratories. Edge effect was checked in an assay plate which was uniformly seeded $9x10^3$ cell/well with 10nM Dihydrotestosterone (DHT). If the case that both %CV of RLU values among all wells measured 24 h after stimulation were less than 10%, the edge effect was decided as negligible.

26. The results of edge effect check were given in Table 5. The %CV of RLU values among all well were less than 10% in all laboratories, therefore the edge effects were considered to be negligible.

Table 5 Results of edge effect test in each laboratory

	CERI	Sumitomo	Hokkaido	NIFDS		
				trial 1	trial 2	trial 3
AVG	3117.8	204832.8	4637.6	302467.5	287767.0	297763.7
SD	101.2	14081.0	195.0	18273.7	23220.0	20411.3
%CV	3.2	6.9	4.2	6.0	8.1	6.9

6.3 Proficiency test

27. The aim of the proficiency test was to confirm the technical transfer status at each participating laboratory by testing the same stock of minimal number of reference chemicals used in the technical transfer meeting.

28. The proficiency test was absolved for the NIFDS, because the NIFDS staff had been technically trained in the technical transfer meeting held in CERI in 2012, and their proficiency was confirmed by the data submitted to CERI.

29. In the proficiency test, each laboratory, excluding NIFDS, tested the minimal number of reference chemicals (DHT for positive and Di(2-ethylhexyl)phthalate (DEHP) for negative in agonist assay, Hydroxyflutamide (HF) for positive and DEHP for negative in antagonist assay) used in the technical transfer meeting in their own laboratories with same plate assignment as in the technical transfer meeting.

30. In the event that at least one run of assay results met the acceptability criteria shown in Table 6, the laboratory was permitted to start the Phase-1 study.

Table 6-1 Acceptability criteria for reference chemicals in AR agonist assay

Fold-induction for PC_{AGO}	> = 6.4		
FI PC10	Greater than 1 (fold-induction of VC) +2SD		
Chemical Name [CAS No.]	logPC10	logPC50	Test range
5α-Dihydrotestosterone (DHT) [521-18-6]	-9.87 ~-12.08	-9.00 ~ -11.03	10^{-6} ~ 10^{-12}M
Mestanolone [521-11-9]	to be set by phase 1 results	to be set by phase 1 results	10^{-6} ~ 10^{-12}M
Di(2-ethylhexyl)phthalate (DEHP) [117-81-7]	-	-	10^{-5} ~ 10^{-11}M

PC_{AGO}: Positive control (10 nM of DHT)
FI PC10: fold-induction of corresponding to the PC10
VC: Vehicle Control

Table 6-2 Acceptability criteria for reference chemicals for AR antagonist assay

Fold induction for AGref [AGref; 500 pM DHT]/[VC]	> = 5.0		
RTA of PC_{ATG} (%)	≤ 46		
Chemical Name [CAS No.]	log linearIC30	Log linearIC50	Test range
Hydroxyflutamide (HF) [52806-53-8]	-6.41 ~ -8.37	-6.17 ~ -7.80	10^{-5} ~ 10^{-10}M
Bisphenol A (BPA) [80-05-7]	-4.48 ~ -7.52	-4.29 ~ -7.05	10^{-5} ~ 10^{-10}M
Di(2-ethylhexyl)phthalate (DEHP) [117-81-7]	-	-	10^{-5} ~ 10^{-10}M

VC: Vehicle control (DMSO);
PC_{AGO}: Positive AR agonist control (10 nM of DHT);
AG ref: AR agonist reference (500 pM DHT, 0.1% DMSO)
PC_{ATG}-: Positive AR antagonist control (500 pM DHT, 0.1 μM of HF)
PC_{CT}: Cytotoxicity control (10 μg/mL of cycloheximide)
RTA : relative transcriptional activity

RTA of PC_{ATG}(%) is calculated by the following equation;

$$\text{RTA of } PC_{ATG}\,(\%) = \text{Mean}\left(\frac{\text{RLU of } PC_{ATG} - \text{Mean RLU of VC}}{\text{Mean RLU of AG ref - Mean RLU of VC}} \times 100\right)$$

31. Results of the Proficiency test for agonist and antagonist assays are shown in Tables 7-1 and 7-2. All results obtained in three domestic laboratories met the requirements for this test, and all passed the acceptability criteria.

Table 7-1 Results of the Proficiency test for agonist assay

		CERI		Sumitomo		Hokkaido	
		Result	Decision	Result	Decision	Result	Decision
FI for PC_{AGO}		8.906	Pass	6.84	Pass	7.35	Pass
FI VC_Mean + 2SD		1.09	Pass	1.19	Pass	1.07	Pass
FI PC10		1.79		1.58		1.64	
DHT	log[PC10]	-10.71	Pass	-10.57	Pass	-10.85	Pass
	log[PC50]	-9.73	Pass	-9.41	Pass	-10.21	Pass
DEHP	log[PC10]	-		-		-	
	log[PC50]	-		-		-	

FI for PC_{AGO}: Fold induction for PC_{AGO}.
FI VC: Fold induction for vehicle control (DMSO).
FI PC10: fold-induction of the PC10

Table 7-2 Results of the Proficiency test for antagonist assay

		CERI		Sumitomo		Hokkaido	
		Result	Decision	Result	Decision	Result	Decision
FI for AG ref		6.823	Pass	5.314	Pass	8.139	Pass
RTA of PC_{AGO}		123.57		137.57		122.52	
RTA of PC_{ATG} (%)		3.32	Pass	4.24	Pass	7.33	Pass
RTA of PC_{CT}		-1.79		-5.07		-2.56	
HF	log[lin.IC30]	-7.36	Pass	-7.88	Pass	-7.18	Pass
	log[lin.IC50]	-6.95	Pass	-7.41	Pass	-6.77	Pass
DEHP	log[lin.IC30]	-		-		-	
	log[lin.IC50]	-		-		-	

RTA of PC_{AGO}: Relative transcriptional activity of PC_{AGO} against AGref
RTA of PC_{ATG} (%): Relative transcriptional activity of PC_{ATG} (500 pM DHT, 0.1 μM of HF)
RTA of PC_{CT}: Relative transcriptional activity of PC_{CT} (10 μg/mL of cycloheximide) against AGref.

6.4 Phase-1 study

32. The aims of the Phase-1 study were to confirm the overall laboratory proficiency by testing the same lot number of reference chemicals and to collect data to set a reference criteria for mestanolone. In addition, data from the NIFDS was used to confirm the validity of the acceptability criteria.

33. In the Phase-1 study, the reference chemicals listed in Table 1-1 and Table 1-2 of the protocol were provided by Chemical Distribution Management, excluding NIFDS where the same lot numbers of chemicals were obtained from their local distributors. Then each laboratory tested the Phase-1 chemicals according to the assay protocol in at least three runs in triplicate.

34. The assay results were stored and locked in the Specified work sheet provided by CERI. Then each laboratory submitted at least 3 sets of assay results meeting all the acceptability criteria shown in the assay protocol, to the Project Coordinator.

6.4.1 *Agonist assay*

35. The results of Phase-1 study for agonist assay in three Japanese laboratories were summarized in Table 8.

36. All FI (Fold induction) values for PC_{AGO} in Japanese laboratories were over 7.40, LogPC10(M) and LogPC50(M) values for DHT were within the range required in acceptability criteria.

Table 8 Results of the Phase-1 study for agonist assay in Japanese laboratories

		FI (PC_{AGO})	FI VC mean + 2SD	FI PC10	DHT (Log PC10) (M)	DHT (Log PC50) (M)	Mestanolone (Log PC10) (M）	Mestanolone (Log PC50) (M)
	1	8.38	1.12	1.74	-10.76	-9.81	-10.65	-9.62
CERI	2	8.64	1.08	1.76	-10.66	-9.70	-10.56	-9.59
	3	8.68	1.14	1.77	-10.71	-9.75	-10.64	-9.65
	1	7.67	1.10	1.67	-10.64	-9.59	-10.47	-9.43
Sumitomo	2	7.35	1.08	1.64	-10.77	-9.82	-10.66	-9.60
	3	8.14	1.12	1.71	-10.69	-9.67	-10.57	-9.53
	1	7.71	1.07	1.67	-10.83	-10.10	-10.79	-9.87
Hokkaido	2	7.84	1.08	1.68	-10.83	-10.08	-10.81	-10.00
	3	7.40	1.08	1.64	-10.83	-10.11	-10.84	-10.08
For 3 labs.				MEAN	-10.75	-9.85	-10.67	-9.71
				SD	0.07	0.20	0.13	0.22
				MEAN+2SD	-10.60	-9.45	**-10.41**	**-9.26**
				MEAN-2SD	-10.89	-10.25	**-10.92**	**-10.15**

37. The aim of the Phase-1 study was to set the primary reference criteria for mestanolone as the mean logPCx±2SD with the data obtained in three Japanese laboratories. The ranges were calculated as -10.41~-10.92 for LogPC10(M) and -9.26~-10.15 for LogPC50(M), respectively.

38. The results of Phase-1study for agonist assay in NIFDS are shown in Table 9 and response curves for reference chemicals in all labs are shown in Fig.2.

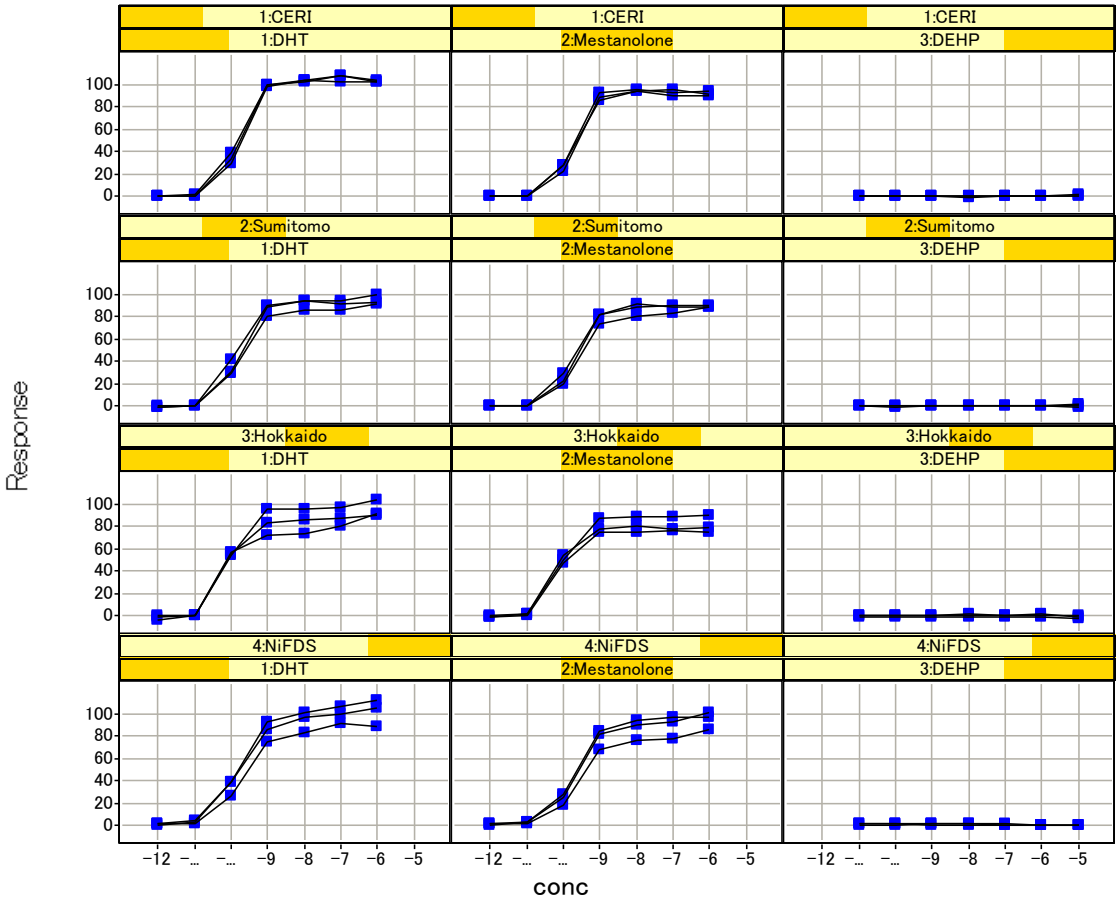
Table 9 Results of the Phase-1 study for agonist assay in NIFDS

		FI (PC_{AGO})	FI VC mean + 2SD	FI of PC10	DHT (Log PC10) (M)	DHT (Log PC50) (M)	Mestanolone (Log PC10) (M）	Mestanolone (Log PC50) (M)
	1	7.44	1.07	1.64	-10.82	-9.75	-10.69	-9.56
NIFDS	2	6.91	1.05	1.59	-10.79	-9.80	-10.70	-9.60
	3	6.94	1.04	1.59	-10.64	-9.50	-10.49	-9.35

FI: Fold induction.
VC: Vehicle control.

39. The results in NIFDS passed all the requirements of the acceptability criteria including mestanolone in three of three trials.

Fig. 2 Response curves for reference chemicals in Phase-1 study-Agonist



40. The PC values of mestanolone (mean logPCx±2SD) including the data from NIFDS were almost the same as the range calculated from the data of the three Japanese laboratories. The Project coordinator therefore decided that the acceptability criteria for mestanolone in Phase-2 study should be as shown in Table 10.

Table 10 Acceptability criteria for reference chemicals in AR agonist assay for Phase-2 study

Fold-induction for PC_{AGO}	**> = 6.4**		
FI PC10	Greater than 1 (fold-induction of VC) +2SD		
Chemical Name [CAS No.]	logPC10	logPC50	Test range
5α-Dihydrotestosterone (DHT) [521-18-6]	-9.87 ~-12.08	-9.00 ~ -11.03	10^{-6} ~ 10^{-12}M
Mestanolone [521-11-9]	**-10.41~-10.92**	**-9.26~-10.15**	10^{-6} ~ 10^{-12}M
Di(2-ethylhexyl)phthalate (DEHP) [117-81-7]	-	-	10^{-5} ~ 10^{-11}M

FI PC10: fold-induction of corresponding to the PC10
VC: Vehicle Control

6.4.2 *Antagonist assay*

41. All Labs conducted three trials (Table 11, Fig 3). The fold induction of Agonist reference (AGref, 500 pM DHT) in the 2nd trial of NIFDS was 4.95. This value was lower than the acceptability criteria (>=5.0). However, the deviation was slight and all other acceptability criteria were met. Thus, the deviation was negligible and the result was judged as acceptable.

