Organisation de Coopération et de Développement Économiques
Organisation for Economic Co-operation and Development

24-Jan-2017

English - Or. English

ENVIRONMENT DIRECTORATE
JOINT MEETING OF THE CHEMICALS COMMITTEE AND THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY

Cancels & replaces the same document of 13 July 2016

Report of the validation of the (anti-) ERα CALUX bioassay: U2-OS cells Transcriptional ERalpha CALUX- assay for the detection of estrogenic and anti-estrogenic chemicals for inclusion in TG455

Series on Testing & Assessment
No. 240

This document is available in pdf format only.

JT03408150

ENV/JM/MONO(2016)35
Unclassified
English - Or. English

OECD Environment, Health and Safety Publications

Series on Testing and Assessment

No. 240

REPORT OF THE VALIDATION OF THE (ANTI-) ERα CALUX BIOASSAY: U2-OS CELLS TRANSCRIPTIONAL ERalpha CALUX - ASSAY FOR THE DETECTION OF ESTROGENIC AND ANTI-ESTROGENIC CHEMICALS FOR INCLUSION IN TG455

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

About the OECD

The Organisation for Economic Co-operation and Development (OECD) is an intergovernmental organisation in which representatives of 35 industrialised countries in North and South America, Europe and the Asia and Pacific region, as well as the European Commission, meet to co-ordinate and harmonise policies, discuss issues of mutual concern, and work together to respond to international problems. Most of the OECD's work is carried out by more than 200 specialised committees and working groups composed of member country delegates. Observers from several countries with special status at the OECD, and from interested international organisations, attend many of the OECD's workshops and other meetings. Committees and working groups are served by the OECD Secretariat, located in Paris, France, which is organised into directorates and divisions.

The Environment, Health and Safety Division publishes free-of-charge documents in twelve different series: **Testing and Assessment**; **Good Laboratory Practice and Compliance Monitoring**; **Pesticides**; **Biocides**; **Risk Management**; **Harmonisation of Regulatory Oversight in Biotechnology**; **Safety of Novel Foods and Feeds; Chemical Accidents; Pollutant Release and Transfer Registers; Emission Scenario Documents; Safety of Manufactured Nanomaterials;** and **Adverse Outcome Pathways.** More information about the Environment, Health and Safety Programme and EHS publications is available on the OECD's World Wide Web site (www.oecd.org/chemicalsafety/).

This publication was developed in the IOMC context. The contents do not necessarily reflect the views or stated policies of individual IOMC Participating Organizations.

The Inter-Organisation Programme for the Sound Management of Chemicals (IOMC) was established in 1995 following recommendations made by the 1992 UN Conference on Environment and Development to strengthen co-operation and increase international co-ordination in the field of chemical safety. The Participating Organisations are FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD. The purpose of the IOMC is to promote co-ordination of the policies and activities pursued by the Participating Organisations, jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment.

This publication is available electronically, at no charge.

For this and many other Environment, Health and Safety publications, consult the OECD's World Wide Web site (www.oecd.org/ehs)

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 validation study of the Transcriptional Estrogen Receptor alpha (ERα) CALUX assay for the detection of estrogenic and anti-estrogenic chemicals. This validation report supported the development of a new test method for inclusion in the Performance Based Test Guideline 455 for Stably Transfected Transactivation In Vitro Assays to Detect Estrogen Receptor Agonists and Antagonists.

The project for the revision of the Performance Based Test Guideline 455 with the inclusion of this new test method was proposed by the Netherlands and included in the work plan of the Test Guidelines Programme in 2014.

The validation report was developed in 2015 and submitted for review to a subgroup of the Validation Management Group for Non Animal Testing (VMG NA). The lead country subsequently revised the validation report to address the comments from the reviewers and developed the related draft test method. The validation report and the revised updated TG 455 were discussed at the meeting of the VMG NA on 1-3 December 2015 (Budapest, Hungary). The documents were subsequently circulated for review and commenting to the Working Group of the National Coordinators of the Test Guidelines Programme (WNT) in December 2015. No comment was received on the validation report which 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 the validation of the (anti-) ERα CALUX bioassay

U2-OS cells Transcriptional ERalpha CALUX- assay for the detection of estrogenic and anti-estrogenic chemicals for inclusion in TG455

Author: Dr.Ir. H.T. Besselink

BioDetection Systems B.V. (BDS)
Science Park 406
1098 XH Amsterdam
Tel.: +31 (0)20 4350 750
Fax: +31 (0)20 4350 757
E-mail: calux@bds.nl

Reviewed by: Prof. Dr. A. Brouwer
Dr. Z Dang
Dr T Bovee

Content

1 Acronyms

%CV	Coefficient of Variation
$\%VC_R$	Reproducibility variation coefficient
BDS	BioDetection Systems BV (the Netherlands)
DMSO	Dimethylsulfoxide
EC	European Commission
EC_{10}	The molar concentration of a chemical which produces 10% of the maximum possible response for that chemical
EC_{50}	The molar concentration of a chemical which produces 50% of the maximum possible response for that chemical
ECVAM	European Centre for the Validation of Alternative Methods
EDCs	Endocrine Disrupting Chemicals
ERα	Estrogen Receptor alpha
ERE	Estrogen Responsive Element
EU	European Union
GD 34	OECD Guidance Document 34 "Guidance document on the validation and international acceptance of new or updated test methods for hazard assessment"
GLP	Good Laboratory Practice
IC_{20}	The molar concentration of a chemical which produces 80% of the maximum possible response for that chemical (20% inhibition)
IC_{50}	The molar concentration of a chemical which produces 50% of the maximum possible response for that chemical (50% inhibition)
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods (U.S.)
IWW	IWW Rheinisch-Westfälisches Institut für Wasserforschung (Germany)
NC	Negative control
NIES	National Institute for Environmental Studies (Japan)
OECD	Organisation for Economic Co-operation and Development
PC	Positive control
PC_{10}	The concentration of a chemical at which its response equals 10% of the maximum response of the reference standard
PC_{50}	The concentration of a chemical at which its response equals 50% of the maximum response of the reference standard.
PC_{80}	The concentration of a chemical at which its response equals 80% of the maximum response of the reference standard.
RIVM	National Institute for Public Health and the Environment (the Netherlands)
SC	Solvent control
SD	Standard Deviation
SOP	Standard Operating Procedure
SPSF	Standard Project Submission Form
TA	Transcriptional Activation
TC_{max}	Maximum relative induction of the test chemical (relative to the maximum induction of reference standard).
TC_{min}	Minimum relative induction of the test chemical (relative to the maximum induction of reference standard).
TCx	Test chemical x
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

2 Summary

1. Two stably transfected human estrogen receptor-α (ERα) transcriptional activation assays for the detection of estrogenic agonist- and antagonistic-activity of chemicals have been validated according to OECD validation guidelines and have been incorporated into the "OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals" as "Level 2" *in vitro* assays. Performance Based Test Guidelines (PBTG; TG455) and Performance Standards (PS) for these assays (BG1Luc ER TA assay; STTA assay) are available.

2. New test methods that are based on similar scientific principles and measure/predict a similar biological effect, can be added to an updated PBTG once such new similar test methods ("me-too" tests) have met available performance standards (e.g. accuracy and reliability). Here, the validation results of the estrogen responsive reporter-gene ERα CALUX bioassay (a stably transfected transactivation *in vitro* assay to detect estrogen receptor agonists and antagonists) are presented. This bioassays was validated as a "me-too' test method according to OECD guidelines for validation and performance standards, for inclusion in relevant guidelines (i.e. TG455). The validation of the ERα CALUX bioassay included an intra- and inter-laboratory validation, both containing pre-screening and comprehensive testing. In total 22 chemicals were tested for their agonistic potency and 11 chemicals for their antagonistic potency.

3. Intra-laboratory comparison of results showed that for the agonistic assay, the reproducibility variation coefficient (%VC_R) of the log[EC50] and log[PC10] for 17 positive test chemicals were less than 4.5%. The intra-laboratory coefficient of variance (%CV) was found to be less than 3.5% for all participating laboratories. The intra-laboratory comparison of results for the antagonistic assay also showed good reproducibility and variance. %VC_R and %CV of the log[IC50] and log[PC80] were also below 4.5% and 3.5% respectively for positive test chemicals. Intra-laboratory concordance of classification, based on the qualitative assessment of test chemicals in 3 independent consecutive runs (1 prescreen run, 2 comprehensive runs), was 100% for both agonism and antagonism.

4. The inter-laboratory comparison of analysis results revealed that for both the agonistic and antagonistic mode of the ERα CALUX bioassay, the reproducibility variation coefficient and coefficient of variance of positive test chemicals was less than 4% (based on log[EC_{50}] and log[PC_{10}] (agonism) and log[IC_{50}] and log[PC_{80}] (antagonism)). Furthermore, comparison of the log[EC_{50}], log[PC_{10}] (agonism) and log[IC_{50}] showed high consistency with data obtained by the STTA and Bg1Luc assays already included in the OECD Performance Based Test Guideline TG455. Inter-laboratory concordance of classification, based on the qualitative assessment of test chemicals in independent consecutive runs (agonism: 8 selected test chemicals tested in 3 independent consecutive runs (1 prescreen run, 2 comprehensive runs) and 14 test chemicals tested in 2 consecutive test runs (2 comprehensive runs); antagonism: all 10 selected test chemicals tested in 3 independent consecutive runs (1 prescreen run, 2 comprehensive runs)), was 95% for agonism and 100% for antagonism

5. Qualitative assessment of analysis results and comparison to the consensus ICCVAM classification showed very good agonistic accuracy and reliability performance values and very good antagonistic accuracy and reliability performance values.

6. The overall assay performance met the criteria as described in OECD Guidance Document No.34 (GD 34) and the OECD performance standards for stably transfected transactivation in vitro assays to detect estrogen agonists (TG455) and antagonists (TG457),

including accuracy and reliability performance values. Thus, the stably transfected ERα CALUX bioassays for the detection of estrogenic agonist- and antagonistic-activity of chemicals has been shown to be a suitable assay for inclusion in the OECD Performance Based Test Guideline for Stably Transfected Transactivation *In vitro* Assays to Detect Estrogen Receptor Agonists and Antagonists (TG455).

3 Introduction

7. Certain chemicals that enter our environment can cause adverse effect by disrupting endocrine processes. It has been proven difficult to identify such endocrine disrupting properties efficiently using existing OECD guideline tests. Therefore, the OECD initiated with high priority the revision and development of new test guidelines and a conceptual framework for screening and testing of such potential endocrine disrupting chemicals. The OECD conceptual framework for testing and assessment of potential endocrine disrupting chemicals was revised in 2012. The conceptual framework comprises 5 levels, each level corresponding to a different level of biological complexity. Several stably transfected transactivation assays have been developed to efficiently detect estrogen receptor agonists and antagonists. According to the OECD conceptual framework, these transactivation assays are placed at level 2.

8. The OECD has established a Performance-Based Test Guideline (PBTG) for in vitro transactivation assays: Performance-Based Test Guideline for Stably Transfected Transactivation In Vitro Assays to Detect Estrogen Receptor Agonists and Antagonists (TG455), allowing use of transactivation assays in a regulatory context [1]. The basis for this PBTG is provided by 2 fully validated reference test methods (the TA (STTA) assay and the BG1Luc ER TA assay) according to OECD validation guidelines [2]. Additional ER transactivation assays (e.g. ERα CALUX) can be included in the OECD updated TG455 following validation ("me-too" tests). The ERα CALUX (Chemically Activated LUciferase eXpression) transcriptional assay is based on the robust and rapidly growing human U2-OS cell line, and can be used for testing estrogenic and anti-estrogenic chemicals. The validation should meet the OECD performance standards for stably transfected transactivation *in vitro* assays to detect estrogen agonists and antagonists [3,4]

9. The transcriptional activation ERα CALUX test has already been pre-validated with an extensive set of chemicals of which 30 had in vivo data available. The results with these chemicals showed an excellent correlation with the Allen-Doisy test [5]. Moreover, using different sets of chemicals, BDS in collaboration with ECVAM and partner laboratories has already performed a successful prevalidation study to measure both estrogenic and anti-estrogenic activity of chemicals, including the assessment of within- and between-laboratory reproducibility [6].

10. Here, the estrogen responsive reporter-gene ERα CALUX bioassay (a stably transfected *in vitro* transactivation assay to detect estrogen receptor agonists and antagonists) was validated as a "me-too' test method according to OECD guidelines for validation and performance standards to be included in TG455.

4 Validation design

11. To include the ERα CALUX bioassay in the OECD PBTG 455, BDS initiated the full validation of the ERα CALUX bioassay (agonistic and antagonistic mode) according to the OECD guidelines for validation [2] and performance standards for stably transfected transactivation *in vitro* assays to detect estrogen agonists [3] and antagonists [4].

According to the performance standards, data for the qualitative assessment of the within- and between- laboratory concordance of classification should be obtained from 3 independent consecutive test runs. The present validation study did not strictly follow this procedure. The pre-screen run was also considered as a test run for classification (positive / negative) of test chemicals. Therefore, the pre-screen run and 2 comprehensive runs were used for the qualitative assessment of the within- and between- laboratory concordance of classification (3 independent consecutive test runs).

The validation of the ERα CALUX bioassay consisted of 2 phases:

1 Intra-laboratory validation phase.
2 Inter-laboratory validation phase.

The objective of the intra-laboratory validation phase was to:

- assess the within-laboratory reproducibility (concordance of classification)
- determine the intra-laboratory variability and reproducibility with respect to the investigated endpoints
- assess the relevance of the proposed test method to detect a range of estrogenic and anti-estrogenic activities
- identify acceptable intra-laboratory variations of the test protocol;

The objective of the inter-laboratory validation phase was to:

- assess the between-laboratory reproducibility (concordance of classification)
- determine the intra- and inter-laboratory variability and reproducibility with respect to the investigated endpoints
- identify acceptable inter-laboratory variations of the test protocol;

Both phases contained a pre-screen run and comprehensive runs.

4.1 Organization

12. The validation of the stably transfected *in vitro* transactivation ERα CALUX bioassay was organized and coordinated by BioDetection System BV (BDS) and supervised by a study director. The NL National Coordinator and experts from the Dutch National Institute for Public Health and the Environment provided advice on the OECD procedures, blindly labelled the performance chemicals and reviewed the draft of the report. The key for the labelled chemicals was released after submission of all data to the organizer of the study. Data handling was performed by the organising party using standardised calculation documents that were validated prior to the study. The calculation documents were protected for modifications. Three laboratories participated in the validation study: BioDetection Sytems BV (BDS), National Institute for Environmental Studies Center for Material Cycles and Waste Management Research Material Lifecycle and Substance Management Section (NIES) from Japan, and Rheinisch-Westfälisches Institut für Wasserforschung (IWW) from Germany. The participating laboratories already implemented the ERα CALUX bioassay and use the bioassay for analysis. All participating laboratories were therefore appropriately facilitated and used materials as indicated in the standard operating procedures. All personnel performing the assays had adequate training and qualifications. The participants did not have prior experience using the standard operating procedures to be used for the validation study. The present validation study was not conducted under GLP. However, both the intra- and inter-laboratory validation of the

ERα CALUX bioassay were performed and managed in line with GLP principles. In figure 1, the organization scheme employed for the intra-and inter-laboratory validation study is given.

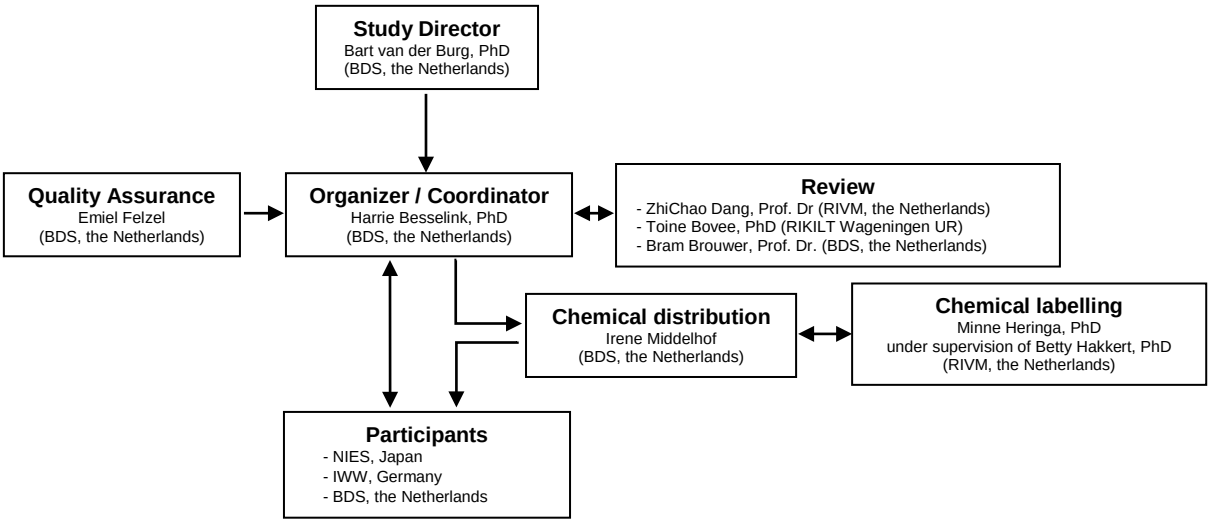


Figure 1. Organization scheme for the intra- and inter-laboratory ERα CALUX validation study.

Table 1 Organization, supporters and participants of the ERα CALUX validation study

Organization /coordination		
BioDetection Systems BV (BDS)	Science Park 406 1098 XH Amsterdam The Netherlands	Contact: Harrie Besselink, PhD harrie.besselink@bds.nl
Advisors/blinding of performance chemicals		
National Institute for Public Health and the Environment (RIVM); Ministry of Health, Welfare and Sport	P.O. Box 1 3720 BA Bilthoven The Netherlands	Contact: Betty C. Hakkert, PhD betty.hakkert@rivm.nl Zhichao Dang zhichao.dang@rivm.nl Minne Heringa, PhD minne.Heringa@rivm.nl
Participants		
BioDetection Systems BV (BDS	Science Park 406 1098 XH Amsterdam The Netherlands	Contact: Harrie Besselink, PhD harrie.besselink@bds.nl
National Institute for Environmental Studies. Center for Material Cycles and Waste Management Research Material Lifecycle and Substance, Management Section (NIES)	Onogawa 16-2 Tsukuba 305-8506 Japan	Contact: Go Suzuki, PhD g-suzuki@nies.go.jp
IWW Rheinisch-Westfälisches Institut für Wasserforschung	Moritzstrasse 26 45476 Mülheim an der Ruhr Germany	Contact: Jessica Richard, PhD j.richard@iww-online.de

Table 2 Overview of the Standard Operating Procedures

	ERα CALUX (agonism)	**Anti-ERα CALUX (antagonism)**
Cell line	ERα CALUX (human U2OS cells stably transfected with hERα)	ERα CALUX (human U2OS cells stably transfected with hERα)
Cell medium (assay medium)	D-MEM/F12 medium without phenol red (DMEM/F12) containing 5% dextran-coated charcoal-treated fetal calf serum (DCC-FCS)	D-MEM/F12 medium without phenol red (DMEM/F12) containing 5% dextran-coated charcoal-treated fetal calf serum (DCC-FCS)
Vehicle	DMSO	DMSO
Vehicle control	1% DMSO final concentration	1% DMSO final concentration
Final concentration vehicle	1%	1%
Reference standard	17β-estradiol	Tamoxifen
Positive control	17α-methyltestosterone	4-hydroxytamoxifen
Negative control	Corticosterone	Resveratrol
No. of test chemicals within intra- and inter-laboratory validation	22	11
No. of runs per test chemical	3 (Pre-screen, comprehensive 1, comprehensive 2, optional comprehensive 3)	3 (Pre-screen, comprehensive 1, comprehensive 2, optional comprehensive 3)
Concentrations tested	Pre-screen run: starting at highest soluble concentration, dilution steps 0f 10 (1 – 10000000 times). Comprehensive runs: see annex C	Pre-screen run: starting at highest soluble concentration, dilution steps 0f 10 (1 – 10000000 times). Comprehensive runs: see annex C
Cell density	10.000/well	10.000/well
Incubation time	22-24 hr.	22-24 hr.

Note. all concentratration of reference standards, positive control, negative control are analysed in triplicate.

4.2 Standard operating procedures

13. A standard protocol for performing the agonistic and antagonistic ERα CALUX bioassay was constructed before the start of the intra-laboratory validation of the bioassay by the organizer of the validation study. Protocols were based on earlier protocols from a successful prevalidation of the assay [6], and further improvements and adaptations to match requirements in OECD TG455. In addition, slight modifications for determination of cytotoxicity were implemented in these standard operating procedures. The final protocol (Transactivation assay for the detection of chemicals with (anti)estrogenic potential using ERα CALUX cells) is given in annex A and was used for inter-laboratory validation of the (anti-) ERα CALUX bioassay by all participating laboratories. An overview of the protocol is given in table 2.

14. In addition to a standard protocol, standard calculation documents were prepared and used throughout the intra- and inter-validation studies. Adequate and efficient quality controls were included. For all measurements, a maximum set of six 96-microtiter plates were exposed in 1 experiment (maximum of 11 test chemicals). Only plate 1 contained a dilution serie of the reference standard. To be able to compare results from the remaining 5 microtiterplates with the microtiterplate containing the reference standard, all microtiterplates contained 3 control samples: DMSO blank, the highest concentration of the reference standard and the approximate EC_{50}/IC_{50} concentration of the reference standard (see figure 2 and 3). Only microtiter plates that fulfil the acceptance criteria for these control samples and the acceptance criteria for the Z-factor for each microtiter plate, are considered valid and can be used to evaluate the response of the test chemicals.

15. The acceptance criteria for both the agonistic and antagonistic ERα CALUX analyses are given in the table 3 and table 4 respectively. In addition, these acceptance criteria are automatically evaluated in the calculation documents.

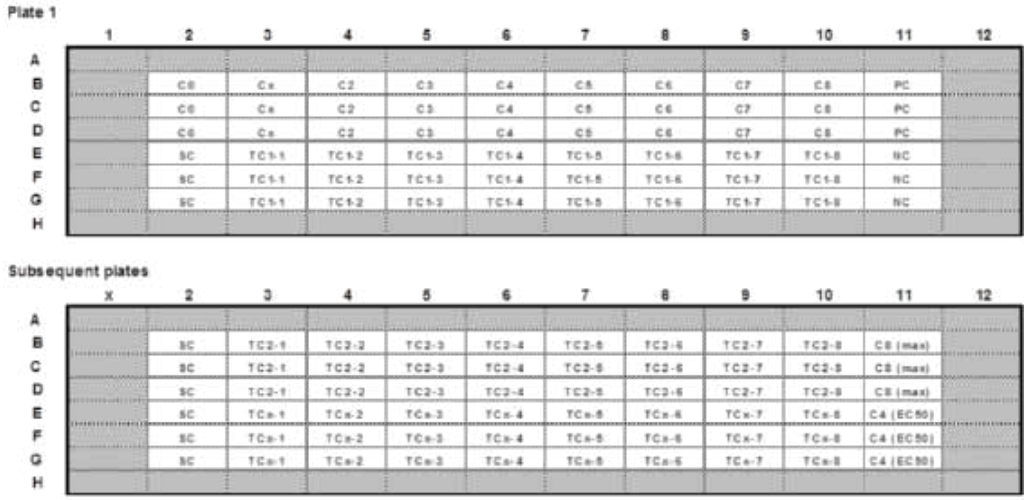


Figure 2 Plate Lay-out of the of the 96 microtiter plates for prescreening and assessment of agonistic effect.

C0	=	solvent reference. Work solution containing solvent reference only.
C(1-8)	=	concentrations (1-8) of reference-work solution.
PC	=	positive control.
NC	=	negative control.
TCx-(1-8)	=	dilutions (1-8) of test chemical-work solution for the pre-screen run and assessment of agonistic effect of test chemical x.
SC	=	Solvent control of the test chemical evaluated on the same rows. Work solution containing solvent for test chemical x only.