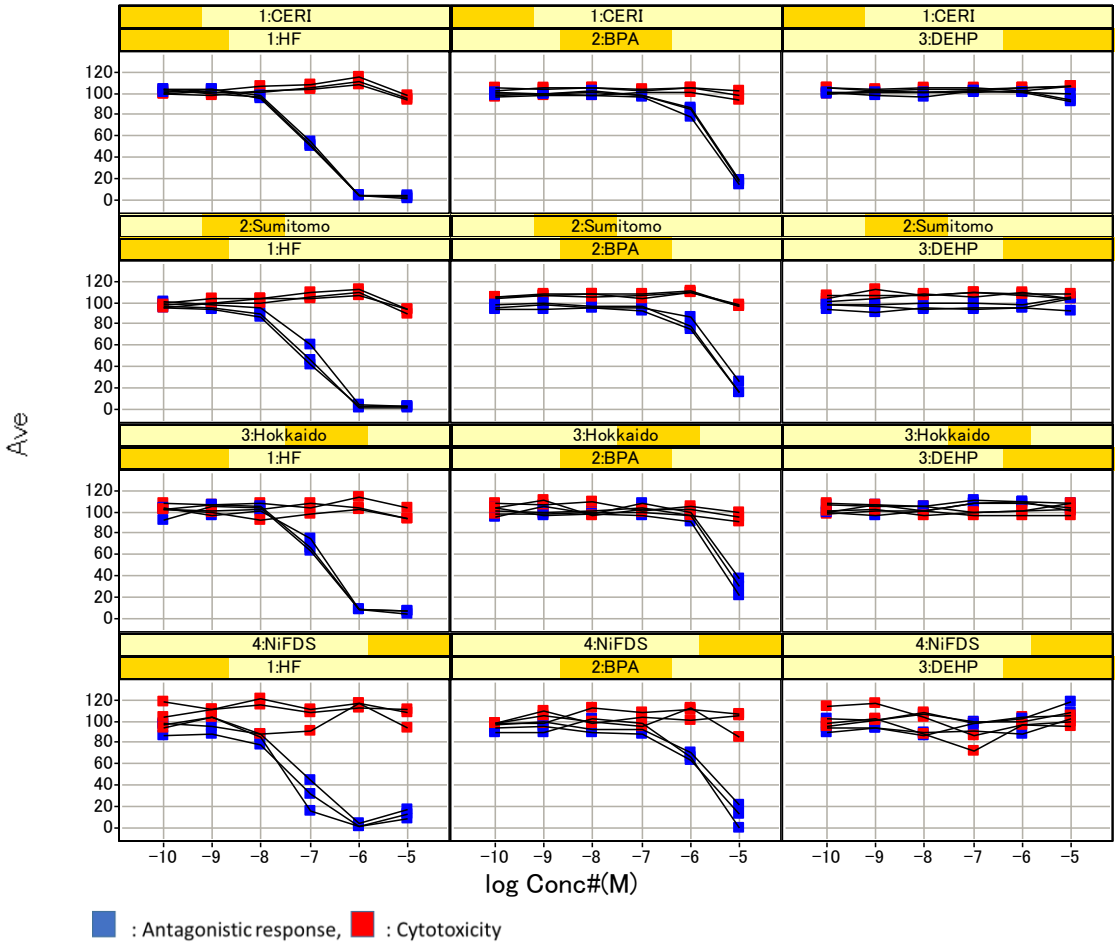
42. Consequently, all laboratories passed all of acceptability criteria in three of three trials, and all four laboratories passed the Phase-1 study.

Table 11 Results of the Phase-1 study for antagonist assay

		FI (AG ref)	RTA of PC_{ATG} (%)	HF (logIC30) (M)	HF (logIC50) (M)	BPA (logIC30) (M)	BPA (logIC50) (M)
CERI	1	7.07	3.91	-7.36	-6.92	-5.76	-5.47
	2	7.29	2.81	-7.44	-6.99	-5.88	-5.56
	3	7.43	3.99	-7.41	-6.97	-5.78	-5.49
Sumitomo	1	5.44	4.02	-7.55	-7.10	-5.92	-5.58
	2	5.54	6.97	-7.28	-6.82	-5.74	-5.40
	3	6.00	2.09	-7.63	-7.19	-5.88	-5.56
Hokkaido	1	6.91	7.19	-6.93	-6.62	-5.53	-5.21
	2	6.56	4.39	-7.10	-6.72	-5.71	-5.42
	3	7.19	4.85	-7.17	-6.76	-5.61	-5.31
NIFDS	1	5.49	6.32	-7.59	-7.14	-6.00	-5.58
	2	4.95*	5.46	-7.78	-7.49	-6.13	-5.76
	3	5.05	7.24	-7.83	-7.40	-6.29	-5.74
		MEAN		-7.42	-7.01	-5.85	-5.51
		SD		0.27	0.27	0.21	0.16
		MEAN+2SD		-6.88	-6.48	-5.42	-5.18
		MEAN-2SD		-7.97	-7.54	-6.28	-5.83

*Value in red letter was deviated from the acceptability criteria.

Fig. 3 Response curves for reference chemicals in Phase-1 study-Antagonist



6.5 Phase-2 study

43. The aims of the Phase-2 study were to provide the supplemental data according to previous peer review comments of this assay and to evaluate the assay performance (within/between-laboratory reproducibility and predictive capacity) by blind testing of 5 coded chemicals for agonistic and 5 for antagonistic activities .

6.5.1 *Study design*

44. The Phase-2 study was conducted with all four laboratories who passed the acceptability criteria in the Phase-1 study.

45. The Phase-2 study was performed with 5 test chemicals for the agonist assay and 5 test chemicals for the antagonist assay. Each laboratory tested the Phase-2 chemicals in triplicate, in a plate at appropriate concentration ranges of each chemical for the assessment of their activity to evaluate the assay performance (within/between-laboratory reproducibility and predictive capacity).

46. All 10 chemicals for Phase-2 study were provided encoded by Chemical Distribution Management. Each laboratory tested these chemicals according to the assay protocol up to the maximum concentration decided according to the diagram for the solubility test.

47. Every run of the Phase-2 study required the simultaneous testing of the reference chemicals used in

Phase-1 study. All participant laboratories tested each of the Phase-2 chemicals in at least three independent assays.

48. The plate assignments/layouts used in Phase-2 study are described in Annex 1.

49. All assay results were stored and password locked by each laboratory to protect data in the Specified work sheet previously provided by CERI. Each laboratory then submitted at least three sets of assay results that met all acceptability criteria shown in Table 10 including newly decided criteria for mestanolone to the Project Coordinator.

50. All results in the validation study were analyzed by NIHS to evaluate the performance of this assay. For this purpose, two-by-two table analyses were employed to evaluate accuracy (concordance) ([a+d]/[a+b+c+d]), sensitivity (a/[a+c]), and specificity (d/[b+d]) of the proposed ARTA assay system by comparing it with an expected result.

		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

51. The inter-laboratory concordance of judgment (positive/negative) of coded test chemicals was required to be more than 80% for each of the agonist and antagonist assays as acceptability criteria. The positive/negative judgments were made by same criteria as described in the first validation study; when PC10 for agonist assay or logIC30 for antagonist assay were derived, the chemical was regarded as positive, respectively, in each run.

6.5.2 *Agonist assay*

52. Phase-2 study was started with the solubility test, and all laboratories decided the maximum dose according to the scheme for the solubility test in the assay protocol.

53. The results of the solubility test are shown in Table 12. Consequently, the dose range for the all test chemicals for in Phase-2 were decided to be 10^{-12}- 10^{-6} M or 10^{-11}- 10^{-5}M.

Table 12 Test concentration range decided by solubility test in agonist assay

Test chemical	Test concentration range (M)			
	CERI	Sumitomo	Hokkaido	NIFDS
Testosterone	10^{-12}- 10^{-6}	10^{-11}- 10^{-5}	10^{-11}- 10^{-5}	10^{-11}- 10^{-5}
17β-estradiol	10^{-12}- 10^{-6}	10^{-11}- 10^{-5}	10^{-11}- 10^{-5}	10^{-11}- 10^{-5}
Medroxyprogesterone 17-acetate	10^{-12}- 10^{-6}	10^{-12}- 10^{-6}	10^{-11}- 10^{-5}	10^{-11}- 10^{-5}
17α-ethinyl estradiol	10^{-12}- 10^{-6}	10^{-11}- 10^{-5}	10^{-11}- 10^{-5}	10^{-11}- 10^{-5}
Butylbenzyl phthalate	10^{-12}- 10^{-6}	10^{-11}- 10^{-5}	10^{-11}- 10^{-5}	10^{-11}- 10^{-5}

54. All laboratories passed all Acceptability criteria shown in Table 10 in the first three runs. The results for reference chemicals are shown in Table 13.

55. The LogPC10(M) and LogPC50(M) for DHT ranged from -10.54 to -10.82 and from -9.56 to -10.04, respectively. The LogPC10(M) and LogPC50(M) for mestanolone were ranged from -10.47 to -10.88

and from -9.49 and -10.02, respectively. The %CV of LogPC10(M) and LogPC50(M) for each parameter was less than 2%.

56. For the test chemicals, the positive candidate chemicals, Testosterone, 17β-estradiol and Medroxyprogesterone 17-acetate, tested positive in all runs of all laboratories, and the negative candidate chemicals, 17α-ethinyl estradiol and Butylbenzyl phthalate, tested negative in all runs of all laboratories. In addition, the %CV of LogPC10(M) and LogPC50(M) for each chemical were less than 5% (Table 13).

57. The results of the two-by-two table analysis with the candidate effects are shown in Table 15. The Accuracy, Sensitivity and Specificity of the assay were all calculated to be 100% in each laboratory. Accuracy, Sensitivity and Specificity across all four laboratories were also 100%.

Table 13 Results for the reference chemicals in agonist assay

	Run No.	FI (PC_{AGO})	FI VC mean+2SD	FI of PC10	DHT Log PC10 (M)	DHT Log PC50 (M)	Mestanolone Log PC10 (M)	Mestanolone Log PC50 (M)
CERI	1	8.19	1.05	1.72	-10.69	-9.70	-10.69	-9.65
		8.92	1.11	1.79				
	2	8.18	1.13	1.72	-10.78	-9.84	-10.72	-9.72
		8.23	1.15	1.72				
	3	8.14	1.04	1.71	-10.71	-9.71	-10.71	-9.66
		7.61	1.12	1.66				
	Mean	8.21	1.10	1.72	-10.72	-9.75	-10.71	-9.68
	SD	0.42	0.04	0.04	0.05	0.08	0.02	0.04
	%CV	5.07%	4.09%	2.42%	0.46%	0.82%	0.15%	0.41%
Sumitomo	1	7.47	1.06	1.65	-10.74	-9.75	-10.62	-9.56
		7.33	1.07	1.63				
	2	7.27	1.08	1.63	-10.73	-9.76	-10.59	-9.55
		7.34	1.04	1.63				
	3	7.56	1.07	1.66	-10.76	-9.77	-10.66	-9.59
		7.15	1.12	1.61				
	Mean	7.35	1.07	1.64	-10.75	-9.76	-10.62	-9.57
	SD	0.15	0.03	0.01	0.01	0.01	0.04	0.02
	%CV	1.97%	2.45%	0.89%	0.13%	0.11%	0.35%	0.23%
Hokkaido	1	7.41	1.11	1.64	-10.82	-10.04	-10.85	-10.02
		6.96	1.09	1.60				
	2	7.49	1.09	1.65	-10.78	-9.87	-10.74	-9.77
		7.22	1.07	1.62				
	3	7.35	1.09	1.64	-10.82	-9.97	-10.82	-9.99
		7.88	1.12	1.69				
	Mean	7.39	1.09	1.64	-10.81	-9.96	-10.80	-9.93
	SD	0.31	0.02	0.03	0.02	0.09	0.05	0.14
	%CV	4.13%	1.64%	1.86%	0.22%	0.89%	0.50%	1.39%
NIFDS	1	7.42	1.04	1.64	-10.54	-9.56	-10.47	-9.49
		7.17	1.04	1.62				
	2	7.51	1.06	1.65	-10.70	-9.71	-10.61	-9.57
		7.84	1.05	1.68				
	3	7.34	1.06	1.63	-10.76	-9.73	-10.88	-9.89
		6.62	1.05	1.56				
	Mean	7.32	1.05	1.63	-10.66	-9.67	-10.66	-9.65
	SD	0.41	0.01	0.04	0.11	0.09	0.21	0.21
	%CV	5.59%	0.67%	2.51%	1.08%	0.93%	1.94%	2.22%
For four labs:								
	Mean	7.57	1.08	1.66	-10.73	-9.78	-10.70	-9.71
	SD	0.49	0.03	0.05	0.08	0.13	0.12	0.18
	%CV	6.53%	2.95%	2.98%	0.71%	1.32%	1.10%	1.84%
	Max	8.92	1.15	1.79	-10.54	-9.56	-10.47	-9.49
	Min	6.62	1.04	1.56	-10.82	-10.04	-10.88	-10.02

Fig. 4-1 Response curves for reference chemicals in Phase-2 study-Agonist

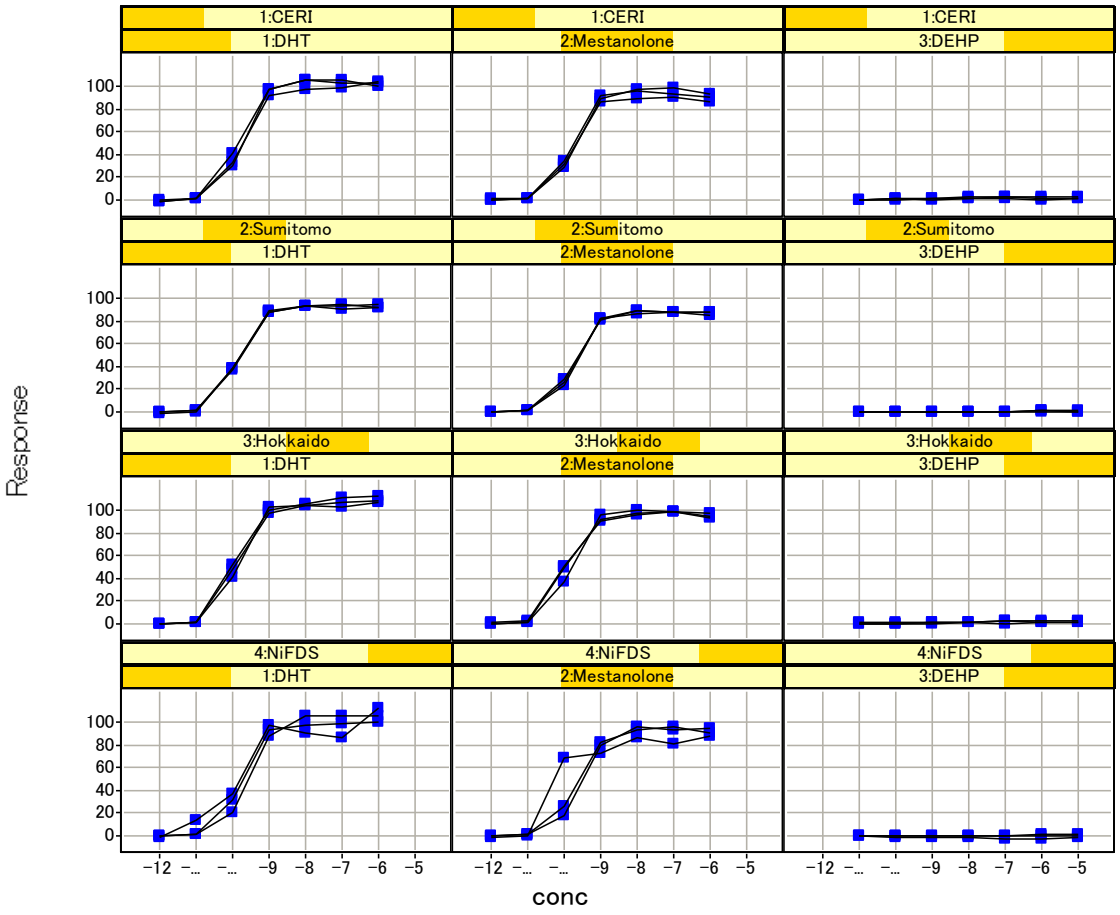


Table 14 Summary of the results for test chemicals in agonist assay

	Lab	Run No.	Log PC10 (M)	Mean SD %CV	Log PC50 (M)	Mean SD %CV	Decision
17a-ethinyl estradiol CASRN:57-63-6	CERI	1	ND		ND		Negative
		2	ND		ND		
		3	ND		ND		
	Sumitomo	1	ND		ND		Negative
		2	ND		ND		
		3	ND		ND		
	Hokkaido	1	ND		ND		Negative
		2	ND		ND		
		3	ND		ND		
	NIFDS	1	ND		ND		Negative
		2	ND		ND		
		3	ND		ND		
	For 4 labs:	Mean	ND		ND		Negative
		SD					
		%CV					
17β-estradiol CASRN:50-28-2	CERI	1	-7.63	-7.63	ND		Positive
		2	-7.67	0.03	ND		
		3	-7.60	0.43%	ND		
	Sumitomo	1	-7.24	-7.23	ND		Positive
		2	-7.19	0.04	ND		
		3	-7.27	0.58%	ND		
	Hokkaido	1	-7.74	-7.72	-5.33	-5.27	Positive
		2	-7.73	0.02	-5.34	0.12	
		3	-7.70	0.30%	-5.13	2.29%	
	NIFDS	1	-7.05	-6.96	-4.93	-4.99	Positive
		2	-7.08	0.19	-4.88	0.15	
		3	-6.75	2.67%	-5.15	2.94%	
	For 4 labs:	Mean	-7.39				Positive
		SD	0.33				
		%CV	4.50%				
Butylbenzyl phthalate CASRN:85-68-7	CERI	1	ND		ND		Negative
		2	ND		ND		
		3	ND		ND		
	Sumitomo	1	ND		ND		Negative
		2	ND		ND		
		3	ND		ND		
	Hokkaido	1	ND		ND		Negative
		2	ND		ND		
		3	ND		ND		
	NIFDS	1	ND		ND		Negative
		2	ND		ND		
		3	ND		ND		
	For 4 labs:	Mean	ND		ND		Negative
		SD					
		%CV					

ND: Not determined.