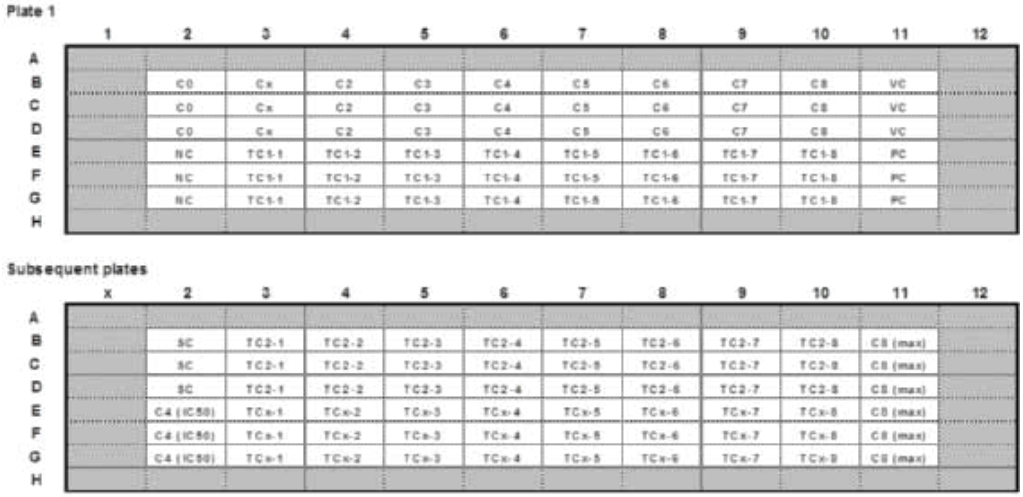


Figure 3 Plate Lay-out of the of the 96 microtiter plates for antagonistic prescreening and assessment of antagonistic effect.

C0 = solvent reference. Work solution containing solvent reference only.
C(1-8) = concentrations (1-8) of reference-work solution.
NC = negative control.
PC = positive control.
TCx-(1-8) = dilutions (1-8) of test chemical-work solution for the pre-screen run and assessment of agonistic effect of test chemical x.
SC = Solvent control of the test chemical. Work solution containing solvent for test chemical x only.
VC = Vehicle control. Work solution prepared using assay medium (not agonist supplemented assay medium!) containing solvent only.

Table 3 Acceptance criteria for the agonistic ERα CALUX bioassay.

	individual samples on a plate	**Criterion**
1	Maximum %SD of triplicate wells (reference, test chemical, PC, NC)	< 15%
2	Maximum %SD of triplicate wells (solvent controls (C0, SC)	< 30%
3	Maximum LDH leakage, as a measure of cytotoxicity.	< 120%
	within a single microtiter plate	
4	Ratio of the reference solvent control (C0; plate 1) and test chemical solvent control (SC; plates 2 to x)	0.5 to 2.0
5	Ratio of the reference concentrations C4 & C8 (plate 1) and reference concentrations C4 & C8 (plates 2 to x)	0.70 to 1.30
6	Z-factor for each plate	>0.6
	within a single series of analyses (all plates within one series)	
7	Sigmoidal curve of reference standards	Yes (17ß-estradiol)
8	EC_{50} range reference standard 17ß-estradiol	$4*10^{-12}$ – $4*10^{-11}$ M
9	Minimum fold induction of the highest 17ß-estradiol concentration, with respect to the reference solvent control.	5
10	Relative induction (%) PC.	> 30%
11	Relative induction (%) NC	<10%

Table 4 Acceptance criteria for the antagonistic ERα CALUX bioassay.

	individual samples on a plate	**Criterion**
1	Maximum %SD of triplicate wells (reference (C0-C7), test chemical, PC, NC, SC)	< 15%
2	Maximum %SD of triplicate wells (vehicle control (VC), reference (C8))	< 30%
3	Maximum LDH leakage, as a measure of cytotoxicity.	< 120%
	within a single microtiter plate	
4	Ratio of the reference solvent control (C0; plate 1) and test chemical solvent control (SC; plates 2 to x)	0.70 to 1.30
5	Ratio of the reference concentrations C4 (plate 1) and reference concentrations C4 (plates 2 to x)	0.70 to 1.30
6	Ratio of the reference concentrations C8 (plate 1) and reference concentrations C8 (plates 2 to x)	0.50 to 2.0
7	Z-factor for each plate	>0.6
	within a single series of analyses (all plates within one series)	
8	Sigmoidal curve of reference standard	Yes (Tamoxifen)
9	IC_{50} range reference standard Tamoxifen	$1*10^{-8}$ - $1*10^{-7}$ M
10	Minimum fold induction of the reference solvent control, with respect to the highest Tamoxifen concentration.	2.5
11	Relative induction (%) PC.	<70%
12	Relative induction (%) NC	>85%

4.3 Cells

16. ERα CALUX cell lines (U2-OS-based CALUX cells) were supplied by BioDetection Systems BV (BDS). ERα CALUX cells originated from the human osteoblastic osteosarcoma U2-OS cell line. Human U2-OS cells were stably transfected with 3xHRE-TATA-Luc and pSG5-neo-hERα using the calcium phosphate co-precipitation method [7,8]. Cells as provided by BDS, Amsterdam, the Netherlands were tested and found to be negative from mycoplasm contamination.

4.4 Chemicals and other materials

17. During both phases of the validation, the ERα CALUX bioassay was evaluated for its ability to detect estrogen agonists and antagonist. For agonistic validation, a set of 22 test chemicals recommended in the performance standards for transactivation *in vitro* assays to detect estrogen agonists [3] was used (see table 5). For antagonistic validation, a set of 11 recommended test chemicals recommended in the performance standards for transactivation *in vitro* assays to detect estrogen antagonists [4] was used (table 6).

Inter-laboratory phase

18. All recommended test chemicals, reference standards and control chemicals to be shipped to the participants, were prepared by the organizer of the validation study. For the pre-screen run, the recommended test chemicals were distributed as pure chemicals. For the comprehensive runs, stock solutions of all test chemicals for each comprehensive run were prepared in DMSO by the organizer. Reference standard and positive and negative control chemicals were distributed as pure chemicals. Stock concentrations of reference standard and control chemicals in DMSO had to be prepared by the participants. DMSO to be used to dissolve and dilute chemicals was also distributed by the organizer. All chemicals and materials distributed were accompanied by detailed instructions on how to store and use the chemicals.

19. Before shipment, all test chemicals for both the pre-screen run and comprehensive runs were labelled blind by an independent expert from the National Institute for Public Health and the Environment (RIVM), who assisted the National Coordinator of The Netherlands). The key for labelling was kept by the RIVM and only released after all analysis result were obtained by the organizer of the study.

Table 5 Selected test chemicals for validation agonistic ERα CALUX

Test chemical	CAS no.	Nature	Supplier	Purity	Lot.no.	coding pre-screen	coding comprehensive
Dimethyl sulfoxide (DMSO; solvent)	67-68-5	liquid	Acros Organics	99.9%	A0324990	**---**	**---**
17β-estradiol (reference standard)	50-28-2	powder	Sigma	>98%	071M0087V	---	---
17α-methyltestosterone (positive control)	58-18-4	powder	Fluka	99.5%	SZBC107XV	---	---
Corticosterone (negative control)	50-22-6	powder	Sigma	>98.5%	BCBK7907V	---	---
Norethynodrel	68-23-5	powder	Sigma	n.s.	88F0192	SPC-1	AEP-1
Bisphenol A	80-05-7	powder	Sigma	>99%	MKBH2096V	SPC-2	AEP-2
Ketoconazole	65277-42-1	powder	Wako	98%	LRAA1641	SPC-3	AEP-3
meso-Hexestrol	84-16-2	powder	Sigma	>98%	071M1242V	SPC-4	AEP-4
Coumestrol	479-13-0	powder	Sigma	>95%	BCBH0742V	SPC-5	AEP-5
4-Cumylphenol	599-64-4	powder	Sigma	99%	14820CHV	SPC-6	AEP-6
Butylbenzyl phtalate	85-68-7	liquid	Wako	>95%	SZBB196XV	SPC-7	AEP-7
Genistein	446-72-0	powder	Wako	98%	WEE4932	SPC-8	AEP-8
p,p'-methoxychlor	72-43-5	powder	Sulpeco	99%	SZBC340XV	SPC-9	AEP-9
Diethylstilbestrol	56-53-1	powder	Sigma	>99%	BCBH3774V	SPC-10	AEP-10
Spironolactone	52-01-7	powder	Sigma	97-103%	SLBC1964V	SPC-11	AEP-11
Reserpine	50-55-5	powder	Sigma	>99%	BCBJ4421V	SPC-12	AEP-12
Linuron	330-55-2	powder	Sigma	99.7%	szb038xv	SPC-13	AEP-13
Atrazine	1912-24-9	powder	Sigma	98.8%	szb8175xv	SPC-14	AEP-14
Kaempferol	520-18-3	powder	Sigma	>90%	BCBJ0059V	SPC-15	AEP-15
Corticosterone	50-22-6	powder	Sigma	>98.5%	BCBK7907V	SPC-16	AEP-16
19-Nortestosterone	434-22-0	powder	Sigma	>99%	BCBJ1311V	SPC-17	AEP-17
17a-Estradiol	57-91-0	powder	Wako	>97%	KWJ3303	SPC-18	AEP-18
17a-Ethinyl estradiol	57-63-6	powder	Sigma	>99%	071M1492V	SPC-19	AEP-19
4-tert-Octylphenol	140-66-9	powder	Sigma	97%	MKBB8667V	SPC-20	AEP-20
Etyl paraben	120-47-8	powder	Sigma	99%	BCBH4047V	SPC-21	AEP-21
Kepone	143-50-0	powder	Sulpeco	99.9%	LC00470V	SPC-22	AEP-22

n.s. not specified

Table 6 Selected test chemicals for validation antagonistic ERα CALUX

Test chemical	CAS no.	Nature	Supplier	Purity	Lot.no.	coding pre-screen	coding comprehensive
Dimethyl sulfoxide (DMSO; solvent)	67-68-5	Fluid	Acros Organics	0.999	A0324990	**---**	**---**
Tamoxifen (reference standard)	10540-29-1	powder	Sigma	0.99	115K1068	---	---
4-hydroxytamoxifen (positive control)	68047-06-3	powder	Sigma	>98%	101M4015V	---	---
Resveratrol (negative control)	501-36-0	powder	Sigma	>99%	SLBC6832V	---	---
Apigenin	520-36-5	powder	Sigma	95%		SPC-51	AEP-51
17a-Ethinyl-estradiol	57-63-6	powder	Sigma	>98%	071M1492V	SPC-52	AEP-52
Genistein	446-72-0	powder	Wako	98%	WEE4932	SPC-53	AEP-53
Flutamide	13311-84-7	powder	Sigma	98%	125K1538	SPC-54	AEP-54
Coumestrol	479-13-0	powder	Sigma	>95%	BCBH0742V	SPC-55	AEP-55
Raloxifen HCl	82640-04-8	powder	Sigma	>99%	031M1303V	SPC-56	AEP-56
Resveratrol	501-36-0	powder	Sigma	>99%	SLBC6832V	SPC-57	AEP-57
Tamoxifen	10540-29-1	powder	Sigma	99%	115K1068	SPC-58	AEP-58
4OH-tamoxifen	68047-06-3	powder	Sigma	>98%	101M4015V	SPC-59	AEP-59
Chrysin	480-40-0	powder	Sigma	>98%	BCBF5239V	SPC-60	AEP-60
Kaempferol	520-18-3	powder	Sigma	>90%	BCBJ0059V	SPC-61	AEP-61

5 Validation process

5.1 Phase 1: Intra-laboratory validation

20. The intra-laboratory validation of the (anti-) the ERα CALUX bioassay started with a pre-screen run followed by 2 comprehensive runs. During the intra-laboratory validation study, the recommended test chemicals were not anonymised. This study was carried out by the lead laboratory.

- *Pre-screen run*

The objective of the pre-screen run is to:

- determine the solubility of test chemicals
- determine cytotoxicity at concentration of test chemical tested
- classify test chemicals for agonism or antagonism (qualitative; positive or negative response)
- obtain a refined concentration-range of test chemicals for comprehensive testing

21. For the agonistic pre-screen runs, 22 recommended test chemicals, a positive control chemical (17α-methyltestosterone), a negative control chemical (corticosterone) and a reference standard (17β-estradiol) were tested. For the antagonistic pre-screen runs, 11 recommended chemicals, a positive control chemical (4-hydroxytamoxifen), a negative control chemical (resveratrol) and a reference standard (tamoxifen) were tested.

22. Test chemicals were initially dissolved in DMSO at a concentration of 0.1 M as described in the standard operating procedures and diluted further if required. Once a clear solution was obtained without turbidity and microscopically visible crystal formation (either in the solvent itself or in the 100-times dilution in culture medium), this concentration was chosen as the maximum stock concentration for conducting the pre-screen run. The maximum stock concentration was used to prepare serial dilutions with dilution steps of 10.

23. Prior to lysing exposed ERα CALUX cells, the viability of the cells was evaluated visually using a phase-contrast reverse microscope. In addition, medium from the incubation was collected and used for a cytotoxicity test (LDH-leakage). Only concentrations of test chemicals not showing signs of cytotoxicity were used for further evaluation of results and comprehensive testing.

24. Following the measurement of luminescence, analysis data are evaluated for compliance of the acceptance criteria. Only data that fulfil these acceptance criteria were used for the selection of a refined series of concentrations for each test chemicals to be used for comprehensive testing. The procedure for the selection of a refined concentration range is given in the standard operating procedures.

25. Results from the pre-screen run were used for the qualitative assessment of the test chemicals (positive or negative).

- *Comprehensive runs*

The objective of the comprehensive runs is to:

- classify test chemicals for agonism or antagonism (qualitative; positive or negative response)

- classify test chemicals for agonism or antagonism (quantitative; agonism: EC_{50}, $EC_{10,}$ PC_{50}, PC_{10}; antagonism: IC_{50}, $IC_{20,}$ PC_{50}, PC_{80})
- assess the performance of the “me-too” test (determination of the estrogenic and anti-estrogenic potency of test chemicals)

26. For the agonistic comprehensive runs, 22 recommended chemicals, a positive control chemical (17α-methyltestosterone), a negative control chemical (corticosterone) and a reference standard (17β-estradiol) were tested. For the antagonistic comprehensive runs, 11 recommended chemicals, a positive control chemical (4-hydroxytamoxifen), a negative control chemical (resveratrol) and a reference standard (tamoxifen) were tested.

27. During a pre-screen run, a refined concentration range for each of the test chemicals was determined. New stock solutions for each test chemical were used to prepare serial dilution in DMSO (dilution: 1, 3, 10, 30, 100, 300, 1000 and 3000). Following exposure and analysis of luminescence, data from each experiment was evaluated for compliance to the acceptance criteria. Microtiter plates or individual test chemicals that were rejected because they did not meet the acceptance criteria, were re-analysed. Only acceptable data was evaluated. Qualitative (positive/negative) and quantitative (e.g. TC_{max}, EC_{50}, EC_{10}, PC_{50}, PC_{10}, TC_{min}, IC50, IC_{20} PC_{50}, $PC_{80,}$) assessment of (anti-) estrogenic activity was based on the guidelines and criteria given in the standard operating procedures.

28. All test chemicals were analysed twice in 2 independent comprehensive runs. In case results of duplicate comprehensive runs of a test chemical did not give reproducible results, a third comprehensive run of the test chemical had to be performed.

5.2 Phase 2: Inter-laboratory validation

29. Before the start of the inter-laboratory validation phase, participating laboratories received the standard operating procedures and all relevant calculation documents. In addition, all participants received detailed instructions for performing the pre-screen and comprehensive phase of the validation study.

- *Prescreen run*

The objectives of the pre-screen runs are similar to the objectives of the intra-laboratory pre-screen runs:

- determine the solubility of test chemicals
- determine cytotoxicity at concentration of test chemical tested
- classify test chemicals for agonism or antagonism (qualitative; positive or negative response)
- obtain a refined concentration-range of test chemicals for comprehensive testing

30. All recommended test chemicals for the pre-screen runs were distributed by the organizer of the validation study as pure chemicals. In addition, the participants received the reference standards and positive and negative controls as pure chemicals and a bottle of DMSO to be used to dissolve all chemicals. Prior to shipment, all test chemicals were labelled blind by the supporter of the validation study (National Institute for Public Health and the Environment (RIVM)).

31. The participants received precisely weighed amounts of test chemicals. The organiser informed the participants about the exact volume of DMSO to be used for preparing a 0.1 M

stock solution of the test chemicals. The participants were instructed to determine the solubility as indicated in the standard operating procedures. The highest concentration prepared not to show turbidity or microscopically visible crystal formation (either in the solvent itself or in the 100-times dilution in culture medium), had to be used as maximum stock concentration from which a dilution serie with dilution steps of 10 had to be prepared for determination of cytotoxicity and range-finding.

32. Following exposure and analysis of luminescence, only data that complied with the acceptance criteria as indicated in the standard operating procedures, was used for the selection of a refined series of concentrations for each test chemicals to be used for comprehensive testing. In case no dose-response was observed because the lowest concentration tested still showed (anti-) estrogenic effects, the pre-screen run had to be repeated using higher serial dilutions of test chemical. In case no full sigmoid dose-response curve was observed or in case the test chemical did not show any (anti-) estrogenicity, the highest concentration tested had to be used for assessment of (anti-) estrogenicity during the comprehensive runs of the inter-laboratory validation study.

33. Results from the pre-screen run were used for the qualitative assessment of the test chemicals (positive or negative).

34. After performing the pre-screen runs and before starting the comprehensive runs, the participants transferred all calculation documents, solubility- and cytotoxicity- data to the organizer of the validation study for data evaluation.

- *Comprehensive runs*

The objectives of the comprehensive runs are similar to the objectives of the intra-laboratory comprehensive runs:

- classify test chemicals for agonism or antagonism (qualitative; positive or negative response)
- classify test chemicals for agonism or antagonism (quantitative; agonism: EC_{50}, $EC_{10,}$ PC_{50}, PC_{10}; antagonism: IC_{50}, $IC_{20,}$ PC_{50}, PC_{80})
- assess the performance of the “me-too” test (determination of the estrogenic and anti-estrogenic potency of test chemicals)

35. Three glass vials per test chemical and per participating laboratory were prepared independently by the organizer, containing an exact amount of test chemical in DMSO. Hence, all participants received an exact same stock concentration for each of the test chemicals in triplicate. Since the assessment of (anti-) estrogenicity had to be determined twice independently, 3 vials per test chemical were sent. Two of them could be used for duplicate assessment of (anti-) estrogenicity, the 3rd vial could be used in case either of the analyses had to be repeated. In addition to test chemicals, participants also received 3 vials containing identical stock concentrations of reference standard, positive- and negative-control (prepared independently). Prior to shipment, all test chemicals were labelled blind by the supporter of the validation study (National Institute for Public Health and the Environment (RIVM)).

36. Refined concentration ranges for each of the test chemicals as determined during the pre-screen run, were prepared in DMSO (dilution: 1, 3, 10, 30, 100, 300, 1000 and 3000). Following exposure and analysis of luminescence, data from each run was evaluated for compliance to the acceptance criteria. Microtiter plates or individual test chemicals that were rejected because they did not meet the acceptance criteria, were re-analysed. Only acceptable data were evaluated. Qualitative (positive/negative) and quantitative (e.g. RPC_{max}, EC50, EC_{10},

PC_{50}, PC_{10}, RPC_{min}, IC_{50}, IC_{20} PC_{50}, PC_{80}; see figure 1) assessment of (anti-) estrogenic activity was based on the guidelines and criteria given in the standard operating procedures.

37. All test chemicals were analysed twice in independent comprehensive runs. In case results of duplicate analysis of a test chemical did not give reproducible results, a third comprehensive run of the test chemical had to be performed.

38. Following the comprehensive runs, all calculation documents were transferred to the organizer of the validation study for further data evaluation and reporting.

5.3 Data evaluation

39. According to the standard operating procedures, results submitted by the participants should comply with the acceptance criteria. This was re-evaluated by the organizer of the validation study upon receipt of the calculation documents containing the raw data. Further data handling and quantitative/qualitative data evaluation of the blinded test chemicals, was performed by the organiser and reviewed by the quality assurance manager of the validation study. Following quantitative/qualitative evaluation of data, the coding of the test chemicals was revealed for determination of accuracy and reliability performance values. Final review of data was performed by the review committee of the validation study.

40. Only data that fulfilled the acceptance criteria was used for further data evaluation. The average of each triplicate analysis was calculated after which the relevant average solvent blank was subtracted. Next, all results were expressed as percentage of response, relative to the analysis results of the reference standard in respective runs (maximum induction of reference standard set at 100% response). The statistical software package Graphpad Prism 5.0 was used to fit raw data (non-linear regression, variable slope, 4 parameters, robust fit). In formula 1, the concentration-response equation (Hill equation) used for curve-fitting, is given. Using the same software package, quantitative assessment of estrogenic activity (TC_{max}, EC_{50}, EC_{10}, PC_{50}, PC_{10}) and anti-estrogenic activity (TC_{min}, IC_{50}, IC_{20} PC_{50}, PC_{80}) was performed (see figure 4).

41. For evaluation of intra-laboratory and inter-laboratory reproducibility, all data was log-transformed. The reproducibility variation coefficient was calculated according to formula (2). For each laboratory, the intra-laboratory reproducibility variation coefficient was determined based on the duplicate comprehensive analysis of all test chemicals. In addition, the coefficient of variation (%CV) of the log[EC_{50}] or log[IC_{50}] concentrations calculated from all reference dose-response series during both the pre-screen run and comprehensive runs, was determined as a measure of intra-laboratory reproducibility. Intra-laboratory comparison was further evaluated by determining the correlation between the duplicate analysis results of all test chemicals. Overall inter-laboratory reproducibility was evaluated following calculation of the inter-laboratory reproducibility variation coefficient per test chemical (13).

42. Qualitative assessment of test chemicals was performed according to the guidelines for data interpretation as stated in the standard operating procedures (agonism: 4.5.9; antagonism: 4.6.11). Following the qualitative assessment and decoding of test chemicals, the concordance of classification and the accuracy and reliability performance values were determined. In table 7, a diagram and formulas are given that were used to calculate the agonistic and antagonistic accuracy and reliability performance values (positive predictive value, negative predictive value, false positive rate, false negative rate, sensitivity, specificity and accuracy).

(1) $$y = Bottom + \frac{(Top - Bottom)}{(1 + 10^{((LogEC_{50} - x) * HillSlope)})}$$

X = Log of dose or concentration
Y = Response (relative induction (%))
Top = Maximum induction (%)
Bottom = Minimum induction (%)
LogEC50 = Log of concentration at which 50% of maximum response is observed
HillSlope = Slope factor of Hill slope

(2) $$VC_R = \sqrt{\frac{\sum_{i=1}^{n} \left(\frac{X_{i1} - Y_i}{0.5(X_{i1} + Y_i)} \right)^2}{2n}}$$

VC_R = reproducibility variation coefficient variation coefficient
X_{i1} = measuring result i^{th} determination first observation
Y_i = measuring result i^{th} determination second (duplo) observation
n = number of determinations

Table 7 Diagram and formulas to calculate agonistic and antagonistic accuracy and reliability performance values

		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

a = positive in both new assay and by reference test classification
b = positive in new assay and negative by reference test classification
c = negative in new assay and positive by reference test classification
d = negative in both new assay and by reference test classification

Accuracy = ([a+d]/[a+b+c+d])
Sensitivity = (a/[a+c])
Specificity = (d/[b+d])
False positive rate = (b/[b+d])
False negative rate = (c/[a+c])
Positive predictivity = (a/[a+b])
Negative predictivity = (d/[c+d])

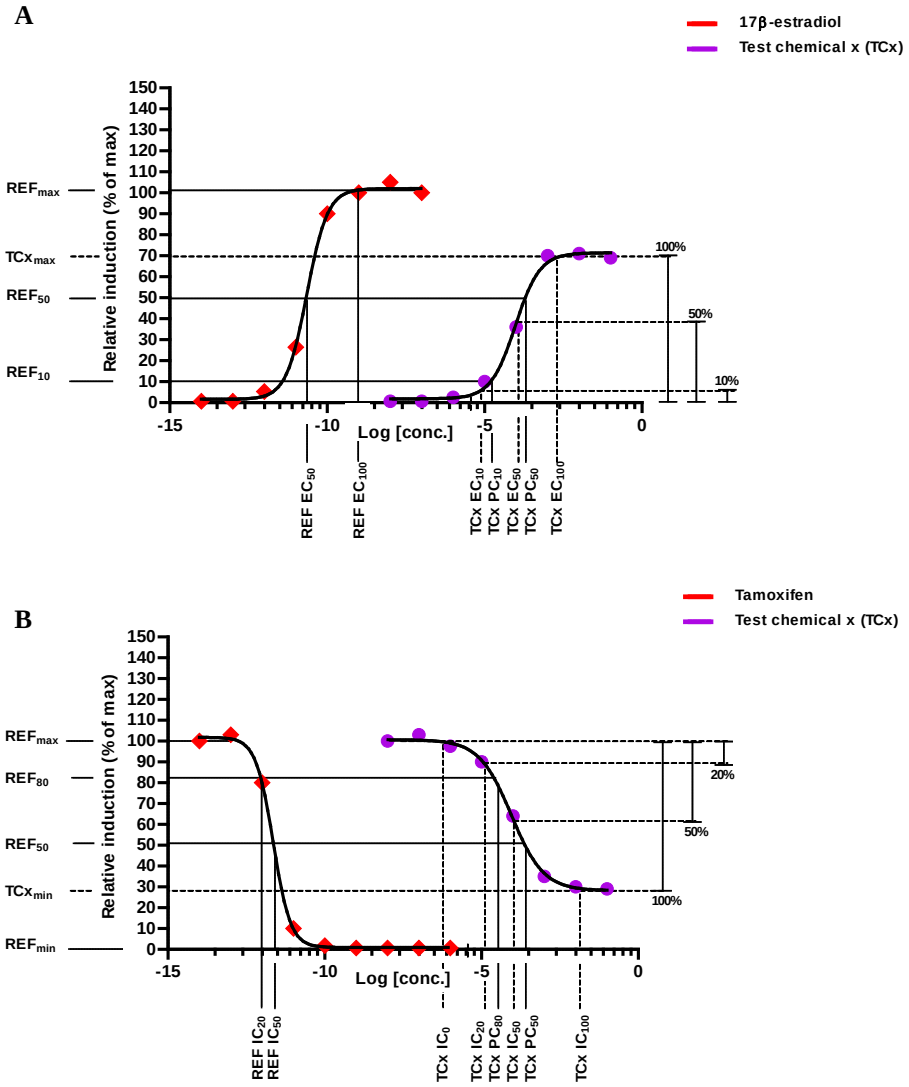


Figure 4 Overview of parameters determined for agonistic (A) and antagonistic (B) evaluation of reference standards (REF) and test chemicals (TC_x).

6 Results

6.1 Solubility

43. For each of the participants, the reported solubility of test chemicals in medium (in the well) is given in annex B. Each of the laboratories reported slightly different solubility concentrations. However, the variation in reported solubility was never more than 1 order of magnitude.

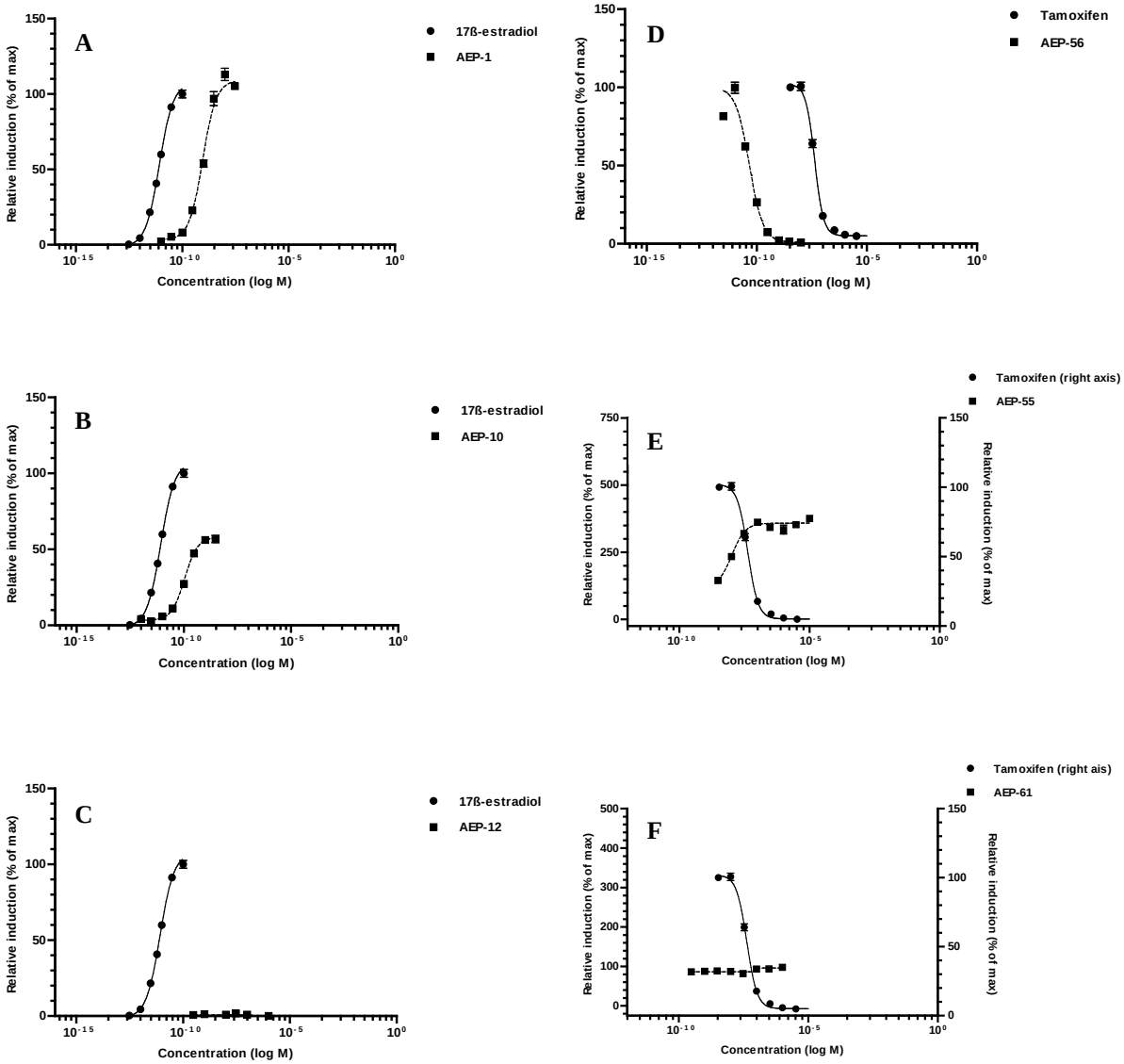


Figure 5 Examples of dose-response curves for test chemicals tested for agonism (A, B and C) and antagonism (D, E and F). In figure A, the test chemicals AEP-1 is an agonist, slightly less potent than the reference standard 17β-estradiol. Test chemical AEP-10 also shows an agonistic effect but the maximum induction is less as compare to 17β-estradiol (B). Test chemical AEP-12 does not show any agonistic effect (C). The antagonistic test chemical AEP-56 shows higher affinity towards the ERα-receptor as compared to the reference standard tamoxifen (D). Test chemical AEP-55 does not show an antagonistic effect but an agonistic effect instead (E). In figure F, the test chemical does not show any antagonistic or agonistic effect.

6.2 Cytotoxicity

44. Cytotoxicity was determined using the LDH leakage test. However, during the intra-laboratory phase of the validation study the lead laboratory observed clear cytotoxicity as observed after visual inspection under a phase-contrast reverse microscope at concentration where no LDH leakage was observed. Therefore, it was decided that the standard operating procedures had to be revised. In addition to the LDH-leakage test as measure for cytotoxicity, visual inspection prior to lysis of cells using a phase-contrast reverse microscope was introduced as additional measure for cytotoxicity. For evaluation of analysis data, only concentrations that did not show LDH-leakage and no visual cytotoxicity, were used.

6.3 Quantitative assessment of estrogenic and anti-estrogenic activity

45. Following both the intra- and inter-laboratory validation phase, the organiser received the calculation documents containing the raw data of the pre-screen run and comprehensive runs from all participating laboratories. Initially, data was evaluated for meeting the acceptance criteria for agonistic ERα CALUX bioassays (table 3) or the acceptance criteria for antagonistic ERα CALUX bioassays (table 4.). All data received complied to the stated performance criteria.

46. All results were transformed into responses relative to the maximum induction of the reference standard used in each series of analysis. In figure 5, dose-response curves for some of the test chemicals are given. The relative induction of test chemicals was used for the quantitative assessment of estrogenic activity (TC_{max}, EC_{50}, EC_{10}, PC_{50}, PC_{10}) and anti-estrogenic activity (TC_{min}, IC_{50}, IC_{20}, PC_{50}, PC_{80}) using the statistical software package Graphpad Prism 5.0 (non-linear regression, variable slope, 4 parameters, robust fit). All analysis data were log-transformed for further evaluation. In annex C, the participant's agonistic and antagonistic analysis results are given for the pre-screen run and both comprehensive runs.

6.4 Intra-laboratory study

6.4.1 Intra-laboratory variability and reproducibility

47. During the intra-laboratory study, the lead laboratory analysed all test chemicals according to the SOP. Three independent test runs (1 pre-screen run and 2 comprehensive runs) were performed. For the assessment of within-laboratory concordance of classification (positive/negative), selected test chemicals as indicated in the performance standards for stably transfected transactivation in vitro assays to detect estrogen agonists and antagonists (3, 4), were classified as positive or negative agonists or antagonists. In Annex E, the classification of the selected test chemicals are given for all runs performed. Based on these results, the concordance of classifications obtained from the three independent consecutive test runs, was determined. The results are presented in table 8.

48. The intra-laboratory coefficient of variance (%CV) was determined based on the average log(EC50) and log(PC10) (agonism), and log(IC50) and log(PC80) (antagonism) derived from both comprehensive runs. In addition, the intra-laboratory reproducibility variation coefficient ($\%VC_R$) was determined based on the log(EC50) and log(PC10) (agonism), and log(IC50) and log(PC80) (antagonism) derived from both comprehensive runs. In annex E, the %CV and $\%VC_R$ data for each test chemical are given. The average intra-laboratory %CV and $\%VC_R$ are given in table 9 and 10 respectively.

Table 8 The agonistic and antagonistic within-laboratory concordance of classifications (positive/negative) obtained in three independent consecutive test runs during the intra-laboratory phase of the validation study.

Concordance of classification (agonism)	(%)
Lead laboratory	100

Concordance of classification (antagonism)	(%)
Lead laboratory	100

Table 9 Intra-laboratory coefficient of variance (%CV) based on log(EC_{50}) and log(PC_{10}) (agonism) and log(IC_{50}) and log(PC_{80}) (antagonism) of all test chemicals during both comprehensive runs of the intra-laboratory phase.

	Agonism		Antagonism	
	%CV (log(EC_{50}))	**%CV (log(PC_{10}))**	**%CV (log(IC_{50}))**	**%CV (log(PC_{80}))**
Lead laboratory	2.4	2.2	1.1	2.4

Table 10 Intra-laboratory reproducibility variation coefficient (%VC_R) based on log(EC_{50}) and log(PC_{10}) (agonism) and log(IC_{50}) and log(PC_{80}) (antagonism) of all test chemicals during both comprehensive runs of the intra-laboratory phase.

	Agonism		Antagonism	
	%VC_R (log(EC_{50}))	**%VC_R (log(PC_{10}))**	**%VC_R (log(IC_{50}))**	**%VC_R (log(PC_{80}))**
Lead laboratory	2.8	2.8	1.5	3.6

6.5 Inter-laboratory study

6.5.1 Intra-laboratory comparison

49. All 3 participating laboratories performed three independent test runs (1 pre-screen run and 2 comprehensive runs). For the assessment of within-laboratory concordance of classifications, selected test chemicals as indicated in the performance standards for stably transfected transactivation in vitro assays to detect estrogen agonists and antagonists [3, 4], were classified as positive or negative agonists or antagonists. In Annex F, the classification of the selected test chemicals are given for each of the participants. Based on these results, the intra-laboratory concordance of classifications (positive/negative) obtained from the three independent consecutive test runs performed by each of the laboratories, was determined. The results are presented in table 11.

50. For each of the participants, the intra-laboratory coefficient of variance (%CV) was calculated based on the log(EC_{50}) and log(IC_{50}) values for the reference standards 17β-estradiol and tamoxifen derived from the pre-screen and comprehensive runs. The agonist reference standard was analysed 14 times in total by each participant whereas the antagonist reference standard was analysed 8 times (participant A) or 6 times (participants B and C). In annex D, the log(EC_{50}) and log(IC_{50}) values for the reference standards are given. From these data, the %CV for the agonistic and antagonistic runs was calculated. The results are given in table 12. In figure 6 and 7, the average percentage of induction of the 17β-estradiol and tamoxifen reference standards obtained from all runs (pre-screen, comprehensive 1 and comprehensive 2) for each of the participants are given. In the tables below, the 95% confidence intervals for the top log[EC_{50}] / log[IC_{50}] and Hill-slope are given. Clearly, a strong intra-laboratory consistency (but also inter-laboratory consistency) is observed.

51. Furthermore, Intra-laboratory comparison was also performed by evaluating the %CV and %VC_R per test chemical. The %CV was determined based on the average log(EC50) and

log(PC10) (agonism), and log(IC50) and log(PC80) (antagonism) derived from both comprehensive runs. The $\%VC_R$ was determined based on the log(EC50) and log(PC10) (agonism), and log(IC50) and log(PC80) (antagonism) derived from both comprehensive runs. In annex F, the %CV and $\%VC_R$ data for each test chemical and each participant are given. The average intra-laboratory %CV and $\%VC_R$ for each participant, is given in table 13 and 14 respectively. In figure 8, the intra-laboratory analysis results are presented graphically (comprehensive 1 vs comprehensive 2).

Table 11 The agonistic and antagonistic within-laboratory concordance of classifications (positive/negative) obtained in three independent consecutive test runs during the inter-laboratory phase of the validation study.

Concordance of classification (agonism)	(%)
Participant A	100
Participant B	100
Participant C	100

Concordance of classification (antagonism)	(%)
Participant A	100
Participant B	100
Participant C	100

Table 12 Intra-laboratory coefficient of variance (%CV) based on log(EC50) and log(IC50) of the agonistic reference standard 17β-estradiol and antagonistic reference standard tamoxifen.

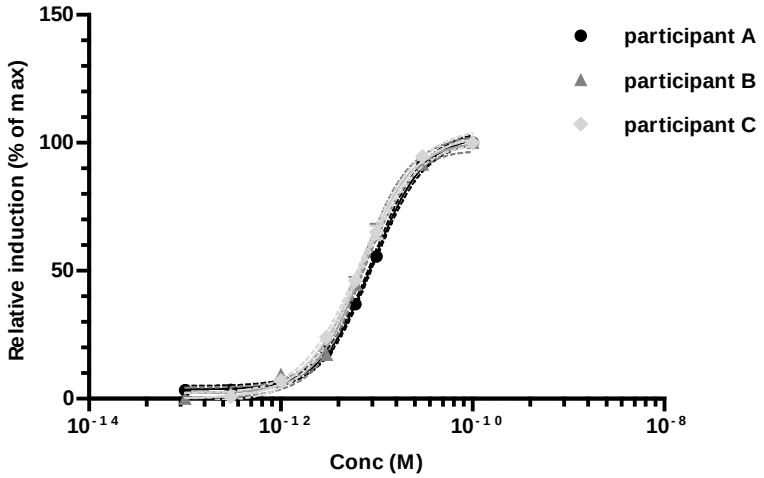
	Agonism %CV	**Antagonism %CV**
Participant A	1.1	2.6
Participant B	1.5	1.1
Participant C	1.3	2.0

Table 13 Intra-laboratory coefficient of variance (%CV) based on log(EC_{50}) and log(PC_{10}) (agonism) and log(IC_{50}) and log(PC_{80}) (antagonism) of all test chemicals during both comprehensive runs.

	Agonism		**Antagonism**	
	%CV (log(EC_{50}))	**%CV (log(PC_{10}))**	**%CV (log(IC_{50}))**	**%CV (log(PC_{80}))**
Participant A	1.2	1.4	0.5	1.3
Participant B	1.9	1.5	1.2	0.8
Participant C	3.1	2.7	1.6	2.5

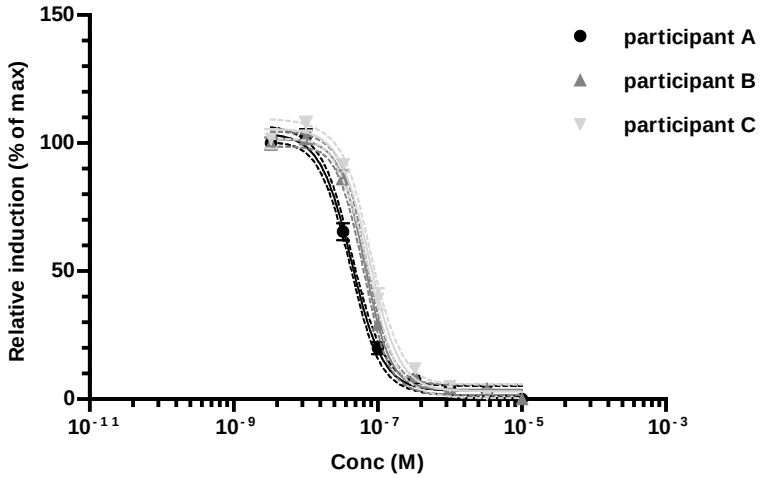
Table 14 Intra-laboratory reproducibility variation coefficient ($\%VC_R$) based on log(EC_{50}) and log(PC_{10}) (agonism) and log(IC_{50}) and log(PC_{80}) (antagonism) of all test chemicals during both comprehensive runs.

	Agonism		**Antagonism**	
	$\%VC_R$ (log(EC_{50}))	**$\%VC_R$ (log(PC_{10}))**	**$\%VC_R$ (log(IC_{50}))**	**$\%VC_R$ (log(PC_{80}))**
Participant A	1.6	1.7	0.5	1.4
Participant B	2.5	2.0	1.5	0.9
Participant C	4.5	4.0	1.8	2.9



	participant A	participant B	participant C
Top	100.5 to 105.6	97.0 to 103.5	100.0 to 106.4
Log[EC_{50}]	-11.1 to -11.0	-11.2 to -11.1	-11.2 to -11.1
Hill Slope	1.5 to 1.8	1.5 to 2.0	1.3 to 1.6
R^2	0.96	0.93	0.95

Figure 6 Average 17β-estradiol dose-response curves from pre-screen, comprehensive 1 and comprehensive 2 runs. Solid lines indicate average fit. Dotted lines indicate 95% confidence. In the table below, the 95% confidence intervals of fitted 17β-estradiol dose-response data are given.



	participant A	participant B	participant C
Bottom	1.4 to 5.2	1.6 to 5.7	0.4 to 5.8
Log[IC_{50}]	-7.4 to -7.3	-7.2 to -7.1	-7.2 to -7.1
Hill Slope	-2.3 to -1.7	-2.8 to -2.1	-2.5 to -1.6
R^2	0.96	0.96	0.95

Figure 7 Average tamoxifen dose-response curves from pre-screen, comprehensive 1 and comprehensive 2 runs. Solid lines indicate average fit. Dotted lines indicate 95% confidence. In the table below, the 95% confidence intervals of fitted tamoxifen dose-response data are given.

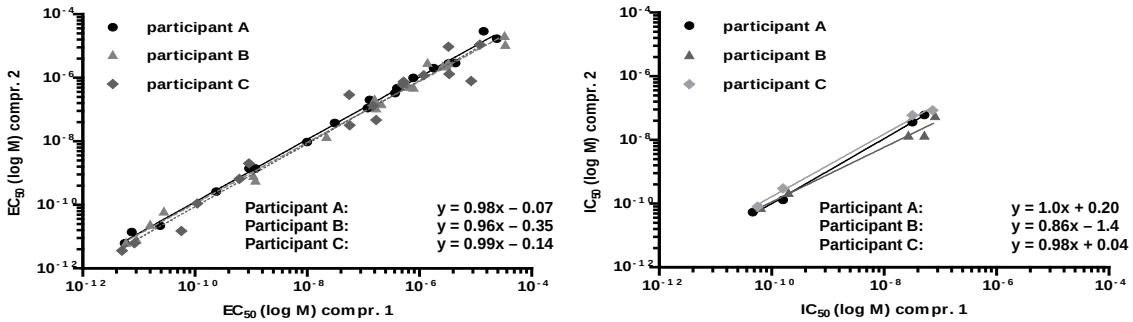


Figure 8 Correlation between calculated EC_{50} values from the comprehensive run 1 and the comprehensive run 2.

6.5.2 Inter-laboratory comparison

52. To assess the between-laboratory concordance of classification, all test chemicals were classified as positive or negative agonists or antagonists (Agonism:, 8 selected test chemicals tested in 3 independent consecutive runs (1 pre-screen run, 2 comprehensive runs) and remaining 14 test chemicals tested in 2 independent consecutive runs (2 comprehensive runs) by each of the participants; Antagonism, all 10 selected test chemicals tested in 3 independent consecutive runs (1 pre-screen run, 2 comprehensive runs) by each of the participants). In Annex G, the classification of the test chemicals is given for all participants. For agonist testing, 1 participant classified 1 test chemical (linuron) different from the other 2 participants. The agonist inter-laboratory concordance of classification was therefore calculated to be 95% (21/22*100%). For antagonist testing, all participating laboratories reported similar classifications of the test chemical. Therefore, the antagonist inter-laboratory concordance of classification was calculated to be 100% (10/10*100%).

53. Inter-laboratory reproducibility was also assessed by evaluating the %CV and $\%VC_R$ per test chemical. The %CV was determined based on the average log(EC50) and log(PC10) (agonism), and log(IC50) and log(PC80) (antagonism) derived from both comprehensive runs. The $\%VC_R$ was determined based on the log(EC50) and log(PC10) (agonism), and log(IC50) and log(PC80) (antagonism) derived from both comprehensive runs. In annex F, the %CV and $\%VC_R$ data for each test chemical are given. The average inter-laboratory %CV and $\%VC_R$ for the (anti-)ERα CALUX bioassay, are given in table 15 and 16 respectively.

54. The comparability between inter-laboratory analysis results can also be visualised by plotting the analysis results from the participating laboratories versus the results reported for the Bg1Luc and STTA assays (see annex G) [1]. In figure 9, the reported Bg1Luc log(EC_{50}) and STTA log(PC_{10}) values for the recommended test chemicals are plotted against the average ERα CALUX log(EC_{50}) and log(PC_{10}) values respectively.

Table 15 Average inter-laboratory coefficient of variance (%CV) for the ERα CALUX bioassay (agonism) and the anti-ERα CALUX bioassay (antagonism).

Agonism	
	%CV
Log[EC_{50}]	3.4
Log[PC_{10}]	3.0

Antagonism	
	%CV
Log[IC_{50}]	1.5
Log[PC_{80}]	2.8

Table 16 Average inter-laboratory reproducibility variation coefficient (%VC_R) for the ERα CALUX bioassay (agonism) and the anti-ERα CALUX bioassay (antagonism).

Agonism	
	%VC_R
Log[EC_{50}]	2.5
Log[PC_{10}]	2.3

Antagonism	
	%VC_R
Log[IC_{50}]	1.3
Log[PC_{80}]	1.6

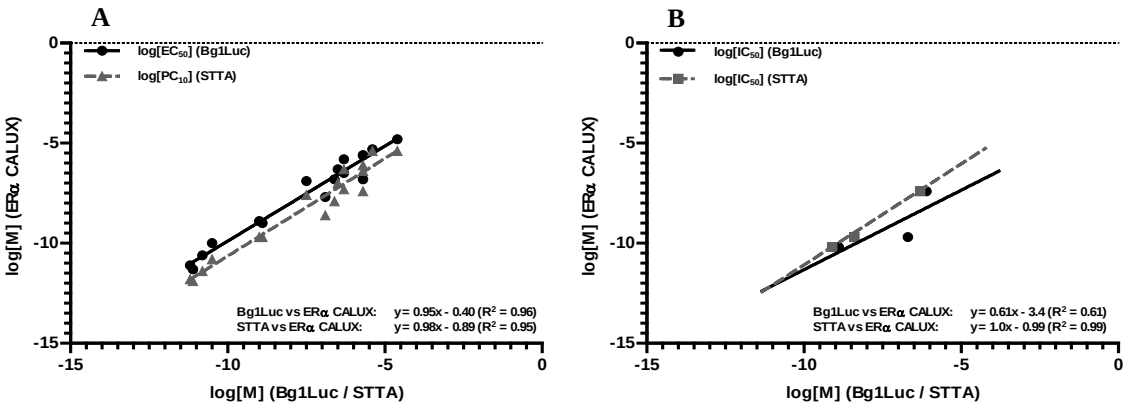


Figure 9 Correlation between reported Bg1Luc log(EC_{50}) / STTA log(PC_{10}) values and calculated ERα CALUX log(EC_{50}) and log(PC_{10}) values (A) and reported Bg1Luc / STTA log(IC_{50}) values and calculated ERα CALUX log(IC_{50}) (B).