Table 14 (continued)

	Lab	ID	Log PC10 (M)	Mean SD %CV	Log PC50 (M)	Mean SD %CV	Decision
Medroxyprogesterone 17-acetate CASRN:71-58-9	CERI	1	-8.94	-8.93	-8.45	-8.46	Positive
		2	-8.93	0.02	-8.50	0.03	
		3	-8.90	0.23%	-8.44	0.38%	
	Sumitomo	1	-8.92	-8.91	-8.44	-8.42	Positive
		2	-8.91	0.02	-8.45	0.04	
		3	-8.89	0.18%	-8.37	0.51%	
	Hokkaido	1	-9.64	-9.38	-8.77	-8.71	Positive
		2	-8.98	0.35	-8.62	0.08	
		3	-9.52	3.76%	-8.72	0.89%	
	NIFDS	1	-8.95	-9.11	-8.51	-8.57	Positive
		2	-9.00	0.24	-8.58	0.06	
		3	-9.39	2.63%	-8.63	0.69%	
	For 4 labs:	Mean	-9.08		-8.54		Positive
		SD	0.27		0.13		
		%CV	2.96%		1.47%		
Testosterone CASRN:58-22-0	CERI	1	-9.83	-9.89	-9.28	-9.30	Positive
		2	-9.98	0.08	-9.35	0.04	
		3	-9.85	0.82%	-9.28	0.41%	
	Sumitomo	1	-9.85	-9.84	-9.24	-9.23	Positive
		2	-9.84	0.00	-9.20	0.02	
		3	-9.84	0.03%	-9.24	0.24%	
	Hokkaido	1	-10.42	-10.32	-9.46	-9.41	Positive
		2	-10.17	0.13	-9.37	0.05	
		3	-10.36	1.24%	-9.39	0.54%	
	NIFDS	1	-9.77	-9.75	-9.13	-9.07	Positive
		2	-9.75	0.02	-9.10	0.09	
		3	-9.73	0.24%	-8.96	0.99%	
	For 4 labs:	Mean	-9.95		-9.25		Positive
		SD	0.24		0.14		
		%CV	2.37%		1.50%		

Fig. 4-2 Response curves for test chemicals in Phase-2 study-Agonist

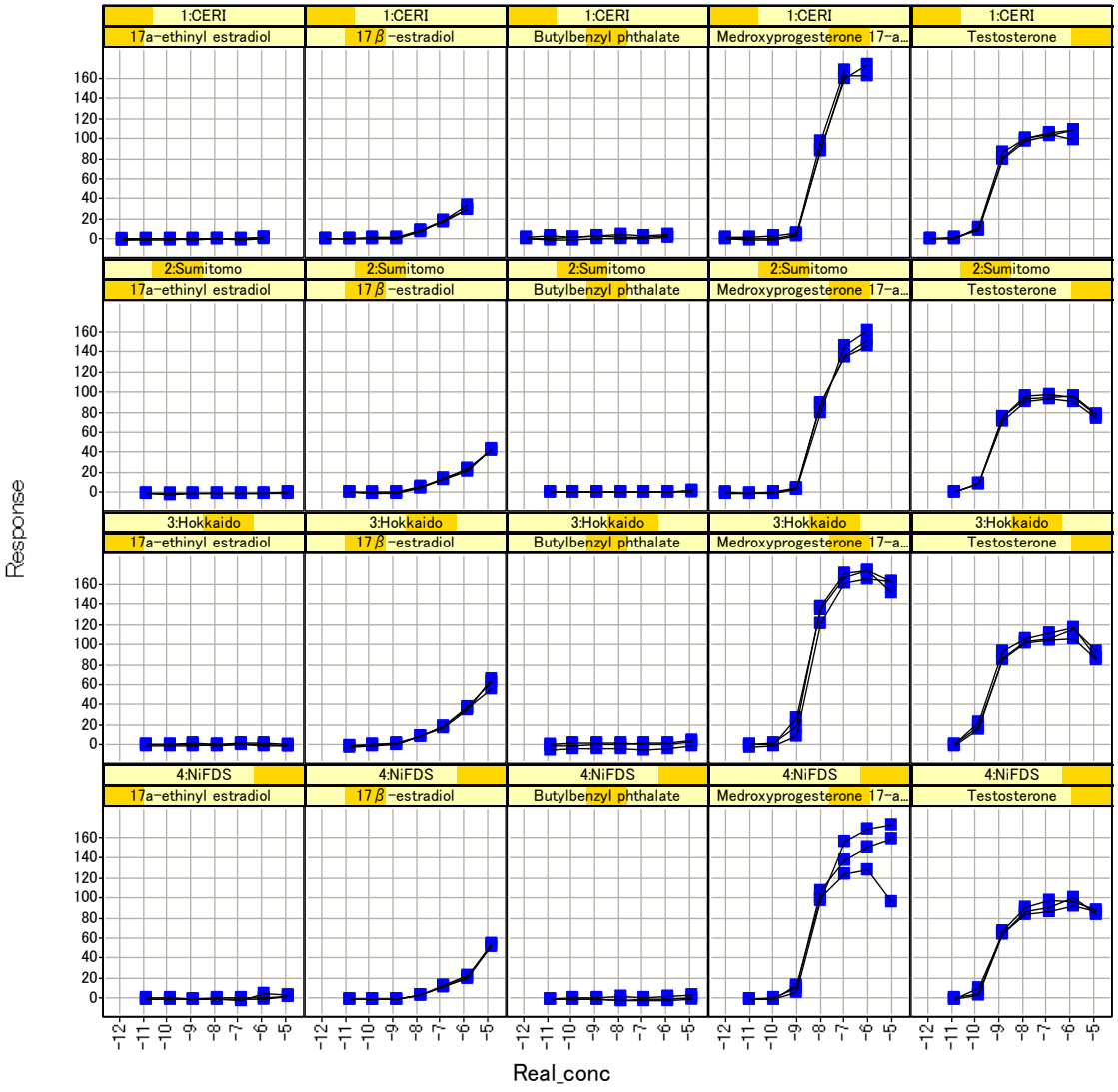


Table 15 Positive/negative outcomes in agonist assay and results of two-by-two table analysis

	Candidate effect	CERI	Sumitomo	Hokkaido	NIFDS	4 Lab
Testosterone	P	P	P	P	P	P
17β-estradiol	P	P	P	P	P	P
Medroxyprogesterone 17-acetate	P	P	P	P	P	P
17α-ethinyl estradiol	N	N	N	N	N	N
Butylbenzyl phthalate	N	N	N	N	N	N
P:Positive						
N:Negative	Accuracy	100%	100%	100%	100%	100%
	Sensitivity	100%	100%	100%	100%	100%
	Specificity	100%	100%	100%	100%	100%

6.5.3 *Antagonist assay*

58. Phase-2 study was started with the solubility test, and all laboratories decided the maximum dose according to the schema for the solubility test shown in the assay protocol.

59. The results of the solubility test are shown in Table 16. Consequently, the dose range for Flutamide, Atrazine, Vinclozolin and Prochloraz in Phase-2 were decided as 10^{-11}- 10^{-6} M or 10^{-10}- 10^{-5}M. The dose range for the 6-Propyl-2-thiouracil was decided as 10^{-9}- 10^{-4} M or 10^{-10}- 10^{-5}M. With these concentration ranges, no cytotoxicity was noted in following assays.

Table 16 Test concentration range decided by solubility test in antagonist assay

Test chemical	Test concentration range(M)			
	CERI	Sumitomo	Hokkaido	NIFDS
Flutamide	10^{-11}- 10^{-6}	10^{-10}- 10^{-5}	10^{-10}- 10^{-5}	10^{-10}- 10^{-5}
Atrazine	10^{-11}- 10^{-6}	10^{-10}- 10^{-5}	10^{-10}- 10^{-5}	10^{-10}- 10^{-5}
Vinclozolin	10^{-11}- 10^{-6}	10^{-10}- 10^{-5}	10^{-10}- 10^{-5}	10^{-10}- 10^{-5}
Prochloraz	10^{-11}- 10^{-6}	10^{-10}- 10^{-5}	10^{-10}- 10^{-5}	10^{-10}- 10^{-5}
6-Propyl-2-thiouracil	10^{-9}- 10^{-4}	10^{-9}- 10^{-4}	10^{-9}- 10^{-4}	10^{-10}- 10^{-5}

60. All laboratories passed all reference criteria in the first three runs. The results for reference chemicals are shown in Table 17 and Fig. 5-1.

61. The LogIC30(M) and LogIC50(M) for HF were ranged from -7.11 to -7.81 and from -6.73 to -7.40, respectively. The LogIC30(M) and LogIC50(M) for BPA ranged from -5.55 to -6.20 and from -5.28 to -5.75, respectively. The %CV for LogIC30(M) and LogIC50(M) was less than 4%.

62. Among the positive candidate chemicals, Flutamide and Vinclozolin, tested positive in all runs of all laboratories, and the negative candidate chemicals, Atrazine and 6-Propyl-2-thiouracil, tested negative in all runs of all laboratories. In addition, the %CV of LogIC10(M) and LogIC50(M) for each chemicals were less than 4% (Table 18, Fig. 5-2).

63. Meanwhile, one of the positive candidate chemicals, Prochloraz, was tested positive in three laboratories, but tested negative in one laboratory for the first three runs.

64. Accordingly, in the results of the two-by-two table analysis in first three runs (Table 19), the Accuracy, Sensitivity and Specificity for all four laboratories were calculated to be 95%, 92% and 100%, respectively.

65. For the discordant chemical, Prochloraz, the concentration range tested by CERI (10^{-11}- 10^{-6}M) in which the chemical was negative, was lower than that of other three laboratories (10^{-10}- 10^{-5}M). This was based on the results of the solubility test conducted previously.

66. The decision on the concentration range was made by the study director in CERI based on the occurrence of precipitation rather than cell viability.

67. To confirm the cause of this discordant result for Prochloraz, an additional trial was conducted by CERI using the same concentration range (10^{-10}- 10^{-5}M) as the other three laboratories.

68. The results of the additional trial by CERI, showed that Prochloraz gave clear positive results in the antagonist assay (Fig. 6). This results showed that the discordant results for Prochloraz were caused by the different concentration range selected by the solubility test rather than assay characteristics.

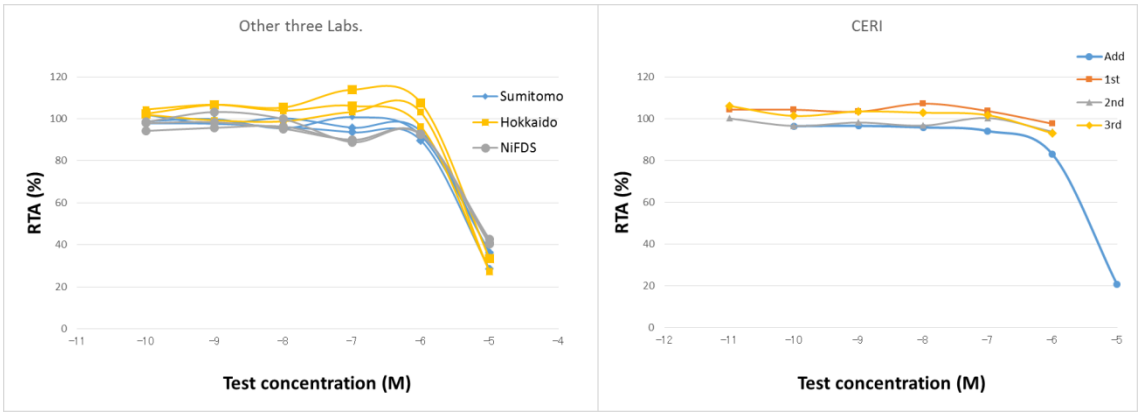


Fig. 6 Comparison of concentration response curves for Prochloraz in each laboratory

69. Consequently with the additional trial, all the positive candidate chemicals tested positive in all laboratories, and the negative candidate chemicals also tested negative in all laboratories. In this case, the %CV of LogIC30(M) and LogIC50(M) for each positive chemicals were also less than 4% (Fig. 5, Table 18).

70. The results of the two-by-two table analysis containing the additional trial are shown in Table 20. The Accuracy, Sensitivity and Specificity of the assay were calculated to be all 100% in all laboratories. The Accuracy, Sensitivity and Specificity for all four laboratories were also 100%.

Table 17 Results for the reference chemicals in antagonist assay

		FI (AG ref)	RTA of PC_{ATG} (%)	HF Log IC30 (M)	HF Log IC50 (M)	BPA Log IC30 (M)	BPA Log IC50 (M)
CERI	1	6.46	3.25	-7.60	-7.18	-5.85	-5.55
	2	6.18	2.87	-7.37	-6.92	-5.92	-5.59
	3	6.28	2.84	-7.40	-6.98	-5.89	-5.58
	Add	5.46	-0.12	-7.48	-7.06	-5.82	-5.52
	Mean	6.10	2.21	-7.46	-7.03	-5.89	-5.57
	SD	0.44	1.56	0.10	0.13	0.04	0.03
	%CV	7.19%	70.67%	1.39%	1.87%	0.60%	0.45%
Sumitomo	1	5.73	3.47	-7.65	-7.21	-5.85	-5.53
	2	5.94	4.33	-7.37	-6.88	-5.81	-5.48
	3	5.37	3.11	-7.62	-7.23	-5.97	-5.63
	Mean	5.68	3.64	-7.54	-7.11	-5.88	-5.55
	SD	0.29	0.62	0.15	0.20	0.08	0.08
	%CV	5.08%	17.15%	-2.05%	2.78%	1.45%	1.37%
Hokkaido	1	6.40	2.24	-7.19	-6.78	-5.55	-5.28
	2	7.66	4.46	-7.31	-6.84	-5.65	-5.38
	3	7.33	5.26	-7.11	-6.73	-5.57	-5.29
	Mean	7.13	3.99	-7.20	-6.78	-5.59	-5.32
	SD	0.66	1.56	0.10	0.06	0.05	0.06
	%CV	9.22%	39.24%	-1.45%	0.88%	0.97%	1.09%
NIFDS	1	5.69	1.26	-7.81	-7.40	-6.20	-5.75
	2	5.43	1.73	-7.77	-7.36	-5.97	-5.64
	3	5.44	2.30	-7.71	-7.32	-5.92	-5.60
	Mean	5.52	1.77	-7.76	-7.36	-6.03	-5.66
	SD	0.15	0.52	0.05	0.04	0.15	0.08
	%CV	2.67%	29.48%	-0.62%	0.56%	2.53%	1.40%
For four labs.							
	MEAN	6.11	2.85	-7.49	-7.07	-5.84	-5.52
	SD	0.73	1.43	0.22	0.23	0.18	0.14
	%CV	11.93%	50.13%	2.96%	3.26%	3.02%	2.49%
	MAX	7.66	5.26	-7.11	-6.73	-5.55	-5.28
	MIN	5.37	-0.12	-7.81	-7.40	-6.20	-5.75

Fig. 5-1 Response curves for reference chemicals in Phase-2 study-Antagonist

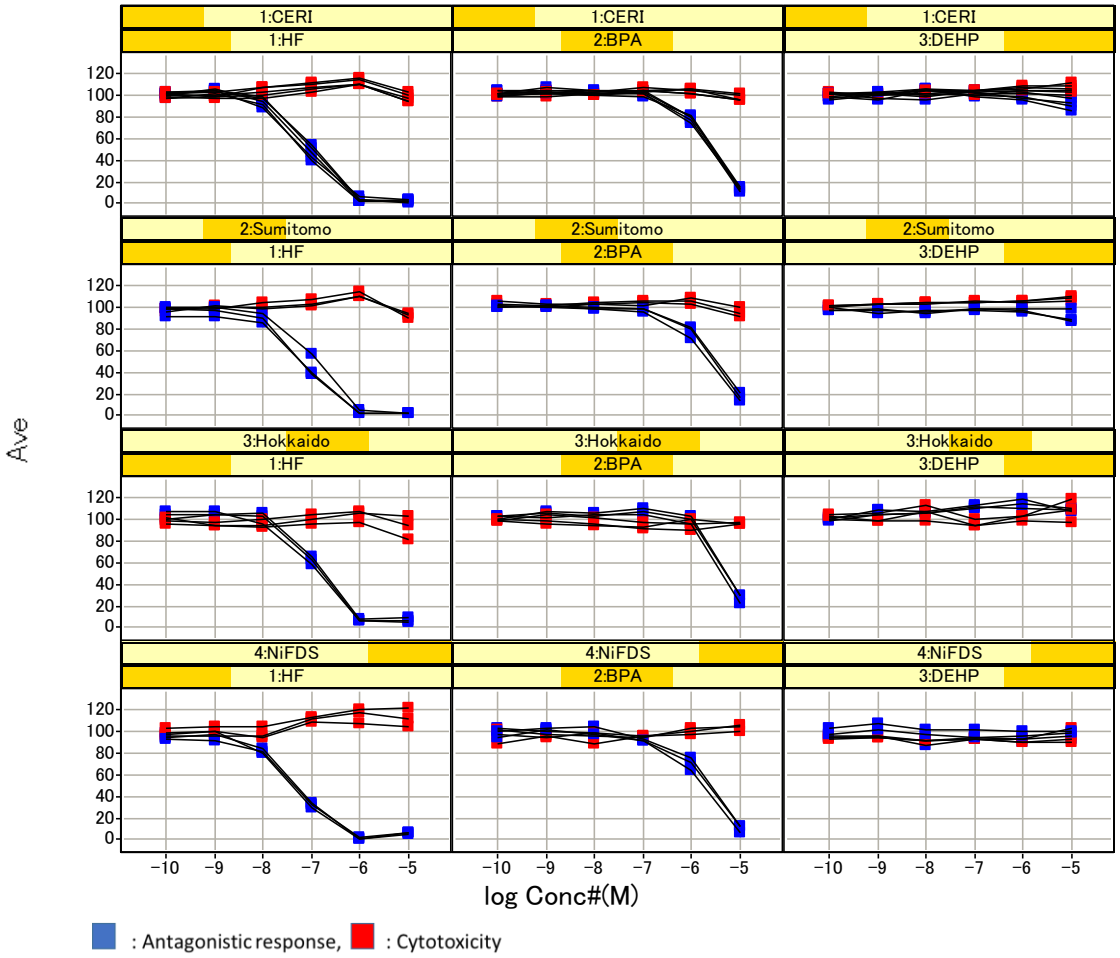


Fig. 5-2 Response curves for test chemicals in Phase-2 study-Antagonist

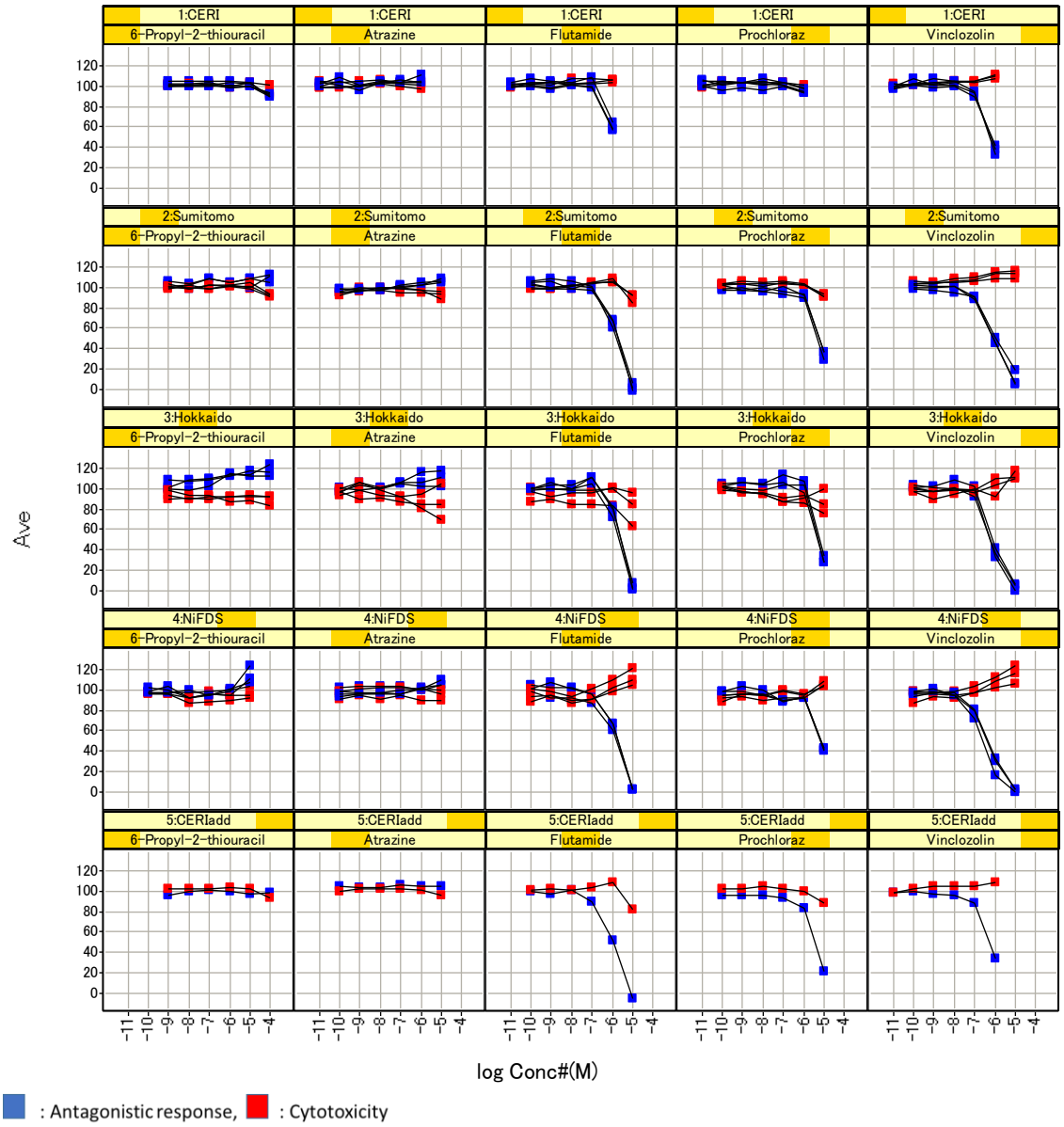


Table 18 Summary of the results for test chemicals in antagonist assay

	Lab	ID	Log IC30 (M)	Mean SD %CV	Log IC50 (M)	Mean SD %CV	Decision
6-Propyl-2-thiouracil CASRN:51-52-5	CERI	1	ND		ND		Negative
		2	ND		ND		
		3	ND		ND		
		Add	ND		ND		Negative
	Sumitomo	1	ND		ND		Negative
		2	ND		ND		
		3	ND		ND		
	Hokkaido	1	ND		ND		Negative
		2	ND		ND		
		3	ND		ND		
	NIFDS	1	ND		ND		Negative
		2	ND		ND		
		3	ND		ND		
	For 4 labs	Mean					Negative
		SD	ND		ND		
		%CV					
Atrazine CASRN:1912-24-9	CERI	1	ND		ND		Negative
		2	ND		ND		
		3	ND		ND		
		Add	ND		ND		Negative
	Sumitomo	1	ND		ND		Negative
		2	ND		ND		
		3	ND		ND		
	Hokkaido	1	ND		ND		Negative
		2	ND		ND		
		3	ND		ND		
	NIFDS	1	ND		ND		Negative
		2	ND		ND		
		3	ND		ND		
	For 4 labs	Mean					Negative
		SD	ND		ND		
		%CV					
Flutamide CASRN:13311-84-7	CERI	1	-5.96	-6.14	ND		Positive
		2	-6.13	0.15	ND		
		3	-6.15	2.45%	ND		
		Add	-6.33		-5.82		Positive
	Sumitomo	1	-5.96	-5.97	-5.57	-5.60	Positive
		2	-5.88	0.09	-5.57	0.05	
		3	-6.07	1.57%	-5.66	0.87%	
	Hokkaido	1	-5.71	-5.74	-5.43	-5.47	Positive
		2	-5.81	0.06	-5.53	0.05	
		3	-5.69	1.10%	-5.44	0.96%	
	NIFDS	1	-6.20	-6.04	-5.66	-5.61	Positive
		2	-5.96	0.14	-5.58	0.05	
		3	-5.95	2.31%	-5.58	0.82%	
	For 4 labs	Mean	-5.96	(-5.98)*	-5.56	(-5.58)	Positive
		SD	0.16	(0.19)*	0.08	(0.11)	
		%CV	2.74%	(3.14%)*	1.44%	(1.99%)	

Table 18 (continued)

	Lab	ID	Log IC30 (M)	Mean SD %CV	Log IC50 (M)	Mean SD %CV	Decision
Prochloraz	CERI	1	ND		ND		
CASRN:67747-09-5		2	ND		ND		Negative
		3	ND		ND		
		Add	-5.77	-5.77	-5.44	-5.44	Positive
	Sumitomo	1	-5.58	-5.60	-5.22	-5.25	
		2	-5.65	0.05	-5.33	0.06	Positive
		3	-5.56	0.89%	-5.21	1.23%	
	Hokkaido	1	-5.54	-5.53	-5.27	-5.26	
		2	-5.59	0.06	-5.30	0.05	Positive
		3	-5.47	1.14%	-5.20	1.04%	
	NIFDS	1	-5.53	-5.53	-5.15	-5.14	
		2	-5.52	0.01	-5.12	0.02	Positive
		3	-5.54	0.16%	-5.16	0.36%	
	For 4 labs	Mean	-5.55	(-5.57)*	-5.22	(-5.24)	
		SD	0.05	(0.08)*	0.07	(0.10)	Positive
		%CV	0.92%	(1.48%)*	1.36%	(1.87%)	
Vinclozolin	CERI	1	-6.44	-6.46	-6.07	-6.10	
CASRN:50471-44-8		2	-6.45	0.03	-6.04	0.05	Positive
		3	-6.46	0.48%	-6.14	0.82%	
		Add	-6.51		-6.14		Positive
	Sumitomo	1	-6.42	-6.38	-5.96	-5.92	
		2	-6.39	0.04	-5.95	0.06	Positive
		3	-6.34	0.62%	-5.85	0.96%	
	Hokkaido	1	-6.46	-6.40	-6.10	-6.07	
		2	-6.42	0.07	-6.12	0.07	Positive
		3	-6.32	1.09%	-6.00	1.09%	
	NIFDS	1	-6.83	-6.70	-6.47	-6.31	
		2	-6.65	0.11	-6.25	0.14	Positive
		3	-6.62	1.67%	-6.21	2.17%	
	For 4 labs	Mean	-6.48	(-6.49)	-6.10	(-6.10)	
		SD	0.17	(0.14)	0.19	(0.16)	Positive
		%CV	2.63%	(2.18%)	3.08%	(2.55%)	

*Values in parenthesis are overall Mean, SD and %CV containing additional trial by CERI.

Table 19 Positive/negative outcomes in antagonist assay and results of two-by-two table analysis

Test chemical	Candidate effect	Result				
		CERI	Sumitomo	Hokkaido	NIFDS	4 Lab
Flutamide	P	P	P	P	P	P
Prochloraz	P	N	P	P	P	P
Vinclozolin	P	P	P	P	P	P
Atrazine	N	N	N	N	N	N
6-Propyl-2-thiouracil	N	N	N	N	N	N
P:Positive						
N:Negative	Accuracy	80%	100%	100%	100%	95%
	Sensitivity	67%	100%	100%	100%	92%
	Specificity	100%	100%	100%	100%	100%

Table 20 Positive/negative outcomes in antagonist assay and results of two-by-two table analysis with consideration of additional trial

Test chemical	Candidate effect	Result				
		CERI	Sumitomo	Hokkaido	NIFDS	4 Lab
Flutamide	P	P	P	P	P	P
Prochloraz	P	P	P	P	P	P
Vinclozolin	P	P	P	P	P	P
Atrazine	N	N	N	N	N	N
6-Propyl-2-thiouracil	N	N	N	N	N	N
P:Positive						
N:Negative	Accuracy	100%	100%	100%	100%	100%
	Sensitivity	100%	100%	100%	100%	100%
	Specificity	100%	100%	100%	100%	100%

7. DISCUSSION

71. The human AR mediated stably transfected TA assay system using AR-EcoScreen™ was developed in Japan, and the assay system consisted of agonist and antagonist assays using a genetically modified stable cell line called AR-EcoScreen™. The cell line employs androgen responsive element (ARE) from prostate C3 gene-responsive element driven by a minimal heat shock protein promoter. This construct is confirmed to have minimal induction of glucocorticoid receptor (GR) mediated responses. AR Ecoscreen has great advantage to provide AR specific response with minimal GR crosstalk. We have compiled a validation report based on the results from the pre-validation study with 40 chemicals and the inter-laboratory validation study performed with the four participating laboratories using the same five chemicals for both androgenic and anti-androgenic activities in 2005.