Table 17 Qualitaitive classification (positive/negative) of recommended test chemicals in agonistic Bg1Luc, STTA and ERα CALUX bioassays

Chemical	CAS no.	ICCVAM Consensus Classification	BG1Luc	STTA	Erα CALUX Participant A	Erα CALUX Participant B	Erα CALUX Participant C
17β-estradiol	50-28-2	Pos	Pos	Pos	Pos	Pos	Pos
Norethynodrel	68-23-5	Pos	Pos	Pos	Pos	Pos	Pos
Bisphenol A	80-05-7	Pos	Pos	Pos	Pos	Pos	Pos
Ketoconazole	65277-42-1	Neg	Neg	Neg	Neg	Neg	Neg
meso-Hexestrol	84-16-2	---	Pos	Pos	Pos	Pos	Pos
Coumestrol	479-13-0	Pos	Pos	Pos	Pos	Pos	Pos
4-Cumylphenol	599-64-4	Pos	Pos	Pos	Pos	Pos	Pos
Butylbenzyl phtalate	85-68-7	Pos	Pos	Pos	Pos	Pos	Pos
Genistein	446-72-0	Pos	Pos	Pos	Pos	Pos	Pos
p,p'-methoxychlor	72-43-5	Pos	Pos	Pos	Pos	Pos	Pos
Diethylstilbestrol	56-53-1	Pos	Pos	Pos	Pos	Pos	Pos
Spironolactone	52-01-7	Neg	Neg	Neg	Neg	Neg	Neg
Reserpine	50-55-5	Neg	Neg	Neg	Neg	Neg	Neg
Linuron	330-55-2	Neg	Neg	Neg	Mild pos	Mild pos	Neg
Atrazine	1912-24-9	Neg	Neg	Neg	Neg	Neg	Neg
Kaempferol	520-18-3	Pos	Pos	Pos	Pos	Pos	Pos
Corticosterone	50-22-6	Neg	Neg	Neg	Neg	Neg	Neg
19-Nortestosterone	434-22-0	---	Pos	Pos	Pos	Pos	Pos
17a-Estradiol	57-91-0	Pos	Pos	Pos	Pos	Pos	Pos
17a-Ethinyl estradiol	57-63-6	Pos	Pos	Pos	Pos	Pos	Pos
4-tert-Octylphenol	140-66-9	Pos	Pos	Pos	Pos	Pos	Pos
Etyl paraben	120-47-8	Pos	Pos	Pos	Pos	Pos	Pos
Kepone	143-50-0	Pos	Pos	Pos	Pos	Pos	Pos

Table 18 Qualitaitive classification (positive/negative) of recommended test chemicals in antagonistic Bg1Luc, STTA and ERα CALUX bioassays

Chemical	CAS no.	ICCVAM Consensus Classification	BG1Luc	STTA	Erα CALUX Participant A	Erα CALUX Participant B	Erα CALUX Participant C
Tamoxifen	10540-29-1	pos	pos	pos	pos	pos	pos
4OH-tamoxifen	68047-06-3	pos	pos	pos	pos	pos	pos
Raloxifen HCl	82640-04-8	pos	pos	pos	pos	pos	pos
17a-Ethinyl-estradiol	57-63-6	neg	neg	neg	neg	neg	neg
Apigenin	520-36-5	neg	neg	neg	neg	neg	neg
Chrysin	480-40-0	neg	neg	neg	neg	neg	neg
Coumestrol	479-13-0	neg	neg	neg	neg	neg	neg
Genistein	446-72-0	neg	neg	neg	neg	neg	neg
Kaempferol	520-18-3	neg	neg	neg	neg	neg	neg
Resveratrol	501-36-0	neg	neg	neg	neg	neg	neg
Flutamide	13311-84-7	neg	neg	neg	neg	neg	neg

Note. Tamoxifen, 4OH-tamoxifen and raloxifen are classified as antagonists of the estrogen receptor. However, it should be noted that these chemicals are SERMs (Selective Estrogen Receptor Modulators). Characteristic of SERMs is that they have different activity in different tissues, from pure agonists to full antagonists.

6.6 Qualitative assessment of estrogenic and anti-estrogenic activity

55. For each participant of the validation study, test chemicals were classified as positive or negative for estrogenic and anti-estrogenic activity in the ERα CALUX bioassay. In table 17, the qualitative classifications for the agonistic analyses are given whereas in table 18, the qualitative classifications for the antagonistic analyses are given. In the same tables, the classification of the OECD approved Bg1Luc and STTA assays as well as the consensus ICCVAM classification is given.

6.7 Accuracy and reliability performance values

56. The qualitative classification of the test chemicals was used to determine the performance values (table 19 (agonism); table 21 (antagonism)). In table 20, the agonistic accuracy and reliability performance values for each of the participating laboratories are given. The antagonistic accuracy and reliability performance values are given in table 22.

Table 19 Diagrams for determination the agonistic positive predictive value, negative predictive value, false positive rate, false negative rate, sensitivity, and specificity and accuracy.

Participant A		**ERα CALUX positive**	**negative**	**total**
ICCVAM classification	**positive**	17	0	17
	negative	1	5	6
	total	18	5	23

Participant B		**ERα CALUX positive**	**negative**	**total**
ICCVAM classification	**positive**	17	0	17
	negative	1	5	6
	total	18	5	23

Participant C		**ERα CALUX positive**	**negative**	**total**
ICCVAM classification	**positive**	17	0	17
	negative	0	6	6
	total	17	6	23

Table 20 Agonistic accuracy and reliability performance values

	Participant A	Participant B	Participant C
Overall accuracy (%)	96	96	100
Sensitivity (%)	100	100	100
Specificity (%)	83	83	100
False positive (%)	17	17	0
False negative (%)	0	0	0
Positive predictivity (%)	94	94	100
Negative predictivity (%)	100	100	100

Table 21 Diagrams for determination the antagonistic positive predictive value, negative predictive value, false positive rate, false negative rate, sensitivity, and specificity and accuracy**.**

Participant A		**ERα CALUX**		
		positive	**negative**	**total**
ICCVAM	**positive**	3	0	3
classification	**negative**	0	8	8
	total	3	8	11

Participant B		**ERα CALUX**		
		positive	**negative**	**total**
ICCVAM	**positive**	3	0	3
classification	**negative**	0	8	8
	total	3	8	11

Participant C		**ERα CALUX**		
		positive	**negative**	**total**
ICCVAM	**positive**	3	0	3
classification	**negative**	0	8	8
	total	3	8	11

Table 22 Antagonistic accuracy and reliability performance values

	Participant A	Participant B	Participant C
Overall accuracy (%)	100	100	100
Sensitivity (%)	100	100	100
Specificity (%)	100	100	100
False positive (%)	0	0	0
False negative (%)	0	0	0
Positive predictivity (%)	100	100	100
Negative predictivity (%)	100	100	100

7 Discussion

57. The ERα CALUX is a stably transfected *in vitro* transactivation assay to detect estrogen receptor agonists and antagonists. ERα CALUX cells express a functioning human estrogen receptor and contain a reporter gene (luciferase) under the control of an estrogen responsive element. An increase or decrease of estrogenic signalling, results in corresponding changes in the expression of luciferase activity in ERα CALUX cells, and is measured with a luminometer. Accordingly, ERα CALUX cells can be used to detect chemicals with (anti)-estrogenic properties. The ERα CALUX bioassay was validated according to OECD guidelines.

58. The validation study consisted of an intra-laboratory phase and an inter-laboratory phase. In both phases, at least 3 consecutive test runs (a pre-screen run and at least 2 comprehensive runs) were conducted. A standard operating procedure (SOP) was constructed that could be used for both phases of the validation study. Following the intra-laboratory phase, the SOP was slightly modified. From the intra-laboratory studies, it became clear that determination of cytotoxicity using the LDH-leakage test was not sufficient. In a number of cases, the LDH-leakage test did not elicit any cytotoxicity whereas a clear reduction of luminescence was found. This was confirmed by visual inspection of the cells prior to lysis. Therefore, in addition to LDH-leakage, visual inspection of cells was introduced in the SOP as measure for cytotoxicity. Alternative and more accurate methods to detect cytotoxicity are available, such as the MTT test and the CALUX cytotoxicity test [9]. However, they require an additional set of exposures since they do not determine cell viability in the same exposure experiment as the exposure experiment to measure luminescence. Nevertheless, the CALUX cytotoxicity test can be a good alternative for cytotoxicity testing in combination with ERα CALUX analyses since this test uses an identical cell: U2OS. It can be used in panels of CALUX assays that measure additional endpoints, e.g. ones involved in endocrine disruption or genotoxicity [10].

59. Evaluation of raw data received form the participants (calculation documents) showed that all data received met the assay's performance criteria. Especially the comparison between analysis results from plate 2-6 and the analysis results from plate 1 in a single analysis series, is of importance. Only the first plate contains a reference dose-response series. Since results obtained from the other plates are expressed as inductions relative to the maximum induction of the reference standard, this comparison is essential for proper quantitative assessment of test chemicals.

60. The within- and between-laboratory reproducibility was assessed by determining the concordance of classification. Each of the participating laboratories showed a within-laboratory concordance of classification of 100% for both agonist and antagonist testing. The agonist between-laboratory concordance of classification showed to be 95% whereas the antagonist between-laboratory concordance of classification was found to be 100%. Therefore, the within- and between-laboratory reproducibility is in accordance to performance standards for stably transfected transactivation in vitro assays to detect estrogen agonists and antagonists [3, 4].

61. During the intra-laboratory phase, the intra-laboratory variation and reproducibility was determined. For both the agonistic and antagonistic mode of the bioassay, the intra-laboratory coefficient of variance (%CV) based on the log(EC_{50}), log(PC_{10}), log(IC_{50}) and log(PC_{80}) was below 3%. The intra-laboratory reproducibility (%VC_R) for both the agonist and antagonistic analyses showed to be below 4% (based on log[EC_{50}] and log[PC_{10}] for agonistic runs and log[IC_{50}] and log[PC_{80}] for antagonistic runs. Based on these results, an inter-laboratory variation and reproducibility of less than 5% should be feasible.

62. The results obtained during the inter-laboratory phase can be used to evaluate the intra- and inter-lab variation and reproducibility. All laboratories showed high intra-laboratory

reproducibility for both the agonist and antagonistic analyses. The $\%VC_R$ of log[EC_{50}] and log[PC_{10}] (agonism) and log[IC_{50}] and log[PC_{80}] (antagonism) for positive samples were less than 4.5%. The %CV for these parameters showed to be even lower: less than 3.5%. This was confirmed by evaluating the intra-laboratory coefficient of variance (%CV) based on log(EC_{50}) and log(IC_{50}) of the agonistic reference standard 17β-estradiol and antagonistic reference standard tamoxifen: <2% and <3% respectively.

63. The inter-laboratory comparison of analysis results also showed high reproducibility for both agonistic and antagonistic measurements. Based on the log[EC_{50}] and log[PC_{10}] (agonism) and log[IC_{50}] and log[PC_{80}] (antagonism), both the %CV and $\%VC_R$ were less than 4%. In this evaluation, linuron was not included. This chemical was classified as positive in 2 laboratories and negative in 1 laboratory. Closer evaluation of the linuron analysis results from both laboratories that classified this test chemical as positive, showed that only the relative induction of the 2 highest concentrations tested, were above the 10% threshold level with maximum inductions of 17 and 25%. Although this chemical was therefore classified as positive, the response can be regarded as weak. A weak induction of luciferase activity was also observed in the OECD validated Bg1Luc cell line [11].

64. The reported Bg1Luc and STTA log(EC_{50}), log(PC_{10}) and log(IC_{50}) values and average ERα CALUX log(EC_{50}), log(PC_{10}) and log(IC_{50}) values are represented in figure 8. For the agonistic mode of the assays, a good correlation was observed between ERα CALUX and both OECD accepted assays. For the antagonistic mode of the assays, a good correlation between the ERα CALUX and the OECD accepted STTA assay was found. The correlation between the ERα CALUX and the OECD accepted Bg1Luc assay was less pronounced. Although only 3 data points (test chemicals) are used for evaluation, it is obvious that the Bg1Luc IC_{50} for hydroxy-tamoxifen is considerable higher as compared to the ERα CALUX and STTA IC_{50} for hydroxy-tamoxifen. In general, hydroxy-tamoxifen is considered to be a more potent ligand for the ERα receptor as compared to tamoxifen [12].

65. Evaluation of the qualitative assessment results of the participating laboratories showed good agonistic accuracy and reliability performance values and very good antagonistic accuracy and reliability performance values. For the agonistic measurements, the average overall accuracy was 95% whereas the average sensitivity, specificity, false positive rate, false negative rate, positive predictivity and negative predictivity were 100%, 89%, 11%, 0%, 96% and 100% respectively. For the antagonistic measurements, the average overall accuracy was 100% and the average sensitivity, specificity, false positive rate, false negative rate, positive predictivity and negative predictivity were 100%, 100%, 0%, 0%, 100% and 100% respectively. These results confirm and extend the excellent assay performance of the (anti-)ERα CALUX bioassay [6].

8 Conclusion

66. The present intra-laboratory and inter-laboratory validation study of the human ERα mediated stably transfected transactivation *in vitro* assay to detect estrogen receptor agonists and antagonists (ERα CALUX) showed the high reproducibility and accuracy of this bioassay. The successful validation qualifies the ERα CALUX bioassay for inclusion in the OECD Performance Based Test Guideline for Stably Transfected Transactivation *In Vitro* Assays to Detect Estrogen Receptor Agonists and Antagonists (TG455).

9 Acknowledgements

67. The present intra-laboratory and inter-laboratory validation study of the human ERα mediated stably transfected transactivation *in vitro* assay to detect estrogen receptor agonists and antagonists (ERα CALUX) was conducted with the cooperation, assistance and support of multiple people.

68. We thank the two additional laboratories for participated in the inter-laboratory validation studies: Jessica Richard (IWW, Germany) and Go Suzuki (NIES, Japan). We also deeply appreciate the assistance of Minne Heringa (RIVM) for anonymizing test chemicals. Finally, we would like to thank Betty Hakkert (RIVM), Zhichao Dang (RIVM) and Toine Bovee (RIKILT Wageningen, UR) for their advice and review of the validation study

10 Literature

1. OECD (2015), *Test No. 455: Performance-Based Test Guideline for Stably Transfected Transactivation In Vitro Assays to Detect Estrogen Receptor Agonists and Antagonists*, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris.

2. OECD (2005), Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment, Series on Testing and Assessment No.34, OECD, Paris. Available at: http://www.oecd.org/officialdocuments/displaydocumentpdf?cote=ENV/JM/MONO(2005)14&doclanguage=en

3. OECD (2012) Performance standards for stably transfected transactivation in vitro assays to detect estrogen agonists for TG455. OECD Environment, Health and Safety Publications, Series on Testing and Assessment No.173. ENV/JM/MONO(2012)18. Available at: [http:// http://www.oecd.org/chemicalsafety/testing].

4. OECD (2012). Performance standards for the BG1Luc estrogen receptor (ER) transactivation in vitro assays to detect ER antagonists for TG457. OECD Environment, Health and Safety Publications, Series on Testing and Assessment No. 174. ENV/JM/MONO(2012)19. Available at: [http:// http://www.oecd.org/chemicalsafety/testing].

5. Sonneveld E, Riteco JAC, Jansen HJ, Pieterse B, Brouwer A, Schoonen WG, Burg van der B. (2006). Comparison of in vitro and in vivo screening models for androgenic and estrogenic activities. Toxicol. Sci. 89(1):173-187.

6. Burg van der B, Winter R, Weimer M, Berckmans P, Suzuki G, Gijsbers L, Jonas A, Linden van der S, Witters H, Aarts J, Legler J, Kopp-Schneider A, Bremer S. (2010). Optimization and prevalidation of the in vitro ERα CALUX method to test estrogenic and antiestrogenic activity of compounds. Reprod Toxicol. 30:73-80.

7. Sonneveld E, Jansen HJ, Riteco JA, Brouwer A, van der Burg B. (2005) Development of androgen- and estrogen-responsive bioassays, members of a panel of human cell line-based highly selective steroid-responsive bioassays. Toxicol. Sci. 2005,Jan;83(1):136-48.

8. Legler J, van den Brink CE, Brouwer A, Murk AJ, van der Saag PT, Vethaak AD, van der Burg B. (1999). Development of a stably transfected estrogen receptor-mediated luciferase reporter gene assay in the human T47D breast cancer cell line. Toxicol Sci. 1999 Mar;48(1):55-66.

9. Van der Linden, SC, Bergh von A, Vugt-Lussenburg van B, Jonker L, Brouwer A, Teunis M, Krul C, Burg van der B. (2014). Development of a panel of high throughput reporter gene assays to detect genotoxicity and oxidative stress, Mutation Res.760:23-32.

10. Piersma AH, Schulpen SHW, Uibel F, Vugt-Lussenburg van B, Bosgra S, Hermsen SAB, Roelofs MJE, Man, H, Jonker, L, Linden van der S, Duursen van MBM, Wolterbeek APM, Schwarz M, Kroese ED, Burg van der B. (2013). Evaluation of an alternative in vitro test battery for detecting reproductive toxicants. Reprod. Toxicol. 38:53-64.

11. Gordon JD, Chu, AC, Chu MD, Denison MS, Clark GC. (2004). Detection of estrogen receptor endocrine disruptor potency of commonly used organochlorine pesticides using the LUMI-CELL bioassay Organohalogen Compounds, Dioxin 2004, 66:169-174.

12. Löser R, Seibel K, Roos W, Eppenberger U. (1985). In vivo and in vitro antiestrogenic action of 3-hydroxytamoxifen, tamoxifen and 4-hydroxytamoxifen. Eur J Cancer Clin Oncol. 21(8):985-90.

13. NEN 7777+C1:2012. Environment and food – Performance characteristics of measurement methods.

ANNEX A

Standard Operating Procedure

Transactivation assay for the detection of chemicals with (anti)estrogenic potential using ERα CALUX® cells

Compiled by: Harrie Besselink
Amended by: Harrie Besselink

Version No.: D
Date: 2 October 2015

Replaced version No. C
Date: 2 June 2014

Authorised by:	Approved by:	Supervisor protocol:
(Head of Quality)	(Head of Laboratory)	(Technical expert)
Emiel Felzel	Harrie Besselink	Irene Middelhof

ANNEX A

Major changes as compared to version

- Addition of visual inspection of cell viability using a phase-contrast reverse microscope

ANNEX A

Transactivation assay for the detection of chemicals with (anti)estrogenic potential using ERα CALUX® cells

Index

ANNEX A

ANNEX A

1 Purpose and Applicability

ERα CALUX® cells express a functioning human estrogen receptor and contain a reporter gene (luciferase) under the control of an estrogen responsive element. An increase or decrease of estrogenic signaling, results in corresponding changes in the expression of luciferase activity in ERα CALUX® cells, and is measured with a luminometer. Accordingly, ERα CALUX® cells can be used to detect chemicals with (anti)-estrogenic properties.

Here, detailed protocols for maintenance of ERα CALUX® cells and for identifying agonistic and antagonistic potency of chemicals using the (anti) ERα CALUX® bioassays, is given.

In the Materials and equipment section (2), all chemicals, reagents, disposables and equipment required for performing the ERα CALUX® bioassay are given. In addition, quality testing for essential chemicals and materials is described.

In section 3 (Preparations), the manual describes procedures for preparing various solutions necessary to maintain the CALUX® cells and to perform (anti) ERα CALUX® bioassays.

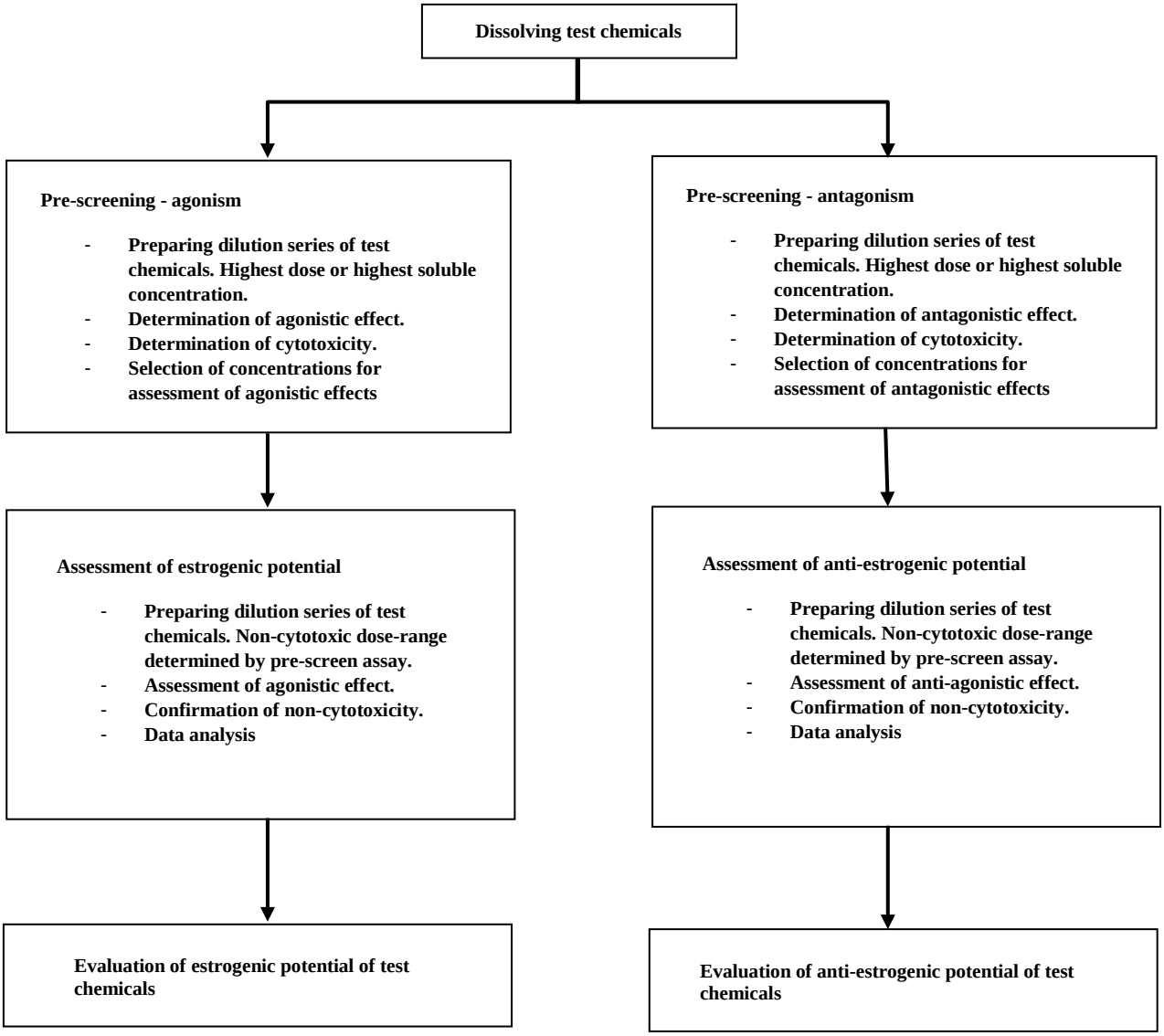
Section 4 (Procedures) contains all procedures and protocols for maintaining ERα CALUX® cells and proper setup for determination of agonistic potency of test chemicals, antagonistic potency of test chemicals and determination of cytotoxicty of test chemicals.

The determination of the agonistic effect of chemicals is described in section 4.5. Prior to the assessment of the agonistic potency of chemicals, a pre-screen has to be conducted to determine the proper concentration range for testing chemicals (4.5.4). Following the determination of the proper dose-range for testing, the exposure of CALUX® cells for the assessment of agonistic potency of chemicals is given in section 4.5.5.. After exposure, cells have to be harvested (4.5.6) and the luciferase activity has to be determined using a luminometer (4.5.7). Data analysis is described in section 4.5.8. and data interpretation in section 4.5.9. For quality control purposes, specific performance criteria have to be fulfilled. In addition, the reference EC_{50} and the relative induction of the positive control and negative control have to be monitored and registered continuously (4.5.10)..