72. The validation report was submitted to OECD in 2010. However the peer review panel report stated that a dedicated inter-laboratory study should be carried out, using the final test protocol to test substances covering a broad range of activity, especially including non-active substances and weak agonists and antagonists. This was an additional inter-laboratory validation study to be performed in accordance with the major peer review comments on the validation report.

73. The additional validation study was conducted with a total of ten test chemicals covering a broad range of agonist and antagonist activities selected by the chemical selection group consisting of OECD VMG-NA members. The study was conducted with three Japanese and one Korean laboratories.

74. The additional validation study consisted of Phase-1 and Phase-2 studies. The Phase-1 study was to confirm the overall laboratory proficiency by testing the same lot number of reference chemicals and to collect data to set reference criteria for mestanolone which was the newly added reference chemical for the agonist study. The Phase-2 study was to provide the supplemental data according to previous peer review comments on this assay and to evaluate the assay performance (within/between-laboratory reproducibility and predictive capacity) by blind testing of 5 coded chemicals for agonistic and 5 for antagonistic activities .

75. In the Phase-1 study, all laboratories passed the reference criteria within the minimum three runs, and the inexperienced Korean laboratory yielded successful results for the additional reference chemical for the agonist assay, mestanolone that met the tentative reference criteria decided based on the results obtained with three Japanese laboratories.

76. In the Phase-2 agonist study, all laboratories passed the reference criteria within the minimum three run, and all laboratories yielded correct positive/negative outcomes corresponding to the candidate effects. Consequently, the Accuracy, Sensitivity and Specificity of the agonist assay were all calculated to be 100% in all laboratories. In addition, the %CV of LogPC10(M) and LogPC50(M) for positive chemicals were less than 5% and high reproducibility of this assay was confirmed.

77. In the Phase-2 antagonist study, all laboratories passed the reference criteria within the minimum three runs, and three out of four laboratories could yield correct positive/negative outcomes corresponding to the candidate effects. However, the remaining one laboratory had a false negative result for the positive candidate chemical, Prochloraz.

78. Accordingly, in the results of the two-by-two table analysis in first three runs, the Accuracy, Sensitivity and Specificity for all four laboratories were calculated to be 95%, 92% and 100%, respectively.

79. However the cause of the false negative response for Prochloraz was considered to be a dose-selection issue rather than a technical issue. An additional trial was conducted using same concentration range as the laboratories that achieved a positive response, in order to confirm the cause of the false negative response. The laboratory then yielded a positive result for Prochloraz.

80. Consequently with the additional trial, all positive candidate chemicals, tested positive in all laboratories, and the Accuracy, Sensitivity and Specificity of the assay was calculated to be100% in all laboratories. In addition, the %CV of LogIC30(M) and LogIC50(M) for positive chemicals containing additional trial were less than 4%, and high reproducibility of this assay was confirmed.

81. The concordance of positive/negative outcomes of coded test chemicals were more than 80% for each of the agonist and antagonist assays, and the high assay performance of this assay was confirmed.

82. The results of the 2nd. validation study show that the original protocol is well established and robust, however the maximum dose selected by the solubility test described in the original protocol may occasionally affect the sensitivity of the assay. Therefore the following sentence should be including in the section of solubility test in the guideline:

    "A solubility test is a very important step to determine the maximum concentration for the assay and it may affect the sensitivity of the assay. Maximum concentration should be selected based on the avoidance of precipitation at highest concentration ranges in culture media. Precipitation observed at any concentration should be noted, but these data should not be included in the dose-response analysis."

83. On the other hand, the presence of increasing levels of cytotoxicity can significantly alter or eliminate the typical sigmoidal response and it should be considered when interpreting the data. Cytotoxicity test provides useful information for discriminating the antagonist effect of chemicals from cytotoxic effect. Accordingly, AR-mediated transcriptional activity and cytotoxicity should be evaluated simultaneously in the same assay plate especially in antagonist assay. For AR agonists, cytotoxicity can also affect the shape of a concentration response curve. In such case, evaluation of cytotoxicity might be considered.

8. CONCLUSIONS

84. Results of the additional inter-laboratory validation study for the human AR mediated stably transfected TA assay system using AR-EcoScreen™ with three Japanese and one Korean laboratories showed high reproducibility of the assay system and good technical transferability of the assay protocols because the concordance of positive/negative outcomes of coded test chemicals were more than 80% for each of agonist and antagonist assay.

85. Accordingly the assay system is well-established and has been shown to be a well-validated assay for the development into an OECD test guideline for the detection of chemicals possessing potential androgenic and anti-androgenic activities through hAR. The assay is, therefore, a promising method to use in the prescreening process of an endocrine disruptor screening strategy.

9. RECOMMENDATIONS

86. The original protocol is well established and robust as the results of the validation and additional validation studies demonstrate. However, the maximum dose selected by the solubility test described in the original protocol may occasionally affect the sensitivity of the assay. Accordingly, the following sentence should be including in the section of solubility test in the guideline:

    "A solubility test is a very important step to determine the maximum concentration for the assay and it may affect the sensitivity of the assay. Maximum concentration should be selected based on the avoidance of precipitation at highest concentration ranges in culture media. Precipitation observed at any concentration should be noted, but these data should not be included in the dose-response analysis."

10. ACKNOWLEDGMENTS

87. The Ministry of Economy Trade and Industry, METI and the Ministry of Health Labour and Welfare, MHLW, Japan supported this additional aspect of the validation programme for the ARTA. We very much appreciate the major contribution of these Japanese authorities, and the participating laboratories in the inter-laboratory validation study, Chemicals Evaluation and Research Institute (CERI), Sumitomo Chemical Co., Ltd., Hokkaido Institute of Public Health and National Institute of Food and Drug Safety Evaluation (NIFDS) in Republic of Korea. The support of the members of OECD VMG-NA is also gratefully acknowledged.

10.1.1 *Appendix 1 Contact List of AR EcoScreen™ 2nd Validation study*

Sponsor

Noritaka Miyasaka (Chief Officer)
Chemical Management Policy Division
Manufacturing Industries Bureau
1-3 Kasumigaseki, Chiyoda-ku, 100-8901 Tokyo, Japan

Study management team

Atsushi ONO
Division of Risk Assessment,
Biological Safety Research Center
National Institute of Health Sciences
Kamiyoga 1-18-1 Setagaya-ku 158-8501 Tokyo, Japan
Tel: +81(3) 3700-1429 Fax: +81(3) 3700-1408
E-mail:atsushi@nihs.go.jp

Anne Marie VINGGAARD
Toxicology and Risk Assessment
Technical University of Denmark
Division of Toxicology and Risk Assessment, National Food Institute
Moerkhoej Bygade 19 DK-2860 Soeborg, Denmark
Tel: +45 35 88 75 49
E-mail: annv@food.dtu.dk

Jenny ODUM
Regulatory Science Associates
39 Sandringham Road Macclesfield SK10 1QB Cheshire, United Kingdom
Tel: +44(0)7720 811615
E-mail: jenny.odum@regulatoryscience.com

Susan LAWS
National Health and Environmental Effects Research Laboratory,
Reproductive Toxicology Division Endocrinology Branch (MD-72)
2525 Highway 54 Research Triangle Park 27713 Durham, United States
Tel: +1 919 541 0173 Fax: +1 919 541 5138
E-mail: Laws.Susan@epa.gov

Masahiro TAKEYOSHI
Chemicals Assessment and Research Center
Chemicals Evaluation and Research Institute (CERI)
1600, Shimotakano Sugito-machi 345-0045 Kitakatsushita-gun, Japan
Tel: +81-(0)480-37-2601 Fax: +81-(0)480-37-2521
E-mail: takeyoshi-masahiro@ceri.jp

Participating Laboratories and responsible persons

Testing facility 1 (Lead laboratory)

Chemicals Assessment and Research Center
Chemicals Evaluation and Research Institute (CERI)
1600 Shimotakano, Sugito-machi, Kitakatsushika-gun

Saitama 3450043, Japan

Study Director: Yosuke Maeda, MS.

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

Study Director: Noriyuki Suzuki, Ph.D.

Testing facility 3 (Core laboratory)
Hokkaido Institute of Public Health
12 Nishi, Kita 19-jyo, Sapporo-shi
Hokkaido 0600819, Japan

Study Director: Hiroyuki Kojima, Ph.D.

Testing facility 4 (Observer laboratory)
Food Safety Risk Assessment Division
National Institute of Food and Drug Safety Evaluation (NIFDS)
Korean Ministry of Food & Drug Safety (MFDS)
Osong Health Technology Administration Complex, 187 Osongsaengmyeong2(i)-ro, Osong-eup,
Cheongwon-gun, Chungcheongbuk-do 363700, Korea

Study Director: Hong, Jin-hwan, Ph.D.

Chemical Distribution Management

Hajime Kojima, Ph.D.
Japanese Center for the Validation of Alternative Methods (JaCVAM),
National Institute of Health Sciences (NIHS), Japan.
1-18 -1 Kamiyouga, Setagaya-ku, 158-8501 Tokyo

10.1.2 *Appendix 2 Point to Point responses to the Peer Review Comments for the First validation report*

The Validation Peer Review Report of the *Androgen Receptor Mediated Stably Transfected Transcriptional Activation (AR-STTA) Assay to Detect Androgenic and Anti-androgenic Activities of Chemicals* was submitted for endorsement to the Working Group of National Coordinators of the Test Guidelines Program (WNT) at its April 2011 meeting.

Considering the major recommendations of the Peer Review Panel (summarized below), i.e.:

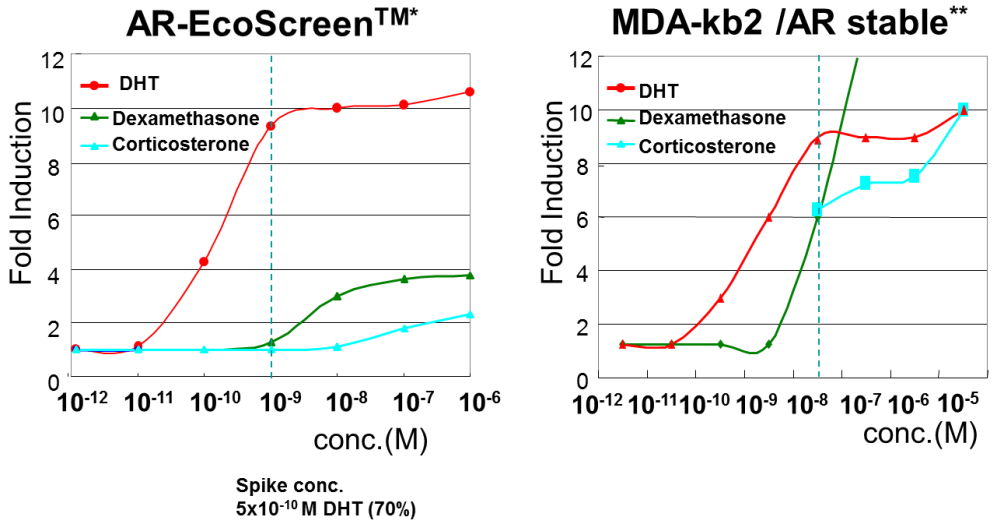
a) A dedicated inter-laboratory study should be carried out, using the final test protocol to test substances covering a broad range of activity, especially including non-active substances and weak agonists and antagonists. The number of substances already tested (five test chemicals) in the inter-laboratory validation, and the affinity range that they cover, is not sufficient;

Japan stated at VMG-9 meeting to conduct an additional validation study, and the study with 10 additional chemicals selected by OECD experts has been completed in 2014.
The results were summarised as additional validation report.

b) The following discussion points should be added to the validation report:
   a. advantages of the AR-STTA assay over similar AR activation assays (i.e., lack of Glucocorticoid receptors in this cell line eliminates cross-talk with AR, and more discussion of positive results in AR-STTA that are negative in AR binding assays),

      Some information about GR was already included in the validation report. The following sentences will be included in the validation report.

      AR Ecoscreen cell employs androgen responsive element (ARE) from prostate C3 gene-responsive element driven by a minimal heat shock protein promoter. This construct is confirmed to have minimal induction of GR mediated responses. AR Ecoscreen has great advantage to provide AR specific response with minimal GR crosstalk.



*K. Satoh *et al.* Journal of Health Science 51, 557-568 2005

**V.Wilson *et al. Toxicological Science*, 66, 2002

b. potential interference of partial agonists with antagonist effects, and proposed solutions to elucidate such interference,

Partial agonistic effect occurs in a dose dependent manner. Most of case, it elicit more than 100% activity at lower concentrations than those observed clear antagonistic effect. In such cases, suspected effect as partial agonist should be noted.

c. potential impacts of differences between protocols used for the pre-validation and the inter-laboratory validation studies

- ❑ Cell line: No impacts. As both pre- and inter-laboratory validations used the same cell line (AR-EcoScreen™)
- ❑ Cytotoxicity Evaluation: No impacts. cLuc-EcoScreen™ cell line was used to evaluate cytotoxicity in the pre-validation study. However, no classification differences were observed between the pre-validation and multi-lab validation studies based on 5 chemicals used tested in the multi-lab validation.

d. the lack of a cytotoxicity measurement in the agonist assay, which masks identification of true negatives from false negatives;

In both pre- and inter-laboratory validations, both agonistic and antagonistic activities were measured for all test chemicals. In the antagonistic assay, the cytotoxicity test is conducted in parallel. Therefore, cytotoxicity can be evaluated based on the data.

- The protocol should be revised to:
  e. add acceptance and assessment criteria for the positive control (5α-Dihydrotestosterone (DHT)),
  f.

When the validation study was started, the acceptance and assessment criteria were not defined. The criteria described in the Test Guideline, was determined by analysing the results of validation study, and these are provided in the Test Guideline.