The determination of the antagonistic effect of chemicals is described in section 4.6. Prior to the assessment of the antagonistic potency of chemicals, a pre-screen has to be conducted to determine the proper concentration range for testing chemicals (4.6.6). The exposure of CALUX® cells to a proper serial dilution range for the assessment of antagonistic potency of chemicals is given in section 4.6.7. Following exposure, cells are harvested (4.6.8) and the luciferase activity is determined using a luminometer (4.6.9). Data analysis is described in section 4.6.10 and data interpretation in section 4.6.11. For quality control purposes, specific performance criteria have to be fulfilled. In addition, the reference IC_{50} and the relative induction of the positive control and negative control have to be monitored and registered continuously (4.6.12).

In addition to the assessment of agonistic or antagonistic potency of chemicals, chemicals are also tested for cytotoxicity. In section 4.7, cytotoxicity testing using a commercially available kit is given.

ANNEX A


Dissolving test chemicals
Pre-screening - agonism
- Preparing dilution series of test chemicals. Highest dose or highest soluble concentration.
- Determination of agonistic effect.
- Determination of cytotoxicity.
- Selection of concentrations for assessment of agonistic effects
Pre-screening - antagonism
- Preparing dilution series of test chemicals. Highest dose or highest soluble concentration.
- Determination of antagonistic effect.
- Determination of cytotoxicity.
- Selection of concentrations for assessment of antagonistic effects
Assessment of estrogenic potential
- Preparing dilution series of test chemicals. Non-cytotoxic dose-range determined by pre-screen assay.
- Assessment of agonistic effect.
- Confirmation of non-cytotoxicity.
- Data analysis
Assessment of anti-estrogenic potential
- Preparing dilution series of test chemicals. Non-cytotoxic dose-range determined by pre-screen assay.
- Assessment of anti-agonistic effect.
- Confirmation of non-cytotoxicity.
- Data analysis
Evaluation of estrogenic potential of test chemicals
Evaluation of anti-estrogenic potential of test chemicals

ANNEX A

1.1 Definitions and Abbreviations

1.1.1 Definitions

Agonist	A chemical that can elicit a similar response as the reference standard in the CALUX® bioassay
Antagonist	A chemical that can elicit suppression of the response of the agonistic reference standard in the CALUX® bioassay
Cell line	A population of cells that propagate indefinitely.
Confluent	A coherent monolayer of cells.
Cross talk	The influence of activity in adjacent wells on the luminescence determination in a specific well.
Cytotoxicity	Cell toxicity.
Work solution	Assay medium supplemented with test-chemical (test chemical-work solution) or reference (reference-work solution) to be tested.
Filter sterilise	Sterilisation by forcing solutions through a 0.2 µm filter
Freezing medium	Supplemented medium used to freeze CALUX cells
Growth medium	Supplemented medium used for culturing CALUX cells.
Harvest	Removal of exposure medium from a microtiter plate and subsequent lysing of cells.
Lyse	Destruction of the membrane structure of cells by chemical, osmotic, physical, or enzymatic treatment.
Monolayer	A single layer of coherent cells
Passage number	The number of times a cell line has been sub-cultured after construction of the cell line
Positive Control	Assay medium supplemented with an active concentration of known agonist/antagonist.
Reference standard	(“control chemical”) Chemical used to provide a basis for comparison with the test chemical
Rounded	Single, round cells that are no longer attached to the bottom of a cell culture flask
Seeding	The transfer of a cell suspension into a microtiter plate.
Solvent Control	Assay medium supplemented only with the solvent used to dissolve the chemicals
Stripped	A process to remove interfering susbstances (e.g.steroids) from the serum by means of charcoal
Subculture	The transfer of a cell suspension into a new culture flask.
Swirl	Gently moving of a flask in a circular movement without splashing
Test Chemical	Chemical that is subject to testing.
Trypsinate	Enzymatic treatment of cells with trypsin to remove intercellular and surface attachment resulting in a discreet single rounded cell suspension.
Vortex	To homogenize using a VORTEX apparatus.

ANNEX A

1.1.2 Abbreviations

ATP	Adenosine-5'-TriPhosphate
BDS	BioDetection Systems BV. The test developer and owner of ERα CALUX® assay and cells
CALUX	Chemically Activated LUciferase eXpression
DCC-FCS	Dextran Coated Charcoal – Foetal Calf Serum
DF	Dulbecco's Modified Eagle Medium (DMEM) supplemented with F12
DMSO	Dimethylsulfoxide
DTT	1,4-dithiothreitol
EC_{50} / EC_{10}	50% / 10% effect concentration
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid
FCS	Foetal Calf Serum
G-418	Geneticin, an aminoglycoside
GLP	Good Laboratory Practice
GMO	Genetically Modified Organism
IC_{50} / IC_{10}	50% / 10% inhibitory concentration
LDH	Lactate dehydrogenase
NEAA	Non-essential amino acids
OD	Pbtical density
PBS	Phosphate Buffered Saline
RPC_{max}	Concentration at which a maximum response of the reference standard or test chemical is observed (agonism)
RPC_{min}	Concentration at which a maximum response of the reference standard or test chemical is observed (antagonism)
RAA	Relative Agonistic Activity
RIA	Relative Inhibitory Activity
RTA	Relative Transcriptional Activation
RTI	Relative Transcritional Inhibition
TRIS	2-amino-2-(hydroxymethyl)-1,3-propanediol

ANNEX A

1.2 Responsibilities

- It is the responsibility of the Study Director and Test Facility Manager to ensure that all study personnel are adequately trained in the following procedures and that these procedures are adhered to
- It is the responsibility of the Study Director that GLP principles are followed.
- It is the responsibility of the Study Director to ensure that all equipment is maintained, calibrated and is functioning properly.

1.3 Health Safety and Environment

While working with cells or chemicals, always wear sufficient personal protective equipment, such as goggles, gloves and laboratory coat

1.3.1 Guidelines for working with GMOs

1.3.1.1 Handling GMOs is usually undertaken in a designated area.

1.3.1.2 Doors and windows must be closed at all times that materials or cells are being manipulated.

1.3.1.3 Workspace must be clean and properly maintained.

1.3.1.4 Workspace must be decontaminated with 70% ethyl alcohol before and at the end of each manipulative activity and at the end of each workday.

1.3.1.5 If a surface is contaminated, it must be cleaned and sterilised with 70% ethyl alcohol immediately.

1.3.1.6 A laboratory coat or other protective clothing must be worn at all times. Work clothing should not be worn outside the designated area. Contaminated clothing must be sterilised before washing. If sterilisation services are not readily available, lab coats can be sterilized in the autoclave. Personal clothing must be stored outside the laboratory.

1.3.1.7 Operators must not eat, drink, smoke, or store food or drinks at the laboratory.

1.3.1.8 Wash hands before leaving and entering the laboratory.

1.3.1.9 Under no circumstances pipette by mouth.

1.3.1.10 Materials that have been in contact with genetically modified organisms can be disposed of as potentially biologically hazardous material or can be disposed of as normal waste after sterilisation for a minimum of 20 minutes at 121^{o}C and 100 PSI.

1.3.1.11 For the removal of potentially biologically hazardous materials, specially designated containers such as containers that can only be closed once must be used.

1.3.1.12 These guidelines apply for all GMO designated areas, which means that even if work is undertaken in these areas which does not involve GMOs, these guidelines still apply.

1.3.1.13 Label culture flasks with the present cell-line and the date which the cells were trypsinated and the name of the cultivator.

1.3.2 Guidelines for working with “coded” and/or toxic chemicals

1.3.2.1 Always consult the Material Safety Data Sheets before handling chemicals, as they may be hazardous.

1.3.2.2 “code” chemicals must be treated as if they were very toxic or having carcinogenic, mutagenic or reproductive properties.

ANNEX A

1.3.2.3 Handling toxic materials is usually undertaken in a designated area.

1.3.2.4 Doors and windows must be closed at all times that materials are being manipulated.

1.3.2.5 Workspace must be clean and properly maintained.

1.3.2.6 When working in the laboratory, a lab coat or other protective clothing must be worn at all times. Work clothing should not be worn outside the designated area. Contaminated clothing must be destroyed. Dispose in containers designated for chemical waste. Personal clothing must be stored outside the laboratory.

1.3.2.7 Operators must not eat, drink, smoke, or store food or drinks at the laboratory.

1.3.2.8 Wash hands before leaving and entering the laboratory.

1.3.2.9 Under no circumstances pipette by mouth.

1.3.2.10 Materials that have been in contact with toxic materials must be disposed of as chemical waste.

1.3.2.11 All organic solvents have to be disposed in special dedicated containers for organic chemical waste. Organic solvent may under no circumstances be disposed of through watersinks!

1.3.2.12 All glassware having been in contact with organic solvent has to be placed in the fume cupboard to allow to dry. Next, glassware has to be soaked in soap-solution before washing in the washing machine. The soap-water has to be discarded through an activated-charcoal-filter.

1.3.2.13 A laboratory log should be maintained at all times which includes as a minimum:

- A list of all toxic chemicals present at the laboratory.
- The storage details of the toxic chemicals

ANNEX A

2 Materials and equipment

It is recommended to use the materials, chemicals, and apparatus from suppliers as stated in this procedure. Materials, chemicals, and apparatus from other suppliers may affect the outcome of the CALUX® assay.

2.1 Forms

FRM01	Exposure information
FRM02	Sub-culturing and seeding of ERα CALUX cells.
FRM03	Preparation of stock solutions of test chemicals, reference standards and control chemicals
FRM04	Quality Control of ERα CALUX cells before and after treatment with chemicals.
FRM05	Cell counting information.
FRM06	Solubility testing to determine solvent and highest soluble dose.
FRM07	Solubility testing under experimental conditions.

DAT01	Doubling time calculation.
DAT02	Calculation of results for agonism.
DAT03	Calculation of results for antagonism.
DAT04	Calculation of results for prescreen - agonism.
DAT05	Calculation of results for prescreen - antagonism.

2.2 Test system

ERα CALUX® cell lines (U2-OS-based CALUX® cells). Supplier is BioDetection Systems BV (BDS)

ERα CALUX® cells originated from the human osteoblastic osteosarcoma U2-OS cell line. Human U2-OS cells were stably transfected with 3xHRE-TATA-Luc and pSG5-neo-hER using the calcium phosphate coprecipitation method.

Before applying this assay, please ensure sufficient cells are available in master and working bank.

Only mycoplasma free cultures shall be used. Cell batches used must either be certified negative for mycoplasma contamination, or a mycoplasma test should be performed before use. Mycoplasma testing shall be performed with either a direct culture method or by DNA extraction followed by realtime PCR method. Cells as provided by BDS are tested and found to be negative from mycoplasm contamination.

ANNEX A

2.3 Chemicals and reagents

Chemicals and reagents	Supplier	Cat. Number
Cytotoxicity testing		
Cytotoxicity kit using the supernatant of exposed cells (e.g. LDH leakage kit)	e.g. Roche	1 644 793 (2000 wells)
Cell maintenance and growth medium		
DF- (D-MEM/F12 medium without phenol red as pH-indicator)	e.g. Gibco	21041-025
DF+ (D-MEM/F12 medium with phenol red as pH-indicator)	e.g. Gibco	31331-028
EDTA Ehtylenediaminetetraacetic acid	e.g. Acros	147855000
FCS (Fetal Bovine Serum) from Australian origin	e.g. Gibco	10099-141
G418 disulphate (CAS: 108321-42-2)	e.g. Duchefa	G0175.0001
MEM (100x) Non-essential amino acids	e.g. Gibco	11140-035
PBS (phosphate buffered saline) pH 7.2; Ca^{2+} and Mg^{2+} free	e.g. Gibco	20012019
Penicillin-streptomycin solution (5000 units/ml; 5000 µg/ml)	e.g. Invitrogen	15070-063
Trypsin stock solution (1:250 activity)	e.g. Gibco	15090
Other reagents		
2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS), ultra pure	Invitrogen	
Activated charcoal	Fluka	05120
Adenosine-5'-trifosfaat (ATP, $C_{10}H_{14}N_5O_{13}P_3Na_2$)	Boehringer	519987
Coenzyme A, free acid grade I	Boehringer Mannheim	103420
demineralized water		
Dextran T500	Amersham Pharmacia	17.0320.01
Dithiothreitol (DTT)	Duchefa	d 1309
D-Luciferine ($C_{11}H_7N_2O_3S_2.H_2O$)	Duchefa	002591-17-5
DMSO (dimethylsulfoxide)	Acros	167852500
Ethanol (100%)		
Ethanol (70%)		
Ethyleendiaminetetraazijnzuur (EDTA)	Jansen chemica	14.785.41
Glycerol	Baker	7044
Hydrogen Chloride (HCl); 37%	Vel	1030
Ice	---	---
Liquid nitrogen	---	---
Magnesium carbonaat 5-hydraat ($(MgCO_3)4Mg(OH)_2.5H_2O$, 99% purity)	Aldrich	22.766.8
Magnesium carbonate hydroxide pentahydrate ($C_4H_2Mg_5O_{14}.5H_2O$)	Aldrich	22.766-8
Magnesium sulfaat-heptahydraat ($MgSO_3.7H_2O$)	Merck	1.05886
Magnesiumsulphate ($MgSO_4$)	Riedel de Haën	31420
Methanol (HPLC grade)	Baker	8402
Sodiumhydroxide (NaOH)	Riedel de Haën	30620
Tetrahydrofuran (THF)		
trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid monohydrate (CDTA)	Fluka	32869
Tricine ($C_6H_{13}NO_5$)	Jansen chemica	117.264.9
Reference standards and control chemicals		
17α-methyltestosterone (CAS: 58-18-4)	Sigma	46444
Corticosterone (CAS: 50-22-6)	Sigma	27840
17ß-estradiol (E2) (CAS: 50-28-2)	Sigma	E2758
Tamoxifen (CAS: 10540-29-1)	Sigma	T5648
4-hydroxytamoxifen (CAS: 68047-06-3)	Sigma	H7904
Resveratrol (CAS: 501-36-0)	Sigma	R5010
Reference standard concentration series in DMSO	BDS	---
Triton® X-100	Sigma	T8787

ANNEX A

2.4 Disposables and equipment

Disposables and equipment	Supplier	Cat. Number
Consumables		
Syringe plus filter (0.2 µm) (sterile)		
5 ml sterile tubes		
Multipipette tips, 10 ml	VWR	613-3533
Multipipette tips, 5 ml	VWR	613-3532
Reagent reservoirs	e.g. Costar	4870
Sterile pipette filter tips, 10µl		
Sterile pipette tips, 200-1000 µl		
Sterile pipette tips, 20-200 µl		
Polystyrene 48-microtiter plate		
Tissue culture plates, (6; 12; 24; 96 well)	Greiner	
Culture flasks, 75 cm2, tissue culture with filter cap	Greiner bio-one	658175
1.5 to 2 ml Cryovials	Greiner	121263
Plastic tubes (50 ml) with cap, sterile		
Sterile pipettes (10 ml)		
Sterile pipettes (5 ml)		
50 ml centrifuge tubes (conical)		
Autoclavable disposable bag		
Equipment		
Wide mouth HDPE bottles	e.g. Nalgene	2106-0004
Anlytical balance	Sartorius	MC210P
Autoclave	PBI-international	AUTO-KOCH
Bottle top filter sterilization unit	Nalgene	
Bürker-Türk counting chamber		
Centrifuge		
CO_2-incubator	Heraeus	Heracel
Erlenmyer; 1 liter		
Freezer, -80°C		
Glass beaker (25, 50, 250, 500, 1000 ml)		
glass bottle ; 1 liter		
Inverted relief contrast microscope (4X, 10X and 20X objective)	Olympus	CK30
Laminar flow cabinet	Heraeus	HS-18
Liquid Nitrogen cell storage facility	Thermolyne	ck509x3
Liquid reservoir		
Luminometer (with 2 injectors) (software: Microwin 2000)	e.g Berthold	Centro
Magnetic stirrer	IKA	RH basic
Microtiter plate shaker		
microtiter shaker	IKA	MTS 2/4
Multichannel pipette (100 µl)		
Multichannel pipette (30 µl)		
Multipipette plus	Eppendorf	4981000.019
pH meter		
Pipette controller	Brand	accu jet
Pipettes (1 ul)		
Pipettes (10 ul)		
Pipettes (20 µl)		
Pipettes (200 ul)		
Refrigerator/freezer combination		
Tweezers		
Water bath (37^{o}C)	GFL	1083
Waterbath		

ANNEX A

2.5 Quality testing of solvents and FCS

A number of chemicals / reagent / materials are subjected to an applicability test prior to usage. These tests include **testing for background contamination or testing for proper characteristics for cell culture**. For DMSO (or other solvent used for dissolving test chemicals) and FCS the procedures for quality testing in this section must be followed.

2.5.1 Quality testing of solvents

Solvents used to dissolve test chemicals, have to be tested for possible background activity. If a solvent has not been used before, please ensure the background activity does not exceed the acceptance criteria in section this section.

2.5.1.1 Seed CALUX® cells according to section 4.4.4.
2.5.1.2 Prepare the appropriate reference-work solution according to section 4.5.2.
2.5.1.3 Prepare work solution containing only the solvent under investigation and work solution containing the same solvent already in use (4.5.1).
2.5.1.4 Following 24 hours of pre-incubation, fill the seeded microtiterplate with the prepared reference-work solution (well C0 – C8 only) according to section 4.5.5.
2.5.1.5 Fill half of the remaining wells [E2-G6] with the work solution containing solvent under investigation and the other half of remaining wells [E7-G11] with work solution containing the solvent already in use.
2.5.1.6 Following 24 hours of incubation in an incubator (37°C, 5% CO2, 100% humidity) the cells can be harvested and luminescence can be determined (see 4.5.6. and 4.5.7.).
2.5.1.7 Calculate the average RLU of the new and old solvent and the average RLU of solvent blank of the reference (C0).
2.5.1.8 Calculate for both the old and new batch of solvent the ratio:
(average solvent RLU) : (average reference blank RLU)
2.5.1.9 Register the ratio of the new solvent in a historical database*.
2.5.1.10 Register the date and lot number.

The new batch of solvent may be used when:

- The ratio of new solvent is within 25% of the average historical ratio.
- The average background activity is not more than 5% of the C8 concentration (1×10^{-10} M) of reference agonist 17ß-estradiol.

*The historical database is constructed by registration of test results of all solvent batches.

2.5.2 Quality testing of FCS

Prior to use, the quality of a new batch of FCS must be tested by cultivation of CALUX® cells in growth medium supplemented with FCS from the new batch, and comparison with results from the old batch.

Before use, FCS has to be treated with Dextran-Coated Charcoal (DCC).
Following preparation of DCC treated FCS, the DCC-FCS serum is tested by comparing the newly prepared DCC-FCS with an old batch of DCC-FCS serum.

2.5.2.1 Minimally subculture cells 3 times and evaluate the cells visually and under the microscope for contamination and viability.
2.5.2.2 Determine the doubling time in accordance with Appendix B under normal culturing conditions.

ANNEX A

2.5.2.3 Perform the ERα CALUX assay by incubating the cells with the reference standards 17ß-estradiol and tamoxifen, using both old and newly prepared batches of FCS and DCC-FCS.

The new batch of FCS or DCC-FCS in the growth or assay medium, respectively, may be used when:

- The doubling time of the ERα CALUX cells, under normal culturing conditions, is within 30 +/- 10 hours.
- The viability of the cells is > 95%.
- There is no contamination.
- The analysis results from the new batch of FCS or DCC-FCS are comparable to the results of the old batch of FCS or DCC-FCS, i.e. the acceptance criteria for plate similarity are met (see table 4.5.4 and 4.6.4).
- The induction factors for reference standards and control chemicals meet the acceptance criteria in table 4.5.4 and 4.6.4.

2.5.3 General quality testing of chemicals, reagents and materials

For other chemicals / reagents / materials used the general quality test given below has to be performed to show applicability of the batch.

2.5.3.1 Treat 1 set of ERα CALUX® cells using chemicals, reagents and materials in use with serial dilutions of the reference standard (see 4.5.2, 4.5.3, and 4.5.5).

2.5.3.2 Similarly, treat 1 set of ERα CALUX® cells using chemicals, reagents and materials from the new batch to be tested with serial dilutions of the reference standard. Only test 1 new chemical, reagent or material at a time (see 4.5.2, 4.5.3, and 4.5.5).

2.5.3.3 Following 24 hours of incubation in an incubator (37°C, 5% CO_2, 100% humidity) the cells must be lysed and luminescence measured (see 4.5.6 and 4.5.7).

2.5.3.4 Determine the EC_{50} of the reference-dose and the relative induction (%) of the positive control (PC) for both plates exposed in accordance with section 4.5.8.

2.5.3.5 Register the date and lot number.

The new batch of chemicals, reagents or materials may be used when:

- The induction factors for reference standards and control chemicals meet the acceptance criteria in table 4.5.4.
- The background activity is not more than 5% of the C8 concentration of reference agonist 17ß-estradiol.
- The relative induction of the PC tested on cells grown, plated and exposed using chemicals, reagents and materials from the new batch is within 25% of the average historical ratio*.
- The relative induction of the PC tested on cells grown, plated and exposed using chemicals, reagents and materials from the a new batch is not more than 25% different from the relative induction of the PC tested on cells grown, plated and exposed in chemicals, reagents and materials from the old batch.

*The historical database is constructed by registration of test results of all chemical, reagent or material batches.

ANNEX A

3 Preparations

All preparations used for cell culture must be prepared and kept under sterile conditions at all times.

3.1 Preparation of FCS

3.1.1 Thaw a bottle of FCS (500 ml) in the waterbath (not warmer as 37°C).
3.1.2 Open the appropriate number of sterile 50 ml tubes in a laminar flow cabinet.
3.1.3 Homogenise the FCS by swirling the flask a few times and open the bottle in the laminar flow cabinet.
3.1.4 Transfer 41 ml portions of the FCS to 50 ml tubes.
3.1.5 Close the tubes and label them. Indicate content, date of preparation and expiration date as indicated on the original FCS bottle.
3.1.6 Store the tubes with FCS at -20°C.

3.2 Preparation of NaOH solution (0.2 M)

3.2.1 Dissolve 8 g NaOH in 1 liter demineralized water.
3.2.2 Close and label the bottle, indicating content, date of preparation and expiration date (6 months from the day of preparation).
3.2.3 Stir with a magnetic stirrer until the NaOH is dissolved completely.
3.2.4 Store at room temperature.

3.3 Preparation of Tris/HCl solution (1 M)

3.3.1 Dissolve 121.1 g Tris in 800 ml demineralized water.
3.3.2 Adjust pH to 8.0 using concentrated HCl.
3.3.3 Adjust to a final volume of 1 liter using demineralized water.
3.3.4 Close the flask but not completely to allow air to escape during autoclavation.
3.3.5 Autoclave at 121^0C for 15 minutes.
3.3.6 Allow Tris/HCl solution to cool down and close the bottle.
3.3.7 Label the bottle, indicating content, date of preparation and expiration date (3 months from the day of preparation).
3.3.8 Store at room temperature.

Note: before using the autoclaved Tris/HCl 1 M solution, check pH.

3.4 Preparation of DCC-FCS

Because steroids present in FCS will lead to background activation in the CALUX® assay, dextran-coated charcoal (DCC) is used to remove steroid from FCS.

3.4.1 Preparing the dextran-coated activated charcoal

3.4.1.1 Rinse 1bottle (1 liter) with demineralized water.
3.4.1.2 Add
- 800 ml demineralised water
- 11.5 ml 1 M Tris/HCl pH8
- 0.625 g Dextran T500

3.4.1.3 Make sure all ingredients are dissolved.
3.4.1.4 Add 6.25 grams of activated charcoal.
3.4.1.5 Fill the bottle completely with demineralized water.
3.4.1.6 Close the bottle tightly with a Teflon coated lid.
3.4.1.7 Stir overnight at 4˚C.

ANNEX A

3.4.2 Stripping the serum

3.4.2.1 Thaw 1 flask (500 ml) of FCS.
3.4.2.2 Heat the FCS for 30 minutes at 56°C in the water bath.
3.4.2.3 Whilst the FCS is warming, distribute the dextran-coated charcoal suspension (0) between twenty 50 ml (plastic) centrifuge tubes. The difference in weight between the tubes must not exceed 0.1 gram.
3.4.2.4 Centrifuge for 10 minutes at 2000 g.
3.4.2.5 Carefully decant the centrifuge tubes, but do not disturb the dextran-coated charcoal pellets. Keep the centrifuge tubes with opening down on a piece of paper to dry. Remove as much fluid as possible without touching the pellets.
3.4.2.6 Add approximately 50 ml of FCS to 10 tubes containing the dextran-coated charcoal pellets and re-suspend the dextran-coated charcoal pellets .
3.4.2.7 Pool the FCS serum suspension from all tubes into the original FCS bottles and incubate in water bath at 45°C for 45 minutes while shaking at such a speed that the serum is gently moving. Do not discard the used tubes.
3.4.2.8 Fill out the 10 used 50 ml tubes with the incubated charcoal-serum suspension and centrifuge the tubes for 10 min at 2000 g.
3.4.2.9 Following centrifugation, carefully transfer the serum into the 10 unused dextran-coated charcoal containing 50 ml tubes and re-suspend the dextran-coated charcoal pellets.
3.4.2.10 Pool the FCS serum suspension from all tubes into the original FCS bottles and incubate in water bath at 45°C for 45 minutes while shaking at such a speed that the serum is gently moving.
3.4.2.11 Following the second incubation, fill out 10 used 50 ml tubes with the incubated charcoal-serum suspension and centrifuge the tubes for 10 min at 2000 g.
3.4.2.12 To remove the last amount of dextran-coated charcoal, transfer the centrifuged serum (supernatant) into 10 clean 50 ml centrifuge tubes and centrifuge for 30 min at 2000 g.
3.4.2.13 Pool the serum into a clean 500 ml bottle.
3.4.2.14 Filter-sterilize the serum using a bottle top filter sterilization unit (Nalgene).
3.4.2.15 Divide the stripped serum into 26.6 ml portions in sterile tubes.
3.4.2.16 Label all tubes as DCC-FCS, indicating their content, date of preparation, and expiration date (6 months from the day of preparation).
3.4.2.17 Store at -20˚C.
3.4.2.18 Test one tube of DCC-FCS as indicated in 2.5.2.

3.5 Preparation of Pen-strep stock solutions

Penicillin Streptomycin solution is added to the medium, to prevent bacterial infections.

3.5.1 Thaw a bottle of penicillin-streptomycin (Pen-Strep) stock solution (containing 5000 units of penicillin and 5000 μg of streptomycin per ml).
3.5.2 Fill out 1 ml portions of the penicillin-streptomycin stock solution.
3.5.3 Label the stocks indicating the content, date of preparation and expiration date as indicated on the original Pen-Strep bottle. Store them at -20°C.

3.6 Preparation of G418 stock solutions

Geneticin (G-418) is used as a selection agent. Cells no longer expressing the vector will die.

3.6.1 Add 20 ml of demineralized water to 1 g of G418 disulphate and mix until the G418 is completely dissolved (50 mg/ml).
3.6.2 Filter sterilize the solution and divide into 2 ml portions.

3.6.3 Label the stocks indicating the content and date of preparation. Store them at -20°C for long term storage. For short term storage (general use), a single tube can be stored at 4°C.

3.7 Preparation of DMEM/F12 containing 7.5% FCS (growth medium)

Growth medium for the ERα CALUX® cells consists of DMEM supplemented with F12 (DF), 7.5% Fetal Calf Serum (FCS), 1% non-essential amino acids, 10 Units/ml Penicillin and 10 μg/ml Streptomycin.

3.7.1 Thaw one tube containing 41 ml FCS in a waterbath (37°C).
3.7.2 Open a 500 ml bottle of DMEM/F12 with phenol red in a laminar flow cabinet.
3.7.3 Add 5.5 ml of the non-essential amino acids (MEM 100x).
3.7.4 Add 41 ml of FCS.
3.7.5 Add 1 ml of penicillin-streptomycin solution.
3.7.6 Close the flask and mix carefully, avoiding bubble formation.
3.7.7 Label the bottle, indicating content, date of preparation and expiration date (8 weeks from the day of preparation).
3.7.8 Store the flask with growth medium at 4°C

3.8 Preparation of DMEM/F12 containing 5% DCC-FCS (assay medium)

Assay medium consists of DMEM without phenol red, supplemented with F12, 5% Dextran-Coated Charcoal treated FCS (DCC-FCS), 1% non-essential amino acids (MEM 1 ×), 10 Units/ml Penicillin and 10 μg/ml Streptomycin.

3.8.1 Thaw one tube containing 26.6 ml DCC-FCS in a waterbath (37°C).
3.8.2 Open a 500 ml bottle of DMEM/F12 without phenol red in a laminar flow cabinet
3.8.3 Add 5.3 ml of non-essential amino acids (MEM 100x).
3.8.4 Add 26.6 ml DCC-FCS.
3.8.5 Add 1 ml of penicillin-streptomycin solution.
3.8.6 Close the flask and mix carefully, avoiding bubble formation.
3.8.7 Label the bottle, indicating content, date of preparation and expiration date (8 weeks from the day of preparation).
3.8.8 Store the flask with assay medium at 4°C

3.9 Preparation of trypsin solution

Trypsin-EDTA is used to detach the cells from the plastic culture flask. It must be used sterile at a concentration of 0.05%.

3.9.1 Dilute the trypsin stock solution to a concentration of 0.05%. Use PBS (without calcium and magnesium!) containing 0.2 g EDTA per liter.
3.9.2 Filter sterilize the trypsin solution and make aliquots of 40 ml.
3.9.3 Close and label the tubes, indicating content, date of preparation and expiration date (3 months from the day of preparation).
3.9.4 Store the tubes of trypsin at -20°C.

3.10 Preparation of lysis mix

Lysis reagent is added to each well to lyse the cells before measurement of luminescence.

3.10.1 Add 500 ml of demineralized water to a 1 liter glass beaker.
3.10.2 Add Tris, DTT and CDTA according to Table 3.10.1.
3.10.3 Add 100 ml (or 112.6 g) glycerol.
3.10.4 Add 10 ml (or 10.7 g) Triton® X-100.
3.10.5 Adjust the pH to 7.8 using 1 M HCl and/or 1 M NaOH solutions.
3.10.6 Adjust the final volume to 1 liter with demineralized water.
3.10.7 Transfer aliquots of approximately 40 ml into 50 ml tubes.
3.10.8 Close and label the tubes indicating content, date of preparation and expiration date. Store at -20°C for a maximum of 1 year, store at 4°C for a maximum of 1 month.

Table 3.10.1 Composition of lysis mix.

Compound	Weight[a] (g)	Volume (ml)	Molecular weight[b]	Molarity
Tris	3.0		121.1	25 mM
DTT	0.31		154.2	2,0 mM
CDTA	0.73		364.35	2,0 mM
Glycerol		100	-	10%
Triton®X-100		10	-	1%

[a]: The amount of compound to weigh out for 1 l lysis mix is given in the second column.
[b]: Check whether the molecular weight of the compounds corresponds to the molecular weight given in table 2. The amount of compound needs to be adjusted when the molecular weight does not correspond, based on the molarity given in the fourth column.

3.11 Preparation of BDS illuminate-mix

BDS illuminate mix solution is added to the cells to measure the luciferase activity. It is added to each well immediately before the measurement of luminescence. Instead of using BDS illuminate-mix, it is possible to determine luciferase in the samples with a commercially available kit.

3.11.1 Add 500 ml demineralized (demi) water to a one liter glass beaker.
3.11.2 Add tricine and magnesiumhydroxidecarbonate pentahydrate, according to Table 3.11.1, and stir with a magnetic stirrer until the solution is clear (this will take approximately one hour).
3.11.3 Take luciferine from the freezer and adjust to room temperature.
3.11.4 Add magnesiumsulphate-heptahydrate, EDTA, DTT and D-luciferine, according to Table 3.11.1. From this point on preparing the BDS illuminate-mix should be performed in the dark and may last no longer than half an hour due to the instability of the compounds used.
3.11.5 Add ATP according to Table 3.11.1.
3.11.6 Add 400 ml demineralized water and adjust the pH to 7.8 using 1 M HCl and/or 1 M NaOH solutions.
3.11.7 Adjust the final volume to 1 liter with demi water and mix carefully.
3.11.8 Divide the BDS illuminate-mix into 100 ml portions in HDPE bottles.
3.11.9 Close and label the bottles indicating content, date of preparation and expiration date. Store at -20°C for a maximum of 3 months or at -80°C for one year.
3.11.10 Check the activity of the BDS illuminate-mix with luciferase standards.

ANNEX A

Table 3.11.1 Composition of BDS illuminate-mix

Compound	Weight (g)[a]	Molecular weight[b]	Molarity
Tricine	3.580	179.2	20.0 mM
$(MgCO_3)_4Mg(OH)_2.5H_2O$	0.520	485.69	1.07 mM
$MgSO_4$ * 7 H_2O	0.658	246.48	2.67 mM
EDTA	0.037	372.23	0.10 mM
DTT	0.231	154.2	1.5 mM
D-Luciferine	0.151	280.3	539 µM
ATP	3.026	551.1	5.49 mM

[a]: The amount of compound needed for 1 l BDS illuminate mix is given in the second column. One liter of BDS illuminate mix is sufficient for approximately 150 microtiter plates.

[b]: Check whether the molecular weight of the compounds used corresponds to the molecular weight given in the table above. If the molecular weight does not correspond, adjust the amount of compound needed based on the molarity given in the fourth column.

3.12 Preparation of freezing medium

3.12.1 Open a 500 ml bottle of DMEM/F12 in the laminar flow cabinet.

3.12.2 Remove 142.5 ml and discard.

3.12.3 Add 5.5 ml of non-essential amino acids and mix gently.

3.12.4 Add 100 ml of FCS.

3.12.5 Add 37.5 ml of DMSO.

3.12.6 Mix gently and place 40 ml aliquots in 50 ml plastic bottles.

3.12.7 Label the plastic tubes indicating content, date of preparation and expiration date (1 year from the day of preparation).

3.12.8 Store freezing medium at -20°C.

3.12.9 Following preparation of freezing medium, incubate a 10 ml aliquot of the prepared freezing medium in the CO_2 incubator for 1 week. Check (microscope) after 1 week for contamination. In case of no contamination, the prepared batch of freezing medium can be used.

ANNEX A

4 Procedures

4.1 Test chemical solubility testing

Stock solutions

4.1.1 Be careful with (unknown) chemicals! Don't inhale, avoid skin contact, wear gloves and glasses, work in the fume hood.

4.1.2 Confirm all test chemicals are clearly labelled e.g. with chemical name / code, run number, CAS number, expiry date etc.

4.1.3 Prepare in a clear glass vial (8-10 ml) a stock solution of the chemical in the solvent of choice (see table 4.1.1).

4.1.4 When molecular weight (Mw) is known, prepare a stock solution of 0.1 M (e.g., 1 ml, sufficient for incubation medium testing later) in solvent. When molecular weight is unknown, prepare a stock solution of 100 mg/ml (approximately 1 ml).

4.1.5 If required, vortex, sonicate and/or gentle heat the solution (30 min; 37^0C).

4.1.6 In case the test chemical appears turbid or insoluble after visual inspection or chrystal formation is observed following microscopical inspection, dilute 3.33 or 10 times. Vortex mix, sonicate or gently heat (30 min; 37^0C) when not immediately soluble.

4.1.7 In case the chemical remains insoluble, repeat step 4.1.3 – 4.1.6. When molecular weight (Mw) is known, prepare a stock solution of 0.01 M (e.g., 1 ml, sufficient for incubation medium testing later) in solvent. When molecular weight is unknown, prepare a stock solution of 10 mg/ml (approximately 1 ml).

4.1.8 In case the chemical remains insoluble, repeat step 4.1.3 – 4.1.7 using the next solvent of choice (see table 4.1.1).

4.1.9 Record the results of solubility testing, including the solvent and concentration (FRM06).

Incubation medium

4.1.10 Check and monitor solubility of stock solution following 100-fold dilution and 24 hours of incubation in assay medium.

4.1.11 Fill 1 well of a clear polystyrene 6 well microtiter plate with 0.5 ml assay medium. Add 5 μl of the prepared stock solution and gently mix. Check for precipitation.

4.1.12 In case the chemical is not soluble in assay medium, dilute the stock solution 3.33 and/or 10-fold using the appropriate solvent and repeat steps 4.1.10 and 4.1.11.

4.1.13 A proper stock solution has been prepared when the test chemical is soluble in an appropriate solvent and remains soluble (does not precipitate) in assay medium at 100-fold dilution.

4.1.14 Divide the entire stock into 150 μl-portions. Use glass vials.

4.1.15 Label the vial. Register the date of preparation, concentration, solvent used and storage conditions (FRM07).

ANNEX A

Table 4.1.1 Solvent for dissolving chemicals

	Weight (g)
1st choice	DMSO
2nd choice	H_20

Note that solvents other than DMSO might need to be used to dissolve test chemicals (e.g. ethanol). If the test chemicals are not dissolved in DMSO, use medium that already contains 1% DMSO, as the DMSO concentration influences the response of the cells. Similarly, for test chemicals on the same 96 well plate that are dissolved in DMSO, the work solutions should be prepared using medium that is supplemented with the other solvent used.

4.2 Preparing dilution series of test chemicals for pre-screening

4.2.1 Freshly prepare a test chemical stock solution at the highest soluble concentration as determined in section 4.1.

4.2.2 Prepare a dilution serie in conical glass vials using the same solvent as used to prepare the stock solution:
1x, 10x, 100x, 1000x, 10000x,100000x, 1000000x and 10000000x dilution.

4.2.3 Label the vials as indicated in Table 4.2.1 to avoid mixing-up dilutions. Use form FRM03 to record data.

Table 4.2.1 Labeling of pre-screen concentration range test chemicals

Label	**Dilution**
TCx-1	10000000x
TCx -2	1000000x
TCx -3	100000x
TCx -4	10000x
TCx -5	1000x
TCx -6	100x
TCx -7	10x
TCx -8	1x

TCx = pre-screen dilution of test chemical x

4.3 Preparing dilution series of test chemicals for assessment of agonistic and/or antagonistic effects

4.3.1 Following pre-screening, the dilution range for the assessment of agonistic and/or antagonistic effects is determined. In Appendix C, the procedure for the selection of concentrations for assessment of agonistic and antagonistic effects is given. Please note that the concentration determined using Appendix C is the concentration in the well. For preparing a stock concentration, the concentration should be a 100x more concentrated!

4.3.2 Freshly prepare the test chemical stock solution.

4.3.3 Dilute the stock solution to the highest dilution to be tested (Appendix C) using the same solvent as used to prepare the stock solution. This dilution is indicated as the TI1-8 dilution (TI1 = test chemical1).

4.3.4 Prepare the selected dilution serie in conical glass vials as using the same solvent as used to prepare the stock solution, starting with the highest dilution (TI1-8):
TI1-1; TI1-2; TI1-3; TI1-4; TI1-5; TI1-6; TI1-7; TI1-8

4.3.5 Label the vials to avoid mixing.

4.3.6 Register test chemical identification and concentration (form FRM03).

ANNEX A

4.4 Maintenance and handling of cells

4.4.1 Freezing of cells

4.4.1.1 Prepare 75 cm^2 (25x) or 175 cm^2 (15x) culture flasks with cells.

4.4.1.2 Start freezing procedure when cells have reached a minimum of 90% confluence.

4.4.1.3 Mark new sterile 1.5 to 2 ml cryovials indicating the type of cells, passage number, date, and name. For each 75 cm^2 flask, mark 3 cryovials; for each 175 cm^2 flask, mark 7 cryovials. Cover the writing with transparent tape to protect it or use cryo proof stickers.

4.4.1.4 Open the cryovials carefully and set aside in the laminar flow cabinet (sterile) until further use (see 4.4.1.11)

4.4.1.5 Trypsinate all cells according to the procedure described in the corresponding cell culture protocol and remove trypsin.

4.4.1.6 Suspend the cells in growth medium (10 ml for each 75 cm2 flasks; 15 ml for each 175 cm2 flasks).

4.4.1.7 Pool and count the viable cells. Calculate how many cryovials can be prepared at a concentration of 1.5 x 10^6 cells/vial.

4.4.1.8 Divide the cells over 50 ml tubes and centrifuge (250 x g, 5 minutes)

4.4.1.9 Following centrifugation, keep the vials and 50 ml tubes on ice at all times and work as quickly as possible to minimise the toxic effect of DMSO at room temperature!

4.4.1.10 Discard medium and re-suspend the cells in freezing medium. The volume in ml of freezing medium to be used, equals the number of vials that can be prepared (4.4.1.7). Each cryovial should contain 1 ml of cell suspension.

4.4.1.11 Divide the re-suspended cells in freezing medium over the number of cryovials calculated (1 ml per cryovial) and close the cryo-vials.

4.4.1.12 Cover the inside of the storage box of the cryo-vials with tissues to facilitate a graduate freezing of the cells and store at -80^{o}C overnight. Alternatively, apply Mr. Frosty freezing containers to achieve a rate of cooling close to -1°C/minute..

4.4.1.13 Transfer cryovials to liquid nitrogen for storage.

4.4.1.14 Thaw one cryovial after at least one day of storage in the liquid nitrogen. Record the recovery rate by determining viability after thawing. Culture the cells for at least one week according to the appropriate protocol. Check the cells for signs of contamination, determine doubling time and check the cell reactivity towards reference standards. Cells may be accepted when acceptance criteria in table 4.5.4 are met.

ANNEX A

4.4.2 Thawing of cells

4.4.2.1 Heat water bath to 37°C.

4.4.2.2 Take a flask of growth medium from the refrigerator and heat it in the water bath.

4.4.2.3 Open two culture flasks (75cm^2) in a safety-cabinet, pipette 10 ml growth medium in each 75 cm^2 culture flask and place them in the CO_2-incubator for 30 minutes.

4.4.2.4 Retrieve a cryovial of CALUX® cells from the liquid nitrogen.
NOTE: Wear safety glasses and protective gloves.

4.4.2.5 Wait briefly to be sure that no liquid nitrogen is present the cryovial.

4.4.2.6 Thaw the cells quickly by gently moving the vial in a water bath of 37°C until the ice has almost melted.

4.4.2.7 Clean the outside of the cryovial with 70% alcohol.

4.4.2.8 Pipette 0.5 ml of growth medium from the culture flask into the cryovial using a sterile pipette.

4.4.2.9 Distribute the cells over the two flasks and label the culture flasks. Indicate the type of cells, date of preparation, name and passage number.

4.4.2.10 Place the culture flasks in the CO_2-incubator. When using non-filter lids, ensure the flasks are not closed completely.

4.4.2.11 Refresh the growth medium after 24 hours. If the flask is 85-95% confluent, sub-culture the cells according to section 4.4.3.

4.4.2.12 After thawing, cells have to be sub-cultured at least 2 times before the can be used to assess the agonistic and/or antagonistic effects of test chemicals in the (anti) ERα CALUX® bioassay.

4.4.3 Sub-culturing of cells

4.4.3.1 Remove the medium from the cells once the cells reached a confluency of 85-95%.

4.4.3.2 Wash the cells carefully two times with 5 ml of PBS.

4.4.3.3 Rinse the cells with 2 ml of trypsin solution and remove the trypsin solution.

4.4.3.4 Place the flaks in an incubator and wait until all cells are rounded and have detached from the bottom (1-3 minutes).

4.4.3.5 Re-suspend the cells in 10 ml of growth medium.

4.4.3.6 Transfer the proper amount of the cell suspension into a new 75 cm2 culture flask according to Table 4.4.1.

4.4.3.7 Add growth medium up to a final volume of 10 ml.

4.4.3.8 Add 40 µl G-418 solution. Addition of G-418 to culture flasks is done once per week.

4.4.3.9 Label the culture flasks. Indicate the type of cells, date of sub-culturing, passage number, dilution factor and your name.

4.4.3.10 Incubate the cells in the CO_2 incubator (37°C, 5% CO_2, 100% humidity). When using non-filter lids, ensure the flasks are not closed completely to allow transfer of gases.

4.4.3.11 The total amount of cell suspension can be used to subculture. The old culture flask and redundant cell suspension must be disposed of as biologically hazardous waste.

4.4.3.12 Cells are sub-cultured twice a week.

Table 4.4.1 Dilution ratio for sub-culturing cells

days to next sub-culturing	Dilution ratio (cell suspension: growth medium) ERα CALUX®
3	1:5
4	1:7

ANNEX A

4.4.4 Seeding of cells

4.4.4.1 Trypsinate cells which are between 85%-95% confluent as described in 4.4.3.

4.4.4.2 Re-suspend the cells gently in 10 ml assay medium and transfer to a sterile 50 ml tube.

4.4.4.3 Count the cells and record the number of living and dead cells on form FRM05.

4.4.4.4 Further dilute the cells with assay medium by sterile pipetting until a cell suspension of 100.000 cells per ml (10.000/well) is obtained.

4.4.4.5 Homogenize the cell suspension in the assay medium by sucking up and releasing the cell-suspension gently with the multipipette at least five times. Avoid foam formation.

4.4.4.6 Fill the 60 inner wells gently with 100 µl of cell suspension (figure 4.4.1). Mix the cell suspension carefully from time to time to keep the suspension homogeneous.

4.4.4.7 Fill the 36 outer wells with 200 µl of PBS (figure 4.4.1). These wells are not used in the assay.

4.4.4.8 Incubate the plates for a minimum of 16 and a maximum of 32 hours in the CO_2 incubator (37°C, 5% CO_2, 100% humidity).

4.4.4.9 To make sure all wells with CALUX® cells have the same conditions during the experiment, following measures have to be taken:

- All microtiter plates must always be placed separately from each other in the CO_2, so not stacked.
- After seeding the cells and during exposure, the disturbance of the climate in the CO_2 incubator must be kept to a minimum by minimizing opening and closing the CO_2 incubator.
- After a minimum of 16 hours of incubation, visually inspect the microtiter plate(s) and check for cloudy wells as an indicator of bacterial infection. Further check the cell morphology and confluence of a few wells. The confluence should be between 50 and 90 %

	1	2	3	4	5	6	7	8	9	10	11	12
A	O	O	O	O	O	O	O	O	O	O	O	O
B	O	I	I	I	I	I	I	I	I	I	I	O
C	O	I	I	I	I	I	I	I	I	I	I	O
D	O	I	I	I	I	I	I	I	I	I	I	O
E	O	I	I	I	I	I	I	I	I	I	I	O
F	O	I	I	I	I	I	I	I	I	I	I	O
G	O	I	I	I	I	I	I	I	I	I	I	O
H	O	O	O	O	O	O	O	O	O	O	O	O

Figure 4.4.1 Schematic representation of a 96 well microtiter plate after seeding. The outer wells (grey) are filled with 200 µl PBS and the inner wells (clear) with 100 µl cell-suspension.

4.4.5 Acceptance criteria for the test system

The following acceptance criteria must be met before the cells are used in the ERα CALUX® assay:

- Cultured cells must be sub-cultured at least twice a week.
- Cells must be sub-cultured as soon as cells reach 85-95% confluence. Cells must be discarded when they are overgrown and many detached cells are visible, under the microscope.
- Cells should have a passage number > 2 and < 30.
- Cells should be free of bacterial, fungal, yeast and mycoplasma contamination.

ANNEX A

The following acceptance criteria must be met during the assay:

- Cell viability for seeding must be > 95 %
- When 24 hour after seeding bacterial infection in any well is observed, it is advised not to use these microtiter plates for bioanalysis.
- Activity of the cells must meet the acceptance criteria for the positive and negative agonistic and antagonistic reference standards and control chemicals in Table 4.5.4.

Raw data, demonstrating that acceptance criteria are met, must be documented.

4.5 Determination of agonistic effect of test chemicals

Note: - Do not expose more than 6 microtiter plates (11 test chemicals) at a time!
- In case more test chemicals have to be evaluated on a single day, work in series of 6 microtiter plates (11 test chemicals).
- In the procedures given below, the volume of various media to be prepared, is sufficient for 6 microtiter plates (11 test chemicals).

4.5.1 Preparing the test chemical-work solution

4.5.1.1 Fill suitable polypropylene (or glass) tubes with assay medium. The amount of test chemical-work solution to be prepared per test concentration, is given in Table 4.5.3.

4.5.1.2 For each of the test concentrations of test chemicals (4.2 (pre-screening); 4.3 (assessment of agonistic effects)), pipette the required volume of test chemical into the amount of assay medium prepared (see Table 4.5.3).

4.5.1.3 Prepare sufficient solvent control-work solution by pipetting the required volume of solvent into the amount of assay medium prepared (see Table 4.5.3 (SC solvent test chemical)).

4.5.1.4 Carefully mix this agonistic test chemical-work solution and solvent control-work solution for at least 10 minutes (300 rpm).

4.5.2 Preparing the agonistic reference-work solution

4.5.2.1 A dilution series of agonistic reference standard has to be prepared in assay medium. In Table 4.5.1, the required dilution series of the agonistic reference standard is given. In Figure 4.5.1 the final position of the reference standard dilution series on the 96 well plate, is indicated.

4.5.2.2 Fill suitable polypropylene (or glass) tubes with assay medium. The amount of agonistic reference-work solution to be prepared per reference concentration, is given in Table 4.5.3.