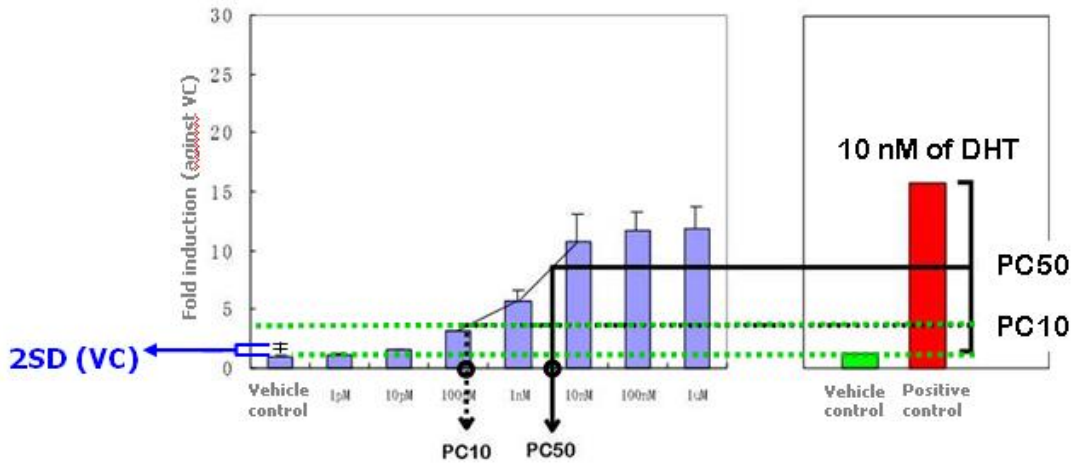
g. precisely define the decision criteria for classification, especially considering cytotoxic effects (e.g. introduce the option of equivocal/not conclusive results, since cytotoxicity can interfere with the detection of androgenic and especially anti-androgenic responses),

Data derived in a concentration where the cytotoxicity was observed will be omitted from the data evaluation. Therefore, cytotoxic effects do not affect the classification. However when equivocal result is suspected, additional run or check with specific antagonist would be recommended.

h. Explore the biological and statistical appropriateness of the PC10 in more detail,

Biological appropriateness:
The PC10 was employed as the criterion of detection of androgenic activity in the ICCVAM list. Our group have confidence about the biological appropriateness of the ICCVAM list. Furthermore, as described in a paragraph 93 of the 1st AR Validation Report, relationship between our results and ICCVAM list was analysed in a two-by-two table, the statistical appropriateness of the result was confirmed.

Statistical appropriateness:
PC10 is a useful simple parameter with significance without complicated statistical processing. In order to make PC10 significant, PC10 must be greater than 1 + 2SD (mean fold-induction of VC + 2SD of VC).



i. Include a list of proficiency chemicals for both the androgenic and the anti-androgenic assay,

These are provided in Table 2-1 and 2-2 in the draft TG.

the WNT agreed that, before finalizing the development of the draft Test Guideline for an AR-STTA assay,

- The Validation Management Group for non-animal testing should address the above recommendations as appropriate, in particular the recommendation to test more substances in a new inter-laboratory validation, while ensuring a good balance of substances with androgenic and anti-androgenic activity, negative and positive control substances;

- The cell line should be made freely available.

The cell line can be obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank as a reference No. JCRB1328, upon signing a Material Transfer Agreement (MTA)", the cell line can be available.

10.1.3 *Appendix 3 Definitions and abbreviations*

Agonist: A substance that binds to a specific receptor and triggers a response in the cell. It mimics the action of an endogenous ligand binds to the same receptor.

AG ref: Agonist reference (500 pM of DHT) in the antagonist assay.

Androgenic activity: the capability of a chemical to mimic 5α-Dihydrotestosterone in its ability to bind to and activate androgen receptors. AR mediated specific androgenic activity can be detected in this Test Guideline.

Antagonist: A type of receptor ligand or chemical that does not provoke a biological response itself upon binding to a receptor, but blocks or dampens agonist-mediated responses.

Anti-androgenic activity: the capability of a chemical to suppress the action of 5α-Dihydrotestosterone mediated through androgen receptors. AR mediated specific anti-androgenic activity can be detected in this Test Guideline.

AR: Androgen receptor

ARTA: Androgen Receptor Transcriptional Activation Assay.

BPA: Bisphenol A

CV: Coefficient of variation

Cytotoxicity: the harmful effects to cell structure or function ultimately causing cell death. It can be the result of a reduction in the number of cells present in the well at the end of the exposure period or a reduction of the capacity for a measure of cellular function when compared to the concurrent vehicle control.

DCC-FBS: Dextran-coated charcoal treated fetal bovine serum.

DEHP: Di(2-ethylhexyl)phthalate

DHT: 5α-Dihydrotestosterone

DMSO: Dimethyl sulfoxide

EC50 value: the concentration of agonist that provokes a response halfway between the baseline (Bottom) and maximum response (Top).

ER: Estrogen receptor

FBS: Fetal bovine serum

HF: Hydroxyflutamide

IC50: the concentration of a test chemical at which the measured activity in an antagonist assay inhibits at level of 50% of the maximum activity induced by 500 nM DHT in each plate

IC30: the concentration of a test chemical at which the measured activity in an antagonist assay inhibits at level of 30% of the maximum activity induced by 500 nM DHT in each plate

PC_{AGO}: Positive control (DHT at 10 nM)

PC_{ATG} : Positive AR antagonist control (500 pM DHT, 0.1 μM of HF)

PC10: the concentration of a test chemical at which the response in an agonist assay is 10% of the response induced by positive control (DHT at 10 nM) in each plate

PC50: the concentration of a test chemical at which the response in an agonist assay is 50% of the response induced by positive control (DHT at 10 nM) in each plate

PCmax: the concentration of a test chemical inducing the RPCmax

RPCmax: maximum level of response induced by a test chemical, expressed as a percentage against the response induced by PC_{AGO}(10 nM DHT) on the same plate

RTA: Relative Transcriptional Activity

RT PCR: Real Time polymerase chain reaction

SD: Standard deviation

STTA: Stably Transfected Transcriptional Activation Assay.

TA: Transcriptional activation

Validation: The process by which the reliability and relevance of a particular approach, method, process or assessment is established for a defined purpose (12).

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

Annex 1

Assay Protocol

For Androgen Receptor (AR) Mediated Stably Transfected Transcriptional Activation (AR-STTA) Assay to Detect Androgenic and Anti-androgenic Activities

Chemicals Evaluation and Research Institute, Japan

2013

1. **Cell Lines**

The stably transfected AR-EcoScreen^{TM} cell line should be used for the assay. The cell line can be obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank as a reference No. JCRB1328, upon signing a Material Transfer Agreement (MTA).

For this validation study, CERI will provide cell stock to each participant laboratory.

2. **Cell Culture and Plating Conditions**

Following mediums should be prepared;

Medium for dilution: Phenol Red Free D-MEM/F-12.

Medium for cell propagation: Phenol Red Free D-MEM/F-12 supplemented with 5% fetal bovine serum, Zeocin (200 μg/mL), Hygromycin (100 μg/mL) , Penicillin (100 units /mL), and Streptomycin (100 μg/ml).

Medium for the assay plate: Phenol Red Free D-MEM/F-12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12) supplemented with 5% DCC-FBS (dextran-coated charcoal-filtered fetal bovine serum), Penicillin (100 units /mL), and Streptomycin (100 μg/ml).

Cell should be maintained in a CO_2 incubator (5% CO_2) at 37±1˚C with Medium for cell propagation. Upon reaching 75-90% confluence, cells can be subcultured at 20 mL of 1.5 – 3.0 x 10^4 cells/mL for 75 cm^2 cell culture flask. To prepare the assay plate, cells should be suspended with the Medium for the assay plate and then plated into wells of a microplate at 90 μL/well at a density of 1 x 10^5 cells/mL. Next, the cells should be pre-incubated in a 5% CO_2 incubator at 37˚±1˚C for 24 hours before the chemical exposure.

To maintain the integrity of the response, the cells should be grown for more than one passage from the frozen stock in the conditioned media and should not be cultured for more than 40 passages. For the AR-EcoScreen^{TM} cell line, this will be less than two months.

The DCC-FBS can be prepared as described in Annex 2, or obtained from commercial sources. The selection of FBS is sometimes critical for the assay performance; therefore, the appropriate FBS should carefully be selected based on the cell response, as generally considered.

3. **Acceptability Criteria**

4.1. Positive and Negative Reference Chemicals

Prior to and during the study, the responsiveness of the test system should be verified using the appropriate concentrations of known reference chemicals as provided in Table 1-1 for AR agonist assay and Table 1-2 for AR antagonist assay. Acceptable range values for the LogPC10, Log PC50, LogIC30, and LogIC50 were derived using data submitted during the initial validation study and are given in Table 1-1 and Table 1-2 (2). All reference chemicals should be included with every AR agonist/antagonist experiments and the results should fall within the given acceptable limits. If this is not the case, the cause for the failure to meet the acceptability criteria should be determined (*e.g.* cell handling, and serum and antibiotics for quality and concentration) and the assay repeated. Once the acceptability criteria have been achieved, to ensure minimum variability of PC50, PC10, linearIC30 and linearIC50 values, consistent use of materials for cell culturing is essential. Thus, all reference chemicals should be included in each experiment (conducted under the same conditions including the materials, passage level of cells and technicians), and can ensure the sensitivity of the assay because the PC10s or linear IC30 of the two positive reference chemicals should fall within the acceptable range, as should the PC50s, or linear IC50 where they can be calculated (see Table 1).

Table 1-1 Performance criteria **for reference chemicals in AR agonist assay**

Fold-induction	> = 6.4		
PC10 value	Greater than 1 (fold-induction of VC) +2SD		
Chemical Name [CAS No.]	logPC10	logPC50	Test range
5α-Dehydrotestosterone (DHT) [521-18-6]	-9.87 ~-12.08	-9.00 ~ -11.03	$10^{-6} \sim 10^{-12}$M
Mestanolone [521-11-9]	to be confirmed	to be confirmed	$10^{-6} \sim 10^{-12}$M
Di(2-ethylhexyl)phthalate (DEHP) [117-81-7]	-	-	$10^{-5} \sim 10^{-10}$M

Table 1-2 Performance criteria for reference chemicals for AR antagonist assay

Fold induction of spike-in [Spike-in of 500 pM DHT]/[Vehicle Control]	> = 5.0		
PC_{ATG} inhibitory ratio	= <0.46		
Chemical Name [CAS No.]	log linearIC30	Log linearIC50	Test range
Hydroxyflutamide (HF) [52806-53-8]	-6.41 ~ -8.37	-6.17 ~ -7.80	$10^{-5} \sim 10^{-10}$M
Bisphenol A (BisA) [80-05-7]	-4.48 ~ -7.52	-4.29 ~ -7.05	$10^{-5} \sim 10^{-10}$M
Di(2-ethylhexyl)phthalate (DEHP) [117-81-7]	-	-	$10^{-5} \sim 10^{-10}$M

4.2 Positive and Vehicle Controls

For agonist assay, positive control (PC) wells (n=6) treated with a natural ligand (10 nM of DHT) and vehicle control (VC) wells (n=6) treated with vehicle alone, should be prepared in each assay plate. **For antagonist assay**, vehicle control (no spike-in*, n=3), positive control for agonistic activity (PC_{AG}, 10 nM of DHT, n=3), positive control for antagonistic activity (PC_{ATG}, 0.1 μM of HF, n=3), positive control for cytotoxicity (PC_{CT}, 10 μg/mL of cycloheximide, n=3) and spike-in control (SPK, 500 pM of DHT, n=12) should be set in each assay plate. As for the inhibitory ratio of PC_{ATG}, it should be greater than 0.46.

* spike-in：SPK, 500 pM of DHT in DMEM/F-12, n=12

4.3 Fold-induction

The mean luciferase activity of the PC (10 nM DHT) should be at least 6.4-fold that of the mean VC on each plate for agonist assay, and at least 5.0-fold for antagonist assay. These criterion was established based on the reliability of the endpoint values from the validation study .

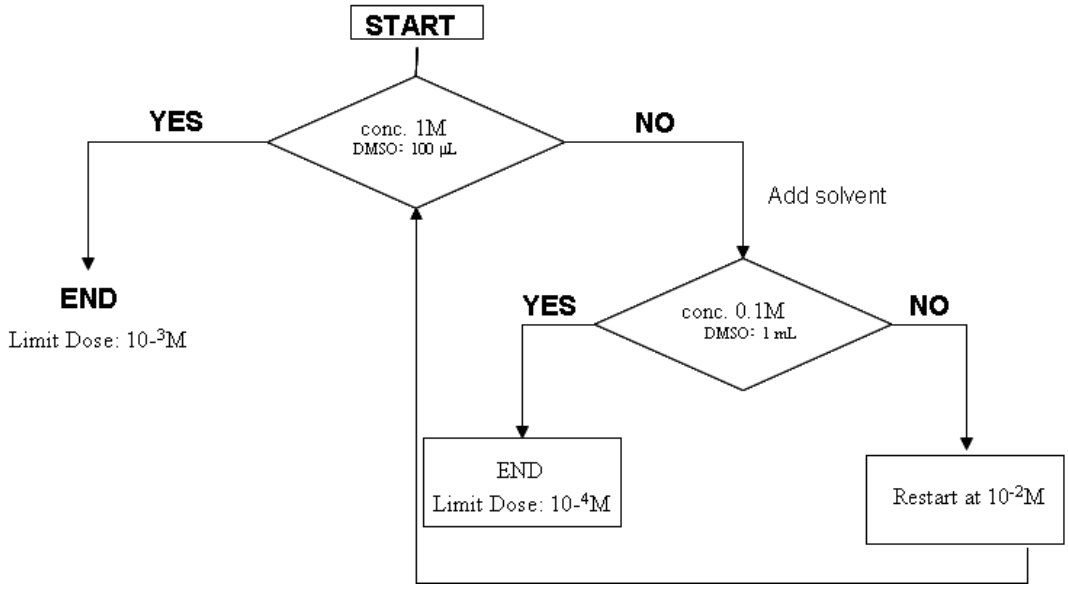
With respect to the quality control of the assay, the fold-induction corresponding to the PC10 value of the concurrent PC (10 nM DHT) should be greater than 1+2SD of the fold-induction value (=1) of the concurrent VC. For prioritization purposes, the PC10 value can be useful to simplify the data analysis required compared to a statistical analysis. Although a statistical analysis provides information on significance, such an analysis is not a quantitative parameter with respect to concentration-based potential, and so is less useful for prioritization purposes.

4. **Vehicle**

Dimethyl sulfoxide (DMSO), or appropriate solvent, at the same concentration used for the different positive and negative controls and the test chemicals should be used as the concurrent VC. Test substances should be dissolved in a solvent that solubilizes that test substance, is miscible with the cell medium and is not cytotoxic at the final test concentration..

5. **Preparation of Test Chemicals**

Generally, the test chemicals should be dissolved in DMSO and serially diluted with the same solvent at a common ratio of 1:10 in order to prepare solutions for dilution with media as the diagram for solubility test indicated below.



The test chemicals dissolved at the maximum concentration in DMSO will be serially diluted with the medium at a common ratio of 1:10. In this serial dilution with the medium, the highest concentration indicating no deposition is assigned as the highest concentration for the test.

6. **Cytotoxicity: Considerations for AR antagonist assay.**

For AR antagonist assay, the presence of increasing levels of cytotoxicity can significantly alter or eliminate the typical sigmoidal response and should be considered when interpreting the data. Cytotoxicity will be evaluated with renilla luciferase activity in AR-EcoScreen^{TM} cell line, which originally was established to expresses renilla luciferase constitutively. Accordingly, AR-mediated transcriptional activity and cytotoxicity will be evaluated simultaneously in same assay plate.

Should the results of the cytotoxicity test show that the concentration of the test substance has

reduced the cell number by 20% or more, this concentration is regarded as cytotoxic, and the concentrations at or above the cytotoxic concentration should be excluded from the evaluation.