4.5.2.3 Add the required volume of each reference concentration into the amount of assay medium pipetted.

4.5.2.4 Prepare the solvent reference work solution (C0) by pipetting the required volume of solvent into the amount of assay medium prepared (see Table 4.5.3 (solvent reference)).

4.5.2.5 Carefully mix this agonistic reference-work solutions and solvent reference work solution for at least 10 minutes (300 rpm).

ANNEX A

Table 4.5.1 Dilution series of reference standard for the agonistic CALUX® bioassay

Position on 96 well plate	ERα CALUX® 17beta-estradiol (E2) conc. in stock (M)	conc. in well (M)
C0	0	0
C1	$1.0*10^{-11}$	1.0*10-13
C2	$3.0*10^{-11}$	3.0*10-13
C3	$1.0*10^{-10}$	1.0*10-12
C4	$3.0*10^{-10}$	3.0*10-12
C5	$6.0*10^{-10}$	6.0*10-12
C6	$1.0*10^{-09}$	1.0*10-11
C7	$3.0*10^{-09}$	3.0*10-11
C8	$1.0*10^{-08}$	1.0*10-10

Note: The stock of C0 contains solvent (e.g. DMSO) only.

Table 4.5.2 Concentrations of positive control (PC) and negative control (NC) for the agonistic CALUX® bioassay

ERα CALUX® – positive control (PC) 17α-methyltestosterone		ERα CALUX® – negative control (NC) corticosterone	
conc. in stock (M)	conc. in well (M)	conc. in stock (M)	conc. in well (M)
$3.0*10^{-04}$	$3.0*10^{-06}$	$1.0*10^{-06}$	$1.0*10^{-08}$

Table 4.5.3 Guidelines for preparing the various work solutions (1% DMSO) sufficient for 1 analysis series of 6 plates (11 test chemicals).

Chemical	Amount assay medium	Volume of chemical per test concentration
Reference standard (C1-C8)	1 ml	10 µl
C0 (solvent reference standard)	1 ml	10 µl (solvent only)
PC	1 ml	10 µl
NC	1 ml	10 µl
Test chemical	1 ml	10 µl
SC (solvent test chemical)	8.0 ml	80 µl (solvent only)
C4 (EC_{50} Ref)	4.0 ml	40 µl
C8 (Max Ref)	4.0 ml	40 µl

Note 1: For pre-screening and the assessment of agonistic effects of test chemicals, various work solutions have to be prepared. In the table, the amount of work solutions for the analysis of a series of 6 plates (11 test chemicals) is given.

Reference standard = 17beta-estradiol
PC (positive control) = 17α-methyltestosterone
NC (negative control) = corticosterone
C4 = appr. EC_{50} of reference standard ($3.0*10^{-12}$ M in well)
C8 = concentration of reference standard ($1.0*10^{-10}$ M in well) inducing maximum luciferase activity

Note 2: In case DMSO is not the solvent used for dissolving test chemicals, the solvent control (C0 or SC) should contain the solvent used instead of DMSO.

ANNEX A

4.5.3 Preparing the agonistic positive control (PC) and negative control (NC)-work solution

4.5.3.1 In each series of analyses, a positive and negative control is measured. In Table 4.5.2, the required concentrations of PC and NC are indicated.

4.5.3.2 Fill suitable polypropylene (or glass) tubes with assay medium. The amount of PC- and NC-work solution to be prepared, is given in Table 4.5.3.

4.5.3.3 Add the required volume of PC and NC stock solution into the amount of assay medium pipetted.

4.5.3.4 Carefully mix these agonistic PC and NC-work solutions for at least 10 minutes (300 rpm).

4.5.4 Filling the microtiter plate for agonistic pre-screening

4.5.4.1 Take the seeded microtiter plate from the incubator (see 4.4.4).

4.5.4.2 Check the cells for visible cytotoxicity, contamination and cell confluence.

4.5.4.3 Carefully remove the medium from the top 30 wells (wells B2-D11).

4.5.4.4 Add 200 µl of the lowest concentration of the reference-work solution (C0) to three wells according to the scheme in Figure 4.5.1. Continue with the reference-work solution (C1) until all the reference concentrations (C0-C8) are transferred to the microtiter plate.

4.5.4.5 Add 200 µl of the positive control-work solution (PC) to the wells as indicated in the scheme in Figure 4.5.1.

4.5.4.6 Carefully remove the medium from the bottom 30 wells (wells E2-G11).

4.5.4.7 Add 200 µl of the solvent test chemical-work solution (SC) to the wells as indicated in the scheme in Figure 4.5.1.

4.5.4.8 Add 200 µl of each concentration of the test chemical-work solution prepared for prescreening to the appropriate wells as indicated in Figure 4.5.1.. Use a new tip for every sample.

4.5.4.9 Add 200 µl of the negative control-work solution (NC) to the appropriate wells (see Figure 4.5.1).

4.5.4.10 Repeat all handlings for all subsequent plates to be analysed, following the plates setup as given in Fig 4.5.1, (subsequent plates).

4.5.4.11 Use FRM01 to register exposure information.

4.5.4.12 Place the microtiter plate(s) in the CO_2-incubator and incubate for 24 hours +/- 2 hours.

4.5.4.13 Proceed with removal of 100 µl medium for cytotoxicity testing (section 4.7), harvesting cells (4.5.6), measurement of luciferase activity (4.5.7) and analysis of results (4.5.8).

4.5.4.14 Determine the proper concentration range for assessing the agonistic potency as described in Appendix C (or use calculation document DAT04).

ANNEX A

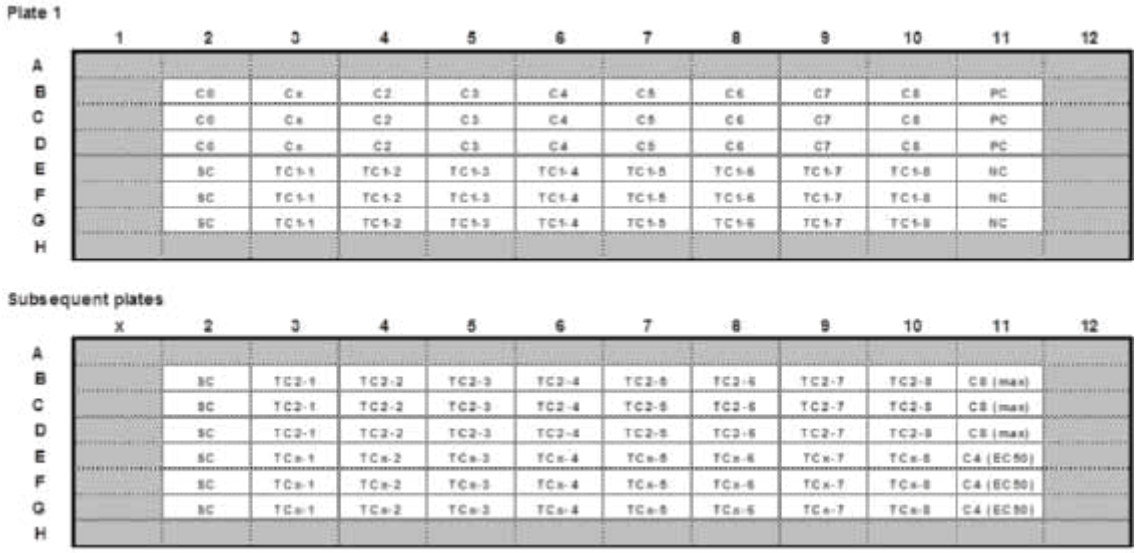


Figure 4.5.1. Plate Lay-out of the of the 96 microtiter plates for prescreening and assessment of agonistic effect.

C0 = solvent reference standard. Work solution containing solvent of reference standard only.

C(1-8) = concentrations (1-8) of reference-work solution.

PC = positive control.

NC = negative control

TCx-(1-8) = dilutions (1-8) of test chemical-work solution for the pre-screen and assessment of agonistic effect of test chemical x.

SC = Solvent control of the test chemical evaluated on the same rows. Work solution containing solvent for test chemical x only.

4.5.5 Filling the microtiter plate for assessment of agonistic effect

4.5.5.1 Take the seeded microtiter plate from the incubator (see 4.4.4).

4.5.5.2 Check the cells for visible cytotoxicity, contamination and cell confluence

4.5.5.3 Carefully remove the medium from the top 30 wells (wells B2-D11).

4.5.5.4 Add 200 µl of the lowest concentration of the reference-work solution (C0) to three wells according to the scheme in Figure 4.5.1. Continue with the reference-work solution (C1) until all the reference concentrations (C0-C8) are transferred to the microtiter plate.

4.5.5.5 Add 200 µl of the positive control-work solution (PC) to the wells as indicated in the scheme in Figure 4.5.1.

4.5.5.6 Carefully remove the medium from the bottom 30 wells (wells E2-G11).

4.5.5.7 Add 200 µl of the solvent test chemical-work solution (SC) to the wells as indicated in the scheme in Figure 4.5.1.

4.5.5.8 Add 200 µl of each concentration of the test chemical-work solution prepared for assessment of agonistic effect to the appropriate wells as indicated in Figure 4.5.1.. Use a new tip for every sample.

4.5.5.9 Add 200 µl of the negative control-work solution (NC) to the appropriate wells (see Figure 4.5.1.).

4.5.5.10 Repeat all handlings for all subsequent plates to be analysed, following the plates setup as given in Fig 4.5.1, (subsequent plates).

4.5.5.11 Place the microtiter plate(s) in the CO_2-incubator and incubate for 24 hours +/- 2 hours.

4.5.5.12 Proceed with removal of 100 µl medium for cytotoxicity testing (section 4.7), harvesting cells (4.5.6), measurement of luciferase activity (4.5.7) and analysis of results (4.5.8).

4.5.6 Harvesting the cells

Note: If cytotoxicity testing is required, ensure that before lysing the cells the appropriate amount of medium is transferred to a fresh 96-well plate for cytotoxicity testing.

4.5.6.1 Check the cells for signs of cytotoxicity and/or contamination under a microscope and mark the wells accordingly on the plate and on form FRM04.
4.5.6.2 Carefully remove all (remaining) medium from the cells.
4.5.6.3 Add 30 µl lysis reagent to the cells in each well.
4.5.6.4 Shake the plate for at least five minutes (300 rpm) without heating the plate.
4.5.6.5 Measure the plates the same day or store the plates at -20°C for a maximum of 4 weeks.

4.5.7 Measuring the luciferase activity.

Note: For this procedure a luminometer equipped with 2 injectors is required. In case the equipment has only one injector, making the stopping with 0.2 M NaOH impossible, additional experiments must be performed demonstrating that there is no carry over of luminescence from one well to the other.

4.5.7.1 Make sure the BDS illuminate-mix and the plates are at room temperature (<30°C).
4.5.7.2 Start the measuring program of the luminometer.
4.5.7.3 Prime the appropriate tubes of the luminometer with BDS illuminate-mix and 0.2 M NaOH.
4.5.7.4 Place the microtiter plate without lid in the luminometer. Make the microtiter plate is orientated correctly.
4.5.7.5 Enter the name of the plate and start the measurement.
Each measurement of a well consists of the injection of 100 µl BDS illuminate-mix, the measurement of emitted light for 4 seconds and injection of 100 µl NaOH to stop the reaction.
4.5.7.6 After the last measurement, empty the tubes and prime/wash them with demineralised water or follow the instructions of the manufacturer.
4.5.7.7 Print the raw data and save the electronic raw data file.

4.5.8 Analysis of results

4.5.8.1 Transfer the raw relative light units (RLU) measured for each of the analysed microtiter plates into the data analysis form DAT02 .
4.5.8.2 Transfer the plate setup used for all plates analyses into the form.
4.5.8.3 For all concentrations of the reference (17ß-estradiol), the average solvent blank of the reference (C0) is subtracted in the form.
4.5.8.4 For all test chemical concentrations, the average solvent control of the samples (SC) is subtracted in the form.
4.5.8.5 Calculate the relative induction of each concentration of the reference; set the induction of the highest concentration of the reference (C8) at 100% (REF_{max}).
4.5.8.6 Calculate the relative induction of each concentration the test chemicals using the highest concentration of the reference as 100% (REF_{max}).

4.5.8.7 Use scientific data analysis software (such as GraphPad Prism) for non-linear regression (variable slope, 4 parameters) and evaluation of data according to the following equation:

Error! Objects cannot be created from editing field codes.

X =	Log of dose or concentration
Y =	Response (relative induction (%))
Top =	Maximum induction (%)
Bottom =	Minimum induction (%)
LogEC50 =	Log of concentration at which 50% of maximum response is observed
HillSlope =	Slope factor of Hill slope

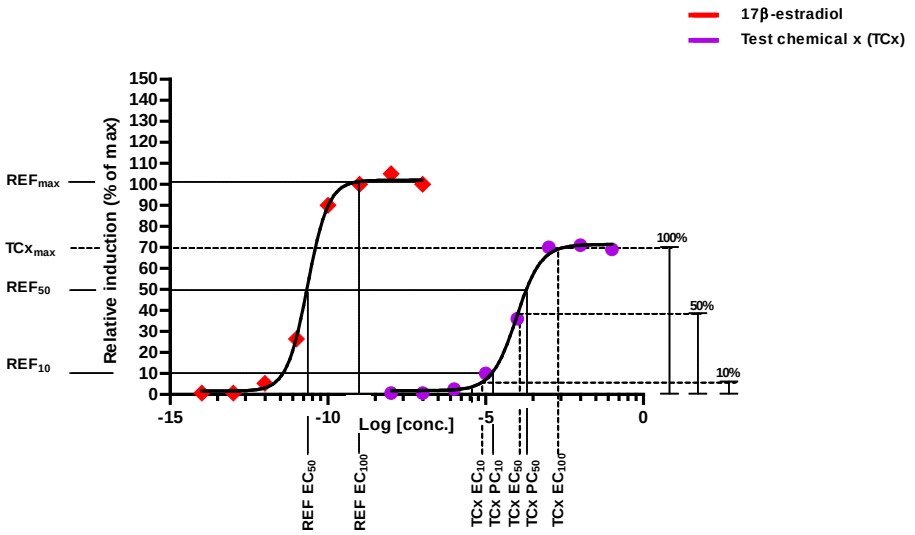


Figure 4.5.2 Overview of parameters determined in the agonist assay.

4.5.8.8 Determine the EC_{50} and EC_{10} of the reference.

4.5.8.9 Determine the EC_{50} and EC_{10} of the test chemical.

4.5.8.10 Determine the maximum relative induction of the test chemical (TC_{max}) using REF_{max} (= 100%).

4.5.7.11 Determine the concentration of the test chemical at which its maximum relative induction (TC_{max}) is reached. This test concentration is indicated at the RPC_{max}.

4.5.7.12 Determine the concentration of the test chemical at which the response of the reference is 50% (REF_{50}). This test concentration is indicated at the PC_{50}.

4.5.8.13 Determine the concentration of the test chemical at which the response of the reference is 10% (REF_{10}). This test concentration is indicated at the PC_{10}.

4.5.8.14 For the reference standard, register the EC_{50} and EC_{10} (form DAT02).

4.5.8.15 For the test chemicals, register the EC_{50}, EC_{10}, PC_{50}, PC_{10}, RPC_{max} and TC_{max} (form DAT02).

Note: For test chemicals, a full dose-response curve may not always be achieved due to e.g. cytotoxicity or solubility problems. Hence, the EC_{50}, EC_{10} and PC_{50} can not be determined. In such case, only the TC_{max}, PC_{10} and RPC_{max} can be determined and registered.

4.5.9 Agonistic data interpretation criteria

ANNEX A

For the interpretation of data and the decision whether a test chemical is considered positive or negative, the following criteria are to be used:

For each pre-screen or comprehensive run, a test chemical is considered **positive** in case:

1 The TC_{max} is equal or exceeds 10% of the maximum response of the reference standard (REF_{10}).

2 At least 2 consecutive concentrations of the test chemical are equal to or exceed the REF_{10}.

For each pre-screen or comprehensive run, a test chemical is considered **negative** in case:

1 The TC_{max} does not exceeds 10% of the maximum response of the reference standard (REF_{10}).

2 Less than 2 concentrations of the test chemical are equal to or exceed the REF_{10}.

To characterise the potency and magnitude of the positive response of a test chemical, the TC_{max}, RPC_{max} and the PC_{10} and if possible the PC_{50} have to be registered.

4.5.10 Performance criteria and quality control.

In Table 4.5.4, acceptance criteria for the agonistic analysis with ERα CALUX® cells are stated. When these criteria are not met, the relevant experiment must be considered as invalid. When the acceptance criteria are consistently not met, investigate the cause systematically.

Table 4.5.4 Acceptance criteria for the agonistic ERα CALUX® bioassay.

	individual samples on a plate	**Criterium**
1	Maximum %SD of triplicate wells (reference standard, test chemical, PC, NC)	< 15%
2	Maximum %SD of triplicate wells (solvent controls (C0, SC)	< 30%
3	Maximum LDH leakage, as a measure of cytotoxicity.	< 120%
	within a single microtiter plate	
4	Ratio of the reference standard solvent control (C0; plate 1) and test chemical solvent control (SC; plates 2 to x)	0.5 to 2.0
5	Ratio of the reference concentrations C4 & C8 (plate 1) and reference concentrations C4 & C8 (plates 2 to x)	0.70 to 1.30
6	Z-factor for each plate	>0.6
	within a single series of analyses (all plates within one series)	
7	Sigmoidal curve of reference standard	Yes (17ß-estradiol)
8	EC_{50} range reference standard 17ß-estradiol	$4*10^{-12}$ – $4*10^{-11}$ M
9	Minimum fold induction of the highest 17ß-estradiol concentration, with respect to the reference solvent control.	5
10	Relative induction (%) PC.	> 30%
11	Relative induction (%) NC	<10%

ANNEX A

4.6 Determination of antagonistic effect of test chemicals

Note: - Do not expose more than 6 microtiter plates (11 test chemicals) at a time!
- In case more test chemicals have to be evaluated on a single day, work in series of 6 microtiter plates (11 test chemicals).
- In the procedures given below, the volume of various media to be prepared, is sufficient for 6 microtiter plates (11 test chemicals).

4.6.1 Preparing agonist supplemented assay medium

4.6.1.1 Prepare 100 ml of assay medium containing 10 µl (0.01%) of $3.0*10^{-8}$ M 17β-estradiol stock solution (= 100x stock EC_{50} of the agonist reference standard):
- The $3.0*10^{-8}$ M 17β-estradiol solution is the stock solution for preparing the highest 17β-estradiol concentration of the reference series (C8).

Hundred ml of agonist supplemented assay medium is sufficient for 6 96-well microtiter plate.

4.6.2 Preparing vehicle control-work solution

4.6.2.1 Fill suitable polypropylene (or glass) tubes with assay medium (**not agonist supplemented assay medium!**). The amount of vehicle control-work solution (VC) to be prepared, is given in Table 4.6.3.

4.6.2.3 Pipette the required volume of solvent into the amount of assay medium prepared (see Table 4.6.3 (VC)).

4.6.2.4 Carefully mix this vehicle control-work solution for at least 10 minutes (300 rpm).

4.6.3 Preparing the test chemical-work solution

4.6.3.1 Fill suitable polypropylene (or glass) tubes with agonist supplemented assay medium. The amount of test chemical-work solution to be prepared per test concentration, is given in Table 4.6.3.

4.6.3.2 For each of the test concentrations of test chemicals (4.2 (pre-screen); 4.3 (assessment of antagonistic effects)), pipette the required volume of test chemical into the amount of agonist supplemented assay medium prepared (see Table 4.6.3).

4.6.3.3 Prepare sufficient solvent control-work solution by pipetting the required volume of solvent control into the amount of agonist supplemented assay medium prepared (see Table 4.6.3 (SC test chemical)).

4.6.3.4 Carefully mix this antagonistic test chemical-work solution and solvent control-work solution for at least 10 minutes (300 rpm).

4.6.4 Preparing the antagonistic reference-work solution

4.6.4.1 A dilution series of antagonistic reference standard has to be prepared in agonist supplemented assay medium. In Table 4.6.1, the required dilution series of the antagonistic reference standard is given. In Figure 4.6.1 the final position of the antagonistic reference standard dilution series on the 96 well plate, is indicated.

4.6.4.2 Fill suitable polypropylene (or glass) tubes with agonist supplemented assay medium. The amount of antagonistic reference-work solution to be prepared per reference concentration, is given in Table 4.6.3..

4.6.4.3 Add the required volume of each antagonistic reference concentration into the amount of agonist supplemented assay medium prepared.

ANNEX A

4.6.4.4 Prepare the solvent reference work solution (C0) by pipetting the required volume of solvent into the amount of assay medium prepared (see Table 4.5.3 (C0; solvent reference)).

4.6.4.5 Carefully mix this antagonistic reference-work solution and solvent reference work solution for at least 10 minutes (300 rpm).

Table 4.6.1 Dilution series of antagonistic reference standard for the antagonistic CALUX® bioassay

Position on 96 well plate	anti-ERα CALUX® Tamoxifen	
	conc. in stock (M)	conc. in well (M)
C0	0	0
C1	$3.0*10^{-07}$	$3.0*10^{-09}$
C2	$1.0*10^{-06}$	$1.0*10^{-08}$
C3	$3.0*10^{-06}$	$3.0*10^{-08}$
C4	$1.0*10^{-05}$	$1.0*10^{-07}$
C5	$3.0*10^{-05}$	$3.0*10^{-07}$
C6	$1.0*10^{-04}$	$1.0*10^{-06}$
C7	$3.0*10^{-04}$	$3.0*10^{-06}$
C8	$1.0*10^{-03}$	$1.0*10^{-05}$

Note: The stock of C0 contains solvent (e.g. DMSO) only.

Table 4.6.2 Concentrations of positive control (PC) and negative control (NC) for the antagonistic CALUX® bioassay

anti-ERα CALUX® – positive control (PC) 4-hydroxytamoxifen		anti-ERα CALUX® – negative control (NC) Resveratrol	
conc. in stock (M)	conc. in well (M)	conc. in stock (M)	conc. in well (M)
$1.0*10^{-07}$	$1.0*10^{-09}$	$1.00*10^{-03}$	$1.00*10^{-05}$

Table 4.6.3 Guidelines for preparing the various work solutions (1% DMSO) sufficient for 1 analysis series of 6 plates (11 test chemicals).

Chemical	Amount agonist supplemented assay medium	Volume of chemical per test concentration
Reference standard (C1-C8)	1 ml	10 µl
C0 (solvent reference standard)	1 ml	10 µl (solvent only)
PC	1 ml	10 µl
NC	1 ml	10 µl
Test chemical	1 ml	10 µl
SC (solvent test chemical)	4 ml	40 µl (solvent only)
C4 (IC_{50} Ref)	4 ml	40 µl
C8 (IC_{100} Ref)	4 ml	40 µl
VC (vehicle control)	1 ml (assay medium, not agonist supplemented assay medium !)	10 µl (solvent only)

Note 1: For pre-screening and the assessment of antagonistic effects of test chemicals, various dose media have to be prepared. In the table, the amount of dose media for the analysis of a series of 6 plates (11 test chemicals) is given.

Reference standard = Tamoxifen
PC (positive control) = 4-Hydroxytamoxifen
NC (negative control) = Resveratrol
C4 = appr. IC_{50} of reference standard ($1.0*10^{-07}$ M in well)
C8 = concentration of reference standard ($1.0*10^{-05}$ M in well) inducing maximum inhibition

Note 2: In case DMSO is not the solvent used for dissolving test chemicals, the solvent control (C0, SC or VC) should contain the solvent used instead of DMSO.

ANNEX A

4.6.5 Preparing the antagonistic positive control (PC) and negative control (NC)-work solutions

4.6.5.1 In each series of analyses, a positive and negative control is measured. In Table 4.6.2., the required concentrations of PC and NC are indicated.

4.6.5.2 Fill suitable polypropylene (or glass) tubes with agonist supplemented assay medium. The amount of PC- and NC-work solution to be prepared, is given in Table 4.6.3.

4.6.5.3 Add the required volume of PC and NC stock solution into the amount of agonist supplemented assay medium pipetted.