7. **Chemical Exposure and Assay Plate Organisation**

For AR agonist assay, the procedure for chemical dilutions (Steps-1 and 2) and exposure to cells (Step-3) can be conducted as follows:

Step-1: Each test chemical should be serially diluted in DMSO, or appropriate solvent, and added to the wells of a microtitre plate to achieve final serial concentrations as determined by the preliminary range finding test (typically in a series of, for example 1 mM, 100 µM, 10 µM, 1 µM, 100 nM, 10 nM, 1 nM, 100 pM, and 10 pM (10^{-3}-10^{-11} M)) for triplicate testing.

Step-2: Chemical dilution: First dilute 10 µL of the test chemical in the solvent into 90 µL of media.

Step-3*: Then 10µL of the diluted chemical prepared in Step-2 should be diluted into 90 µL of the media for dilution.

Step-4: Chemical exposure of the cells: Add 10 µL of diluted chemical solution (prepared in Step-3) to an assay well containing 9×10^3 cells/90 µL/well.
The recommended final volume of media required for each well is 100 µL.

* In case the precipitation of the test chemical occurred in or after Step-3, the data of the relevant concentration range should be neglected in the analysis.

Test samples and reference chemicals can be assigned as shown in Table 3-1.

Table 3-1.: Example of plate concentration assignment of the reference chemicals in the assay plate for agonist assay

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

VC: Vehicle control (DMSO);

PC: Positive control (10 nM of DHT)

For AR antagonist assay, the procedure for chemical dilutions (Steps-1 and 2) and exposure to cells (Step-3) can be conducted as follows:

Step-1: Each test chemical should be serially diluted in DMSO, or appropriate solvent, and added to the wells of a microtitre plate to achieve final serial concentrations on the test plate as determined by the preliminary range finding test (typically in a series of, for example 1 mM, 100 μM, 10 μM, 1 μM, 100 nM, 10 nM, 1 nM and 100 pM (10^{-3}-10^{-10} M)) for triplicate testing.

Step-2: Chemical dilution: First add 10 μL of the test chemical in the solvent prepared in Step 1 to 90 μL of media containing 56nM DHT/0.1% DMSO.

Step-3*: Dilution of chemical and DHT in media: Add 10μL of the diluted chemical prepared in Step-2 to 90 μL of the media (without DHT).

Step-4: Chemical exposure of the cells: Add 10 μL of diluted chemical solution (prepared in Step-3) to an assay well containing 9×10^3 cells/90 μL/well.
The recommended final volume of media required for each well is 100 μL.

* In case the precipitation of the test chemical occurred in or after Step-3, the data of the relevant concentration range should be neglected in the analysis.

Test samples and reference chemicals can be assigned as shown in Table 3-2.

Table 3-2 Example of plate concentration assignment of the reference chemicals in the assay plate for antagonist assay

Row	HF			Bisphenol A			DEHP			Chemical 1		
	1	2	3	4	5	6	7	8	9	10	11	12
A	10 μM	→	→	10 μM	→	→	10 μM	→	→	10 μM	→	→
B	1 μM	→	→	1 μM	→	→	1 μM	→	→	1 μM	→	→
C	100 nM	→	→	100 nM	→	→	100 nM	→	→	100 nM	→	→
D	10 nM	→	→	10 nM	→	→	10 nM	→	→	10 nM	→	→
E	1 nM	→	→	1 nM	→	→	1 nM	→	→	1 nM	→	→
F	100 pM	→	→	100 pM	→	→	100 pM	→	→	100 pM	→	→
G	SPK	→	→	→	→	→	→	→	→	→	→	→
H	VC	→	→	PC_{AG}	→	→	PC_{ATG}	→	→	PC_{CT}	→	→

VC: Vehicle control (DMSO);

PC_{AG}: Positive control (10 nM of DHT);

PC_{ATG}-: Positive control (0.1 μM of HF) ;

PC_{CT}: Cytotoxicity control (10 μg/mL of cycloheximide);

SPK (DMSO at 0.1% spiked with $5x10^{-10}$ M DHT)

** Gray colored wells are spiked with $5x10^{-10}$M DHT

The reference chemicals (DHT, Mestanolone and DEHP for agonist assay; HF, BisA and DEHP for antagonist assay) should be tested in every run (as exampled in Table 3-1 and 3-2). PC wells treated with 10 nM of DHT that can produce maximum induction of DHT and VC wells treated with DMSO (or appropriate solvent) alone should be included in each test assay plate for agonist assay (Table 4-1). In case of antagonist assay, PC_{ATG}: Antagonist positive control (0.1 μM of HF), PC_{CT}: Cytotoxicity control (10 μg/mL of cycloheximide) and SPK-in control (DMSO at 0.1% spiked with 500 pM DHT) should be prepared additionally (Table 4-2). If cells from different sources (*e.g.* different passage number, different lot, etc.,) are used in the same experiment, the reference chemicals should be tested for each cell source.

Table 4-1.: Example of plate concentration assignment of test and plate control chemicals in the assay plate for agonist assay

Row	Test Chemical 1			Test Chemical 2			Test Chemical 3			Test Chemical 4		
	1	2	3	4	5	6	7	8	9	10	11	12
A	conc 1 (10 µM)	→	→	1 mM	→	→	1 µM	→	→	10 nM	→	→
B	conc 2 (1 µM)	→	→	100 µM	→	→	100 nM	→	→	1 nM	→	→
C	conc 3 (100 nM)	→	→	10 µM	→	→	10 nM	→	→	100 pM	→	→
D	conc 4 (10 nM)	→	→	1 µM	→	→	1 nM	→	→	10 pM	→	→
E	conc 5 (1 nM)	→	→	100 nM	→	→	100 pM	→	→	1 pM	→	→
F	conc 6 (100 pM)	→	→	10 nM	→	→	10 pM	→	→	0.1 pM	→	→
G	conc 7 (10 pM)	→	→	1 nM	→	→	1 pM	→	→	0.01 pM	→	→
H	VC	→	→	→	→	→	PC	→	→	→	→	→

Plate controls = VC: Vehicle control (DMSO); PC: Positive control (10 nM of DHT)

Table 4-2.: Example of plate concentration assignment of test and plate control chemicals in the assay plate for antagonist assay

Row	Test Chemical 1			Test Chemical 2			Test Chemical 3			Test Chemical 4		
	1	2	3	4	5	6	7	8	9	10	11	12
A	conc 1 (10 µM)	→	→	1 mM	→	→	1 µM	→	→	10 nM	→	→
B	conc 2 (1 µM)	→	→	100 µM	→	→	100 nM	→	→	1 nM	→	→
C	conc 3 (100 nM)	→	→	10 µM	→	→	10 nM	→	→	100 pM	→	→
D	conc 4 (10 nM)	→	→	1 µM	→	→	1 nM	→	→	10 pM	→	→
E	conc 5 (1 nM)	→	→	100 nM	→	→	100 pM	→	→	1 pM	→	→
F	conc 6 (100 pM)	→	→	10 nM	→	→	10 pM	→	→	100 pM	→	→
G	SPK	→	→	→	→	→	→	→	→	→	→	→
H	VC	→	→	PC_{AG}	→	→	PC_{ATG}	→	→	PC_{CT}	→	→

VC: Vehicle control (DMSO);

PC_{AG}: Positive control (10 nM of DHT);

PC_{ATG}-: Positive control (0.1 µM of HF) ;

PC_{CT}: Cytotoxicity control (10 µg/mL of cycloheximide);

SPK (DMSO at 0.1% spiked with $5x10^{-10}$ M DHT)

** Gray colored wells spiked with $5x10^{-10}$M DHT

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

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

8. **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) can be used for the agonism detection. And Dual-Glo (Promega, E2920 and its equivalents) can be used for

the antagonism detection, as long as the acceptability criteria are met. The assay reagents should be selected based on the sensitivity of the luminometer to be used. When using the standard luciferase assay system, Cell Culture Lysis Reagent (Promega, E1531, or equivalents) should be used before adding the substrate. In case of using Steady-Glo Luciferase Assay System (Promega, E2510) in the agonist assay, 40 μL of prepared reagent should be directly added into the assay wells. In case of using Dual-Glo system (Promega, E2920) in the antagonist assay, 40 μL of the first substrate should be added into the assay wells after removing 60 μL of supernatant to detect Firefly luciferase activity. Then 40 μL of the second substrate should be added into the assay wells to detect Renilla luciferase activity.

9. **ANALYSIS OF DATA**

For Agonist assay, to obtain the relative transcriptional activity to PC (10 nM of DHT), the luminescence signals from the same plate can be analyzed according to the following steps (other equivalent mathematical processes are also acceptable):

Step 1. Calculate mean value for the VC.

Step 2. Subtract the mean value of the VC from each well value to normalize the data.

Step 3. Calculate the mean for the normalised PC.

Step 4. Divide the normalized value of each well in the plate by the mean value of the normalized PC (PC=100%).

The final value of each well is the relative transcriptional activity for that well compared to the PC response.

Step 5. Calculate the mean value of the relative transcriptional activity for each concentration group of the test chemical. There are two dimensions to the response: the averaged transcriptional activity (response) and the concentration at which the response occurs (see following section).

To evaluate cytotoxicity in the antagonist assay, cell viability should be expressed as the percentage of renilla luciferease activity of the chemical treated wells to the mean renilla luciferase activity of the VC wells.

For each test chemical, the following should be provided:

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

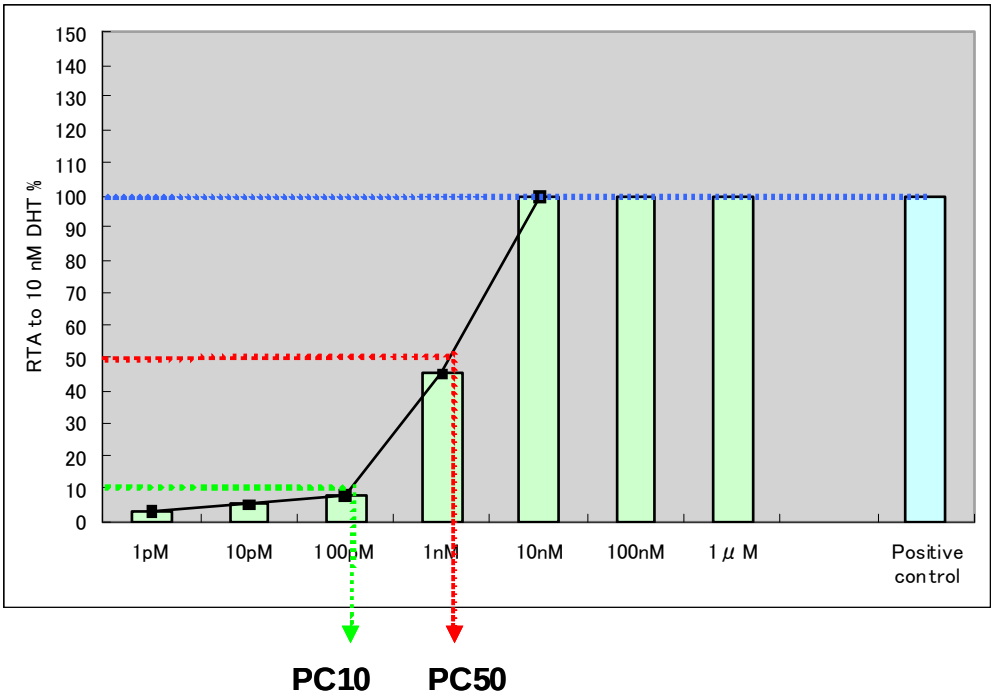
The PCx value can be calculated by interpolating between 2 points on the X-Y coordinate, one immediately above and one immediately below a PCx value. Where the data points lying immediately above and below the PCx value have the coordinates (a,b) and (c,d) respectively, then

the PCx value may be calculated using the following equation:

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

Descriptions of PC values are provided in Figure 1 below.

Figure 1: Example of how to derive PC-values in agonist assay.
The PC (Positive control; 10 nM of DHT) is included on each assay plate



For Antagonist assay, to obtain the relative transcriptional activity, the luminescence signals from the same plate can be analyzed according to the following steps (other equivalent mathematical processes are also acceptable):

Step 1. Calculate mean value for the VC.

Step 2. Subtract the mean value of the VC from each well value to normalize the data.

Step 3. Calculate the mean for the normalized SPK.

Step 4. Divide the normalized value of each well in the plate by the mean value of the normalized mean SPK (SPK=100%).

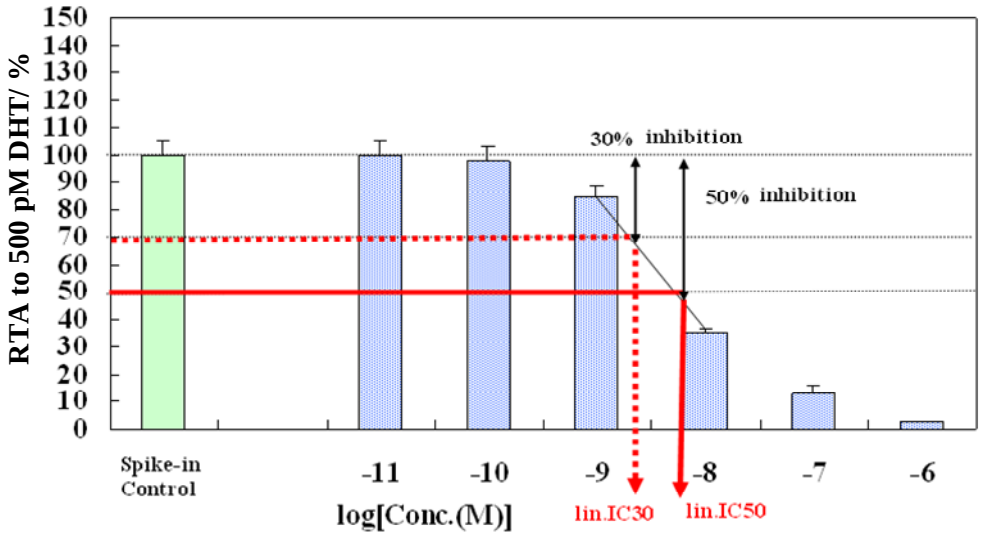
The final value of each well is the relative transcriptional activity for that well compared to the maximum SPK response.

Step 5. Calculate the mean value of the relative transcriptional activity for each concentration group of the test chemical. There are two dimensions to the response: the averaged transcriptional activity (response) and the concentration at which the response occurs (see following section).

In case of antagonist assay, (i) The RICMax which is the maximum inhibition level of luciferase induced by a test chemical, expressed as a percentage of the response induced by 500 pM DTH on the same plate, as well as the PCMax (concentration associated with the RPCMax); and if the RICmax is exist, the lin.IC50 and lin.IC30 values should be calculated. These lin.IC50 and lin.IC30 values can be defined as the test chemical concentrations estimated to elicit either a 50% or a 30% inhibition of transcriptional activity induced by 500 pM DHT that can be calculated same as PC values. Each lin.ICx value can be calculated by a simple linear regression using two variable data points in the transcription activity same as PCx values.

Descriptions of lin.ICx values are provided in Figure 2 below.

<u>Figure 2.:</u> Example of how to derive lin.IC-values in antagonist assay.
The SPK-in control (DMSO at 0.1% spiked with 500 pM DHT) is included on each assay plate.



To distinguish pure antagonism and cytotoxicity related decrease of luciferase activity, AR-EcoScreen is designed to express two kinds of luciferase, one is firefly luciferase inducibly expressed by AR response element and other is Renilla luciferase stably and non-inducibly expressed.

By using Dual-luciferase reporter assay technology, both cell viability and the antagonism can be evaluated with the same cells in a single plate run. PCct (10μg/mL of cycloheximide) is made for adjusting renilla activity by taking PCct values “renilla activities” away from that of all sample wells. To evaluate the true cytotoxicity of chemicals with AR Ecoscreen cell, such revised cell viability should be used. RICMax and lin.ICx values can be accepted if cell viability is recorded as more than 80% at the test concentration.

ANNEX 2

2nd Validation Plan

For Androgen Receptor (AR) Mediated Stably Transfected Transcriptional Activation (AR-STTA) Assay to Detect Androgenic and Anti-androgenic Activities

(Version 131121)

Prepared by

Chemicals Evaluation and Research Institute, Japan
National Institute of Health Sciences, Japan

Approved by

Study management team of the 2nd validation study of AR STTA

2013

BACKGROUND

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 a global concern regarding endocrine disruption effects resulting from chemical exposure, particularly those mediated by the estrogen receptor (ER) and androgen receptor (AR). 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.The EDSTAC recommended that in vitro assays, such as receptor binding and reporter gene assays, be used to screen chemicals for hormone receptor agonist and antagonist activity as part of a tier 1 screening battery, then many efforts have been taken to develop reporter gene assay systems for evaluating ER and AR mediated effects of chemicals.

2 Several reporter gene assay systems 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. Currently, “Stably Transfected Transcriptional Activation (TA) using HeLa-9903 cell line for detecting estrogenic activity of chemicals” has been adopted as OECD test guideline (TG 455) in 2009. Although the need for AR in vitro assays are also urgent, at the present time there are no in vitro screening assays for androgenic activity that have been peer reviewed for potential test guideline development, to enable use for OECD regulatory purposes.

3 We have developed the reporter gene assay system using AR Ecoscreen cell and compiled validation report based on results from the pre-validation study with 40 chemicals and the inter-laboratory validation study performed with the four participating laboratory using 5 same chemicals for both androgenic and anti-androgenic activities.

4 The validation report has submitted to OECD in 2010. However the Peer review panel report stated that a dedicated inter-laboratory study should be carried out, using the final test protocol to test substances covering a broad range of activity, especially including non-active substances and weak agonists and antagonists.

5 According to the peer review comment, we make a plan of the additional inter-laboratory validation study.

OBJECTIVES

6 The aim of this study is to test intralaboratory repeatability and intra- and inter-laboratory reproducibility of Androgen Receptor (AR) EcoScreen protocol using additional chemicals in according to the OECD peer review comments for the previously conducted 1st validation study.

VALIDATION DESIGN

7 The validation study for the stably transfected TA assay using AR-EcoScreen^{TM} cell line to detect androgenic/anti-androgenic activities is consisted of the **Phase-1 and Phase-2 studies**. Prior to start the validation study, each laboratory will conduct the proficiency test following the technical training.

ORGANIZATION

8 Validation study sponsor and supporters

Sponsor*

- Ministry of Economy, Trade and Industory, Japan.

Supporters

- Ministry of Health, Labour and Welfare, Japan.
- National Institute of Health Sciences (NIHS)
- Japanese Center for the Validation of Alternative Methods (JaCVAM)

9 Participating laboratories*

The validation study is conducted by four participating laboratories as follows;

- Chemicals Evaluation and Research Institute (CERI, Lead Laboratory)
- Environmental Health Science Laboratory of Sumitomo Chemical Co. Ltd,
- Hokkaido Institute of Public Health
- National Institute of Food and Drug Safety Evaluation, Korea (NIFDS)

The lead laboratory representing the test method is responsible for providing the test method protocol and the necessary assay datasheets (MSExcel format) and worksheets (MSword format), etc. The lead laboratory is also responsible for providing, if necessary, new versions of the protocols during the entire validation trail. The lead laboratory and the other participating laboratory might be contacted by the Project Coordinator for technical issues.

* See Appendix 1 for detailed contact address

10 The Study management team (SMT) [*]

SMT is organized with following members to support the validation process;

Dr. Atsushi Ono (NIHS, Japan)	Project Coordinator Quality assurance
Dr. Masahiro Takeyoshi (CERI-Japan)	Expertise of this assay Quality assurance
Dr. Anne Marie Vinggaard (Technical University of Denmark) Dr. Jenny Odum (Regulatory Science Associate) Dr. Susan Laws (US EPA)	Validation study expertise

11 Chemical Distribution Management[*]

- Dr. Hajime Kojima (JaCVAM, Japan)

PROTOCOL

12 In this validation study, the protocol will be used (attached Document #A). The protocol will make up a draft by the lead laboratory and be finalized by SMT.

[*] See Appendix 1 for detailed contact address

PROFICIENCY TEST

The aim of the proficiency test is to confirm the technical transfer status at each participating laboratory by testing same stock of minimal reference chemicals used in the technical transfer meeting.

13 Prior to start the Phase-1 study, the technical transfer meeting will be held at CERI for domestic two laboratories, and Edge effect check and Proficiency test will be conducted to check technical transfer status in each laboratory. Edge effect check shall be conducted in all participating laboratory.

14 As for the NIFDS, the proficiency test would be absolved because the NIFDS staff had been technically trained in the technical transfer meeting held in CERI in 2012, and their proficiency was confirmed by the report submitted to CERI. .

15 AR-EcoScreen^{TM} cell is distributed to each participating laboratory excluding NIFDS by CERI. And the information with regard to the lot of serum and the list of the other materials used in the validation will be also announced by CERI prior to the start of validation study.

16 Edge effect will be checked by assay plate which was uniformly seeded $9x10^3$ cell/well with 10nM Dihidrotestosterone (DHT). If the case that both CV% of RLU values between columns and laws measured 24 h after stimulation are less the 10%, the edge effect would be decided as negligible.

17 In the proficiency test, each laboratory, excluding NIFDS, shall test the minimal reference chemicals (DHT and Di(2-ethylhexyl)phthalate (DEHP) for agonist assay, Hydroxyflutamide (HF) and DEHP for antagonist assay) used in the technical transfer meeting in own laboratories with same plate assignment at the technical transfer.

18 At least one run of assay results that meet the performance criteria shown in the protocol is minimum requirement for each laboratory to start the Phase-1 study.

19 Each laboratory should submit the results of the edge effect check and the proficiency test to the Project Coordinator before start the Phase-1 study.

PHASE–1 STUDY

The aims of the Phase-1 study are to confirm the overall laboratory proficiency by testing same lots of reference chemicals and to collect data to set a reference criteria for mestanolone. In addition, data from the NIFDS will be used to confirm the validity of performance criteria.

20 In the Phase-1 study, the reference chemicals listed in Table 1-1 and table 1-2 of the protocol will be provided by Chemical Distribution Management excluding NIFDS, Each laboratory shall test the Phase-1 chemicals according to the assay protocol.

21 NIFDS will obtain same maker of reference chemicals in Korea by own and test all chemicals in same manner to the Japanese laboratories.

22 The Phase-1 study is conducted with participating laboratories that passed the performance criteria in Proficiency test. Each laboratory shall test the reference chemicals at least three runs in triplicate.

23 The plate assignments for Phase-1 study should comply with the assay protocol.

24 The assay results should be stored and locked in the Specified work sheet provided by CERI. Then each laboratory will required to submit at least 3 sets of assay results that meet the all performance criteria shown in the assay protocol to the Project Coordinator. If the any laboratories will not produce 3 sets of successful assay results within 5 runs, the laboratory should suspend subsequent assay steps and comply with the instruction from the SMT.

25 NIHS will set the performance criteria for Mestanolone as mean logPCx±2SD based on the assay results in the Phase I study obtained in three Japanese laboratories. The data from the NIFDS for this chemical will be used to confirm the validity of performance criteria.

26 An acceptance criterion of Phase-1 is that all participating laboratory perform more than 3 sets of assay which meet the performance criteria excluding Mestanolone.

PHASE-2 STUDY

The aims of the Phase-2 study is to provide the supplemental data according to previous Peer review comments of this assay and to evaluate the assay performance (within/between-laboratory reproducibility and predictive capacity) by testing 10 coded chemicals (each five for agonist and antagonist).

27 The Phase-2 study is conducted with participating laboratories that passed all the performance criteria in the Phase-1 study.

28 The Phase-2 study is performed with 5 test chemicals for the agonist assay and 5 test chemicals for the antagonist assay. Each laboratory shall test the Phase-2 chemicals in triplicate at appropriate concentration range of each chemical for the assessment of their activity.

29 The totally 10 chemicals for Phase-2 study will be provided by Chemical Distribution Management in coded manner and each laboratory shall test the chemicals according to the assay protocol from the maximum concentration decided according to the diagram for solubility test .

30 At every runs of the Phase-2 study, simultaneously test reference chemicals used in Phase-1 study is required.

31 The plate assignments for Phase-2 study should comply with the assay protocol.

32 The assay results should be stored and locked in the Specified work sheet provided by CERI. Then each laboratory will required to submit at least 3 sets of assay results that meet the all performance criteria shown in the assay protocol including newly decided criteria for Mestanolone to the Project Coordinator. If the any laboratories will not produce 3 sets of successful assay results within 5 runs, the laboratory should suspend subsequent assay steps and comply with the instruction from the SMT.

33 All results in the validation study will be analyzed in NIHS to evaluate the assay performance of this assay. The interlaboratory concordance of judgment (positive/negative) of coded test chemicals should be more than 80% for each of agonist and antagonist assay as acceptance criteria.

CHEMICALS

34 Reference chemicals, and test chemicals will be shipped according to proper regulatory procedures. Each participating laboratory will be notified by Chemical Distribution Management when any reference chemicals, and test chemicals are shipped. Upon receipt, chemicals should be stored in appropriate storage conditions as per recommendations provided by Chemical Distribution Management. Each participating laboratory should notify the SMT Project Coordinator upon receipt.

35 Reference chemicals

36 Reference chemicals and solvent (DMSO) used in the validation study will distribute from Distribution Management (JaCVAM) prior to start phase1 study to Japanese participant laboratory and prior to start phase2 to NIFDS. Japanese participant laboratory conduct phase 1 and 2 studies used distributed chemicals. NIFDS conduct phase 1 study used chemicals obtaining in local (Korea) and conduct phase 2 study used distributed chemicals.

37 Test chemicals

38 Test chemicals have been selected based on the suggestion from the voluntary chemical selection team of OECD validation management group of non-animal (VMG-NA) by considering the following criteria.

39 + ICCVAM recommendations: ICCVAM Evaluation of In Vitro Test Methods for Detecting Potential Endocrine Disruptors: Estrogen and Androgen Receptor Binding and Transcriptional Activation Assays (Addendum, Tables 6-1 and 6-2)

40 + appropriate negative and positive effects on published AR Ecoscreen assay results

41 + historical data of lead laboratory

42 + availability

43 + costs

44 Coded test chemicals will be packaged so as to conceal their identities and shipped prior to start phase 2. Coded test chemicals, along with a sealed health and safety information package will be shipped to the designated Safety Officer. The Safety Officer should retain the safety information package and pass the coded test chemicals to the Study Director. The safety information package will contain necessary information about the substance hazards and provide instructions for emergency actions. A disclosure key for identifying the test chemicals by code will also be included in the package. If the health and safety package must be opened during the course of the validation study, the Safety Officer should immediately notify the SMT Project Coordinator.

STUDY TIMELINE

45 All laboratories should summit data to NIHS by the fixed deadline as follows;

Edge effect test:	October 31, 2013
Proficiency test:	November 15, 2013
Phase-1 study:	November 30, 2013
Phase-2 study:	January 17, 2014
Draft validation report submission:	September 30, 2014

RECORDS AND ARCHIVES

46 Copy of assay datasheets (MSExcel format) and worksheets (MSword format), signed by the Study Director, should be submitted to the Project Coordinator at the completion of each study phase (i.e., Phases-1,-2).

47 At the end of the validation study, the original raw and derived assay data, as well as copies of other raw data not exclusive to this validation study (instrument logs, calibration records, facility logs, etc.), should be stored and archived for at least five years. At the end of this five year-storage and archiving period, these stored/archived materials should be submitted to sponsor for storage and archiving.

Appendix 1

Contact List of AR EcoScreen 2nd Validation study.

Sponsor

Chemical Management Policy Division
Manufacturing Industries Bureau
1-3 Kasumigaseki, Chiyoda-ku, 100-8901 Tokyo, Japan

Noritaka Miyasaka (Chief Officer)

Study management team

Atsushi ONO
Division of Risk Assessment,
Biological Safety Research Center
National Institute of Health Sciences
Kamiyoga 1-18-1 Setagaya-ku 158-8501 Tokyo, Japan
Tel: +81(3) 3700-1429 Fax: +81(3) 3700-1408
E-mail:atsushi@nihs.go.jp

Anne Marie VINGGAARD
Toxicology and Risk Assessment
Technical University of Denmark
Division of Toxicology and Risk Assessment, National Food Institute
Moerkhoej Bygade 19 DK-2860 Soeborg, Denmark
Tel: +45 35 88 75 49
E-mail: annv@food.dtu.dk

Jenny ODUM
Regulatory Science Associates
39 Sandringham Road Macclesfield SK10 1QB Cheshire, United Kingdom
Tel: +44(0)7720 811615
E-mail: jenny.odum@regulatoryscience.com

Susan LAWS
National Health and Environmental Effects Research Laboratory,
Reproductive Toxicology Division Endocrinology Branch (MD-72)
2525 Highway 54 Research Triangle Park 27713 Durham, United States
Tel: +1 919 541 0173 Fax: +1 919 541 5138
E-mail: Laws.Susan@epa.gov

Masahiro TAKEYOSHI
Chemicals Assessment and Research Center
Chemicals Evaluation and Research Institute (CERI)
1600, Shimotakano Sugito-machi 345-0045 Kitakatsushita-gun, Japan
Tel: +81-(0)480-37-2601 Fax: +81-(0)480-37-2521
E-mail: takeyoshi-masahiro@ceri.jp

Participating Laboratories and responsible persons

Testing facility 1 (Lead laboratory)
Chemicals Assessment and Research Center
Chemicals Evaluation and Research Institute (CERI)
1600 Shimotakano, Sugito-machi, Kitakatsushika-gun
Saitama 3450043, Japan

Study Director: Yosuke Maeda, MS.

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

Study Director: Noriyuki Suzuki, Ph.D.

Testing facility 3 (Core laboratory)
Hokkaido Institute of Public Health
12 Nishi, Kita 19-jyo, Sapporo-shi
Hokkaido 0600819, Japan

Study Director: Hiroyuki Kojima, Ph.D.

Testing facility 4 (Observer laboratory)
Food Safety Risk Assessment Division
National Institute of Food and Drug Safety Evaluation (NIFDS)
Korea Ministry of Food & Drug Safety (MFDS)
Osong Health Technology Administration Complex, 187
Osongsaengmyeong2(i)-ro, Osong-eup, Cheongwon-gun,
Chungcheongbuk-do 363700, Korea

Study Director: Hong, Jin-hwan, Ph.D.

Chemical Distribution Management

Hajime Kojima, Ph.D.

Japanese Center for the Validation of Alternative Methods (JaCVAM),
National Institute of Health Sciences (NIHS), Japan.
1-18 -1 Kamiyouga, Setagaya-ku, 158-8501 Tokyo