4.6.5.4 Carefully mix this antagonistic PC- or NC-work solution for at least 10 minutes (300 rpm).

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		C0	Cx	C2	C3	C4	C5	C6	C7	C8	VC	
C		C0	Cx	C2	C3	C4	C5	C6	C7	C8	VC	
D		C0	Cx	C2	C3	C4	C5	C6	C7	C8	VC	
E		NC	TC1-1	TC1-2	TC1-3	TC1-4	TC1-5	TC1-6	TC1-7	TC1-8	PC	
F		NC	TC1-1	TC1-2	TC1-3	TC1-4	TC1-5	TC1-6	TC1-7	TC1-8	PC	
G		NC	TC1-1	TC1-2	TC1-3	TC1-4	TC1-5	TC1-6	TC1-7	TC1-8	PC	
H												

Subsequent plates

	x	2	3	4	5	6	7	8	9	10	11	12
A												
B		SC	TC2-1	TC2-2	TC2-3	TC2-4	TC2-5	TC2-6	TC2-7	TC2-8	C8 (max)	
C		SC	TC2-1	TC2-2	TC2-3	TC2-4	TC2-5	TC2-6	TC2-7	TC2-8	C8 (max)	
D		SC	TC2-1	TC2-2	TC2-3	TC2-4	TC2-5	TC2-6	TC2-7	TC2-8	C8 (max)	
E		C4 (IC50)	TCx-1	TCx-2	TCx-3	TCx-4	TCx-5	TCx-6	TCx-7	TCx-8	C8 (max)	
F		C4 (IC50)	TCx-1	TCx-2	TCx-3	TCx-4	TCx-5	TCx-6	TCx-7	TCx-8	C8 (max)	
G		C4 (IC50)	TCx-1	TCx-2	TCx-3	TCx-4	TCx-5	TCx-6	TCx-7	TCx-8	C8 (max)	
H												

Figure 4.6.1. Plate Lay-out of the of the 96 microtiter plates for antagonistic prescreening and assessment of antagonistic effect.

- C0 = solvent reference standard. Work solution containing solvent of reference standard only.
- C(1-8) = concentrations (1-8) of reference-work solution.
- NC = negative control.
- PC = positive control.
- TCx-(1-8) = dilutions (1-8) of test chemical-work solution for the pre-screen and assessment of agonistic effect of test chemical x.
- SC = Solvent control of the test chemical. Work solution containing solvent for test chemical x only.
- VC = Vehicle control. Work solution prepared using assay medium (not agonist supplemented assay medium!) containing solvent for only.

4.6.6 Filling the microtiter plate for antagonistic prescreening

4.6.6.1 Take the seeded microtiter plate from the incubator (see 4.4.4).

4.6.6.2 Check the cells for visible cytotoxicity, contamination and cell confluence.

4.6.6.3 Carefully remove the medium from the top 30 wells (wells B2-D11).

4.6.6.4 Add 200 µl of the lowest concentration of the antagonistic reference-work solution (C0) to three wells according to the scheme in Figure 4.6.1. Continue with the

antagonistic reference-work solution (C1) until all the antagonistic reference concentrations are transferred to the microtiter plate.

4.6.6.5 Add 200 µl of the vehicle control-work solution (VC) to the wells as indicated in the scheme in Figure 4.6.1.

4.6.6.6 Carefully remove the medium from the bottom 30 wells (wells E2-H11).

4.6.6.7 Add 200 µl of the negative control-work solution (NC) to the appropriate wells (see Figure 4.6.1.).

4.6.6.8 Add 200 µl of each concentration of the test chemical-work solution prepared for antagonistic prescreening to the appropriate wells as indicated in Figure 4.6.1.. Use a new tip for every sample.

4.6.6.9 Add 200 µl of the positive control-work solution (PC) to the appropriate wells (see Figure 4.6.1.).

4.6.6.10 Repeat all handlings for all subsequent plates to be analysed, following the plates setup as given in Fig 4.6.1 (subsequent plates).

4.6.6.11 Place the microtiter plate(s) in the CO_2-incubator and incubate for 24 hours +/- 2 hours.

4.6.6.12 Proceed with removal of 100 µl medium for cytotoxicity testing (section 4.7), harvesting cells (4.6.8), measurement of luciferase activity (4.6.9) and analysis of results (4.6.10).

4.6.6.13 Determine the proper concentration range for assessing the antagonistic potency as described in Appendix C (or use calculation document DAT05).

4.6.7 Filling the microtiter plate for assessment of antagonistic effect

4.6.7.1 Take the seeded microtiter plate from the incubator (see 4.4.4).

4.6.7.2 Check the cells for visible cytotoxicity, contamination and cell confluence.

4.6.7.3 Carefully remove the medium from the top 30 wells (wells B2-D11).

4.6.7.4 Add 200 µl of the lowest concentration of the antagonistic reference-work solution (C0) to three wells according to the scheme in Figure 4.6.1. Continue with the antagonistic reference-work solution (C1) until all the antagonistic reference concentrations (C0-C8) are transferred to the microtiter plate.

4.6.7.5 Add 200 µl of the vehicle control-work solution (VC) to the wells as indicated in the scheme in Figure 4.6.1.

4.6.7.6 Carefully remove the medium from the bottom 30 wells (wells E2-H11).

4.6.7.7 Add 200 µl of the negative control-work solution (NC) to the appropriate wells (see Figure 4.6.1.).

4.6.7.8 Add 200 µl of each concentration of the test chemical-work solution prepared for antagonistic prescreening to the appropriate wells as indicated in Figure 4.6.1.. Use a new tip for every sample.

4.6.7.9 Add 200 µl of the positive control-work solution (PC) to the appropriate wells (see Figure 4.6.1.).

4.6.7.10 Repeat all handlings for all subsequent plates to be analysed, following the plates setup as given in Fig 4.6.1 (subsequent plates).

4.6.7.11 Place the microtiter plate(s) in the CO_2-incubator and incubate for 24 hours +/- 2 hours.

4.6.7.12 Proceed with removal of 100 µl medium for cytotoxicity testing (section 4.7), harvesting cells (4.6.8), measurement of luciferase activity (4.6.9) and analysis of results (4.6.10).

ANNEX A

4.6.8 Harvesting the cells

Note: If cytotoxicity testing is required, ensure that before lysing the cells the appropriate amount of medium is transferred to a fresh 96-well plate for cytotoxicity testing.

4.6.8.1 Check the cells for signs of cytotoxicity and/or contamination under a microscope and mark the wells accordingly on the plate and on form FRM04.
4.6.8.2 Carefully remove all (remaining) medium from the cells.
4.6.8.3 Add 30 µl lysis reagent to the cells in each well.
4.6.8.4 Shake the plate for at least five minutes (300 rpm) without heating the plate.
4.6.8.5 Measure the plates the same day or store the plates at -20^{o}C for a maximum of 4 weeks.

4.6.9 Measuring the luciferase activity.

Note: For this procedure a luminometer equipped with 2 injectors is required. In case the equipment has only one injector, making the stopping with 0.2 M NaOH impossible, additional experiments must be performed demonstrating that there is no carry over of luminescence from one well to the other.

4.6.9.1 Make sure the BDS illuminate-mix and the plates are at room temperature (<30°C).
4.6.9.2 Start the measuring program of the luminometer.
4.6.9.3 Prime the appropriate tubes of the luminometer with BDS illuminate-mix and 0.2 M NaOH.
4.6.9.4 Place the microtiter plate without lid in the luminometer. Make the microtiter plate is orientated correctly.
4.6.9.5 Enter the name of the plate and start the measurement.
Each measurement of a well consists of the injection of 100 µl BDS illuminate-mix, the measurement of emitted light for 4 seconds and injection of 100 µl NaOH to stop the reaction.
4.6.9.6 After the last measurement, empty the tubes and prime/wash them with demineralised water or follow the instructions of the manufacturer.
4.6.9.7 Print the raw data and save the electronic raw data file.

4.6.10 Analysis of results.

4.6.10.1 Transfer the raw relative light units (RLU) measured for each of the analysed microtiter plates into the data analysis form DAT03.
4.6.10.2 Transfer the plate setup used for all plates analyses into the form.
4.6.10.3 For all antagonistic reference-dose concentrations, the average of the highest antagonistic reference concentration (C8) is subtracted in the form.
4.6.10.4 For all test chemical concentrations, the average of the highest antagonistic reference concentration (C8) is subtracted in the form.
4.6.10.5 Calculate the relative induction of each concentration of the reference; set the induction of the lowest concentration of the reference (C0) at 100% (REF_{max}).
4.6.10.6 Calculate the relative induction of each concentration the test chemicals using using REF_{max} (= 100%).
4.6.10.7 Use statistical software (such as GraphPad Prism) for non-linear regression (variable slope, 4 parameters; tick option Automatic outlier elimination) and evaluation of data according to the following equation:

ANNEX A

Error! Objects cannot be created from editing field codes.

X =	Log of dose or concentration
Y =	Response (relative induction (%))
Top =	Maximum induction (%)
Bottom =	Minimum induction (%)
LogEC50 =	Log of concentration at which 50% of maximum response is observed
HillSlope =	Slope factor of Hill slope

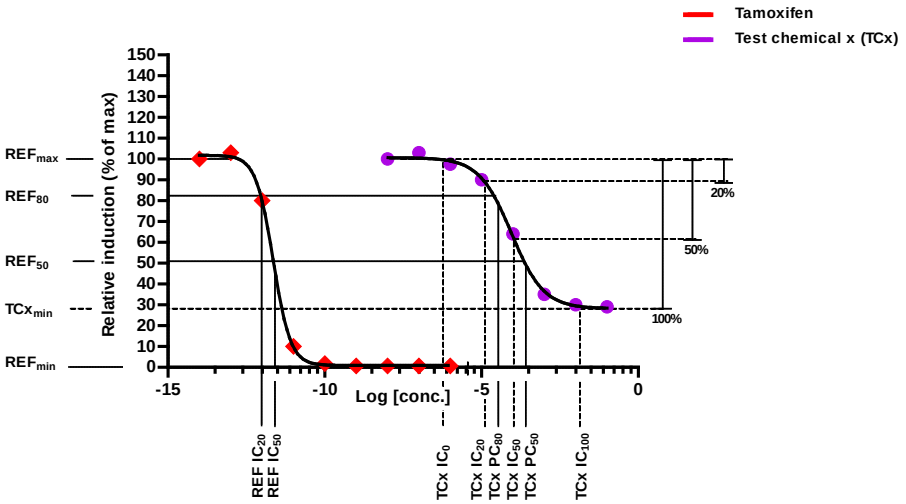


Figure 4.6.2 Overview of parameters determined in the agonist assay.

4.6.10.8 Determine the IC_{50} and IC_{20} of the reference.

4.6.10.9 Determine the IC_{50} and IC_{20} of the test chemical.

4.6.10.10 Determine the minimum relative induction of the test chemical (TC_{min} = maximum inhibition) using REF_{max} (= 100%).

4.5.7.11 Determine the concentration of the test chemical at which its minimum relative induction (TC_{min}) is reached. This test concentration is indicated at the RPC_{min}.

4.5.7.12 Determine the concentration of the test-chemical at which the response of the reference is 50% (REF_{50}). This test concentration is indicated at the PC_{50}.

4.6.10.12 Determine the concentration of the test chemical at which the response of the reference is 80% (REF_{80}). This test concentration is indicated at the PC_{20}.

4.6.10.13 For the reference standard, register the IC_{50} and IC_{20} (form DAT03).

4.6.10.14 For the test chemicals, register the IC_{50}, IC_{20}, PC_{50}, PC_{20}, RPC_{min} and TC_{min} (form DAT03).

Note: For test chemicals, a full dose-response curve may not always be achieved due to e.g. cytotoxicity or solubility problems. Hence, the IC_{50}, IC_{20} and PC_{50} can not be determined. In such case, only the PC_{20}, RPC_{min}, and TC_{min} can be determined and registered.

ANNEX A

4.6.11 Antagonistic data interpretation criteria

For the interpretation of data and the decision whether a test chemical is considered positive or negative, the following criteria are to be used:

For each pre-screen and comprehensive run, a test chemical is considered **positive** in case:

1 The TC_{min} is equal or lower than 80% of the maximum response of the reference standard (REF_{80} = 20% inhibition).

2 At least 2 consecutive concentrations of the test chemical are equal to or lower than the REF_{80}.

For each pre-screen and comprehensive run, a test chemical is considered **negative** in case:

1 The TC_{min} exceeds 80% of the maximum response of the reference standard (REF_{80} = 20% inhibition).

2 Less than 2 concentrations of the test chemical are equal to or lower than the REF_{80}.

To characterise the potency and magnitude of the positive response of a test chemical, the TC_{min}, RPC_{min}, PC_{20} and if possible the PC_{50} have to be registered.

4.6.12 Performance criteria and quality control.

In Table 4.6.4 acceptance criteria for the agonistic and antagonistic analysis with ERα CALUX® cells are stated. When these criteria are not met, the relevant experiment must be considered as invalid. When the acceptance criteria are consistently not met, investigate the cause systematically.

Table 4.6.4 Acceptance criteria for the antagonistic ERα CALUX® bioassay.

individual samples on a plate		**Criterium**
1	Maximum %SD of triplicate wells (reference standard (C0-C7), test chemical, PC, NC, SC)	< 15%
2	Maximum %SD of triplicate wells (vehicle control (VC), reference (C8))	< 30%
3	Maximum LDH leakage, as a measure of cytotoxicity.	< 120%
within a single microtiter plate		
4	Ratio of the reference standard solvent control (C0; plate 1) and test chemical solvent control (SC; plates 2 to x)	0.70 to 1.30
5	Ratio of the reference concentrations C4 (plate 1) and reference concentrations C4 (plates 2 to x)	0.70 to 1.30
6	Ratio of the reference concentrations C8 (plate 1) and reference concentrations C8 (plates 2 to x)	0.50 to 2.0
7	Z-factor for each plate	>0.6
within a single series of analyses (all plates within one series)		
8	Sigmoidal curve of reference standard	Yes (Tamoxifen)
9	IC_{50} range reference standard Tamoxifen	$1*10^{-8}$ - $1*10^{-7}$ M
10	Minimum fold induction of the reference solvent control, with respect to the highest Tamoxifen concentration.	2.5
11	Relative induction (%) PC.	<70%
12	Relative induction (%) NC	>85%

ANNEX A

4.7 Cytotoxicity testing

Note: In addition to the visual inspection of cells, LDH leakage must be measured.The procedure described utilises the LDH kit from Roche. Please refer to the suppliers manual in case another LDH kit is used. The enzyme reaction must take place at room temperature for all solutions.

4.7.1 Following exposure, remove exposed microtiter plate(s) from the CO_2 incubator, visually inspect the microtiter plates for cytotoxcity and check for cloudy wells as an indicator of bacterial infection.

4.7.2 Prepare a LDH leakage test plate by transferring 100 µl exposure medium of relevant triplicate test-wells to a new clear 96-well microtiter plate.

4.7.3 Reconstitute solution 1 “catalyst” with 1 ml demineralized water for 10 minutes and mix thoroughly (the reconstituted solution is stable for several weeks if stored at 2-8^{o}C).

4.7.4 Thaw 1 bottle of 45 ml solution 2 “dye solution” (the dye solution is stable for several weeks if stored at 2-8^{o}C).

4.7.5 For 100 wells: mix shortly before use 250 µl of solution 1 with 11.25 ml of solution 2 (this reaction mixture can not be stored and should be used immediately).

4.7.6 Add 100 ul reaction mixture to every well of the LDH leakage test plate.

4.7.7 Incubate in the dark and measure the OD490 every 10 minutes until the OD490 values for the solvent control wells (wells B2-D2 and E11-G11 for reference standards and test chemicals respectively; see figures 4.5.1 and 4.6.1) are higher than 0.3.

4.7.8 Determine the absorbance at 490 nm (+/-80).

4.7.9 Calculate the average OD values for triplicate solvent control wells and the average OD values of triplicate test wells.

4.7.10 Calculate the percentage LDH leakage by:

Error! Objects cannot be created from editing field codes.

4.7.11 The concentration of the chemical/sample is regarded cytotoxic when the percentage LDH leakage is higher than 120%.

Note: The relative SD for triplicate wells should be lower than 20%.

ANNEX A

Appendix A

General information

This protocol describes the ERα CALUX® method, a quantitative *in vitro* methods to measure (anti)estrogenicity of chemicals. In short, the principle of the method is as follows. Upon estrogen binding, the estrogen receptor becomes activated, and binds to recognition sequences in promoter regions of target genes, the so-called estrogen responsive elements (EREs). Repeats of these EREs have been linked to a minimal promoter element (the so-called TATA box) and the gene of an easily measurable protein (luciferase). The thus obtained reporter gene together with a gene coding for human ERα was stably introduced in human U2-OS cells (Sonneveld et al, 2005, Toxicol. Sci., 83, 136-48). In these cells the ligand-activated receptor by a so-called agonist will activate luciferase transcription, and the transcribed luciferase protein will emit light when a substrate is added. The signal dose-dependently increases as a result of increasing concentrations of ligand. The luciferase activity in cellular lysates is measured with a luminometer, allowing reliable, sensitive and quantitative measurements. To measure antagonistic activity of chemicals, cells are activated by a modest amount of agonist and dose-dependent repression of this signal is measured.

ANNEX A

Appendix B

Doubling time calculation

- Seed ERα CALUX® cells at 70000 cells/ml in 25 cm^2, 3 ml per flask. Count after 24h, 48h, 72h and 96h .
- Record the results on FRM02.
- Transfer the results to form DAT04, which calculates the approximate doubling time as follows.

For each 24 hour period, calculate the doubling time using the following formula (i.e. for the periods between 0 and 24 hours, 24 and 48 hours, 48 and 72 hours and 72 and 96 hours):

$$\text{Doubling time} = 24 \times \frac{\log_{10}(2)}{\log_{10}(conc_{high}) - \log_{10}(conc_{low})}$$

The approximate doubling time for the cell culture is the average of the four calculated doubling times.

ANNEX A

Appendix C

Selection of concentrations for assessment of agonistic and antagonistic effects.

Following removal of medium for cytotoxicity testing (see 4.7), harvesting cells (4.5.6), measurement of luciferase activity (4.5.7) and analysis of pre-screen analysis results (4.5.8), a refined series of concentrations is selected for further assessment of the agonistic and/or antagonistic potency of chemicals. For selecting the refined series of concentrations, the following procedure must be followed:

1. Determine cytotoxicity and agonistic/antagonistic effects.
2. When cytotoxicity is observed, the pre-screen procedure must be repeated with lower non-cytotoxic concentrations.
3. In case the pre-screen of test chemical x does not show a full-dose curve because the concentrations tested generate maximum induction (agonism) or maximum inhibition (antagonism), the pre-screen has to be repeated using lower concentrations of the test chemical (see figure C.1).

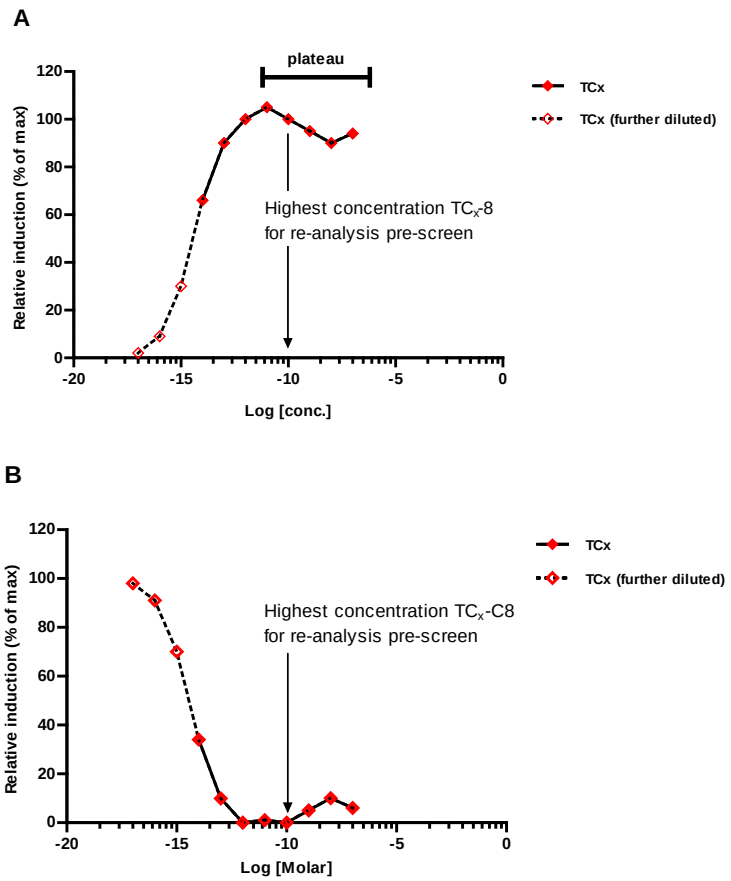


Figure C.1 Agonistic (A) and antagonistic (B) pre-screen analysis results not showing a full dose-response (solid line). The pre-screen has to be repeated using lower concentration of TCx until a full dose-response effect can be observed (dotted line).

4. When a dose-response curve can be generated, determine the concentration of the test chemical at which maximum induction (RPCmax; agonism) or inhibition (RPCmin; antagonism) occurs and does not show cytotoxicity yet (see figure C.2 and figure).
5. In case the concentration at which maximum induction or inhibition occurs is part of a plateau, select the lowest concentration tested that is part of the plateau (see figure C.3).

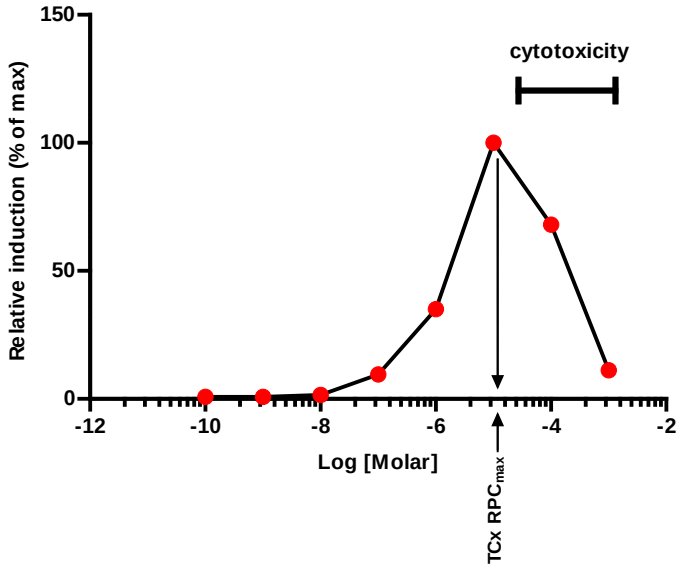


Figure C.2 Selection of the highest concentration of test chemical x (RPC_{max}) to be used for the selection of the dose-range for assessment of agonism following pre-screen.

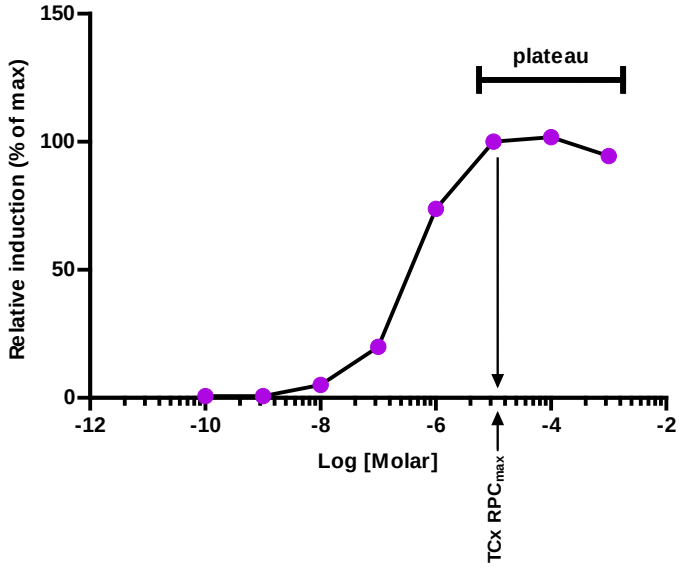


Figure C.3 Selection of the highest concentration of test chemical x (RPC_{max}) to be used for the selection of the dose-range for assessment of agonism following pre-screen.

6. Based on the RPC_{max} (agonism) or RPC_{min} (antagonism), 8 concentrations of test chemical x are selected: TCx-1; TCx-2; TCx-3; TCx-4; TCx-5; TCx-6; TCx-7; TCx-8.

ANNEX A

7. The highest concentration of test chemical (TCx-8) to be tested in the comprehensive runs, is 3-times the TCx-RPC_{max} (agonism) or 3-times the TCx-RPC_{min} (antagonism) determined in the pre-screen run.
   Example: During pre-screening, the TCx-RPC_{max} (agonism) was determined to be $1*10^{-09}$ M (in the well). Therefore, the highest concentration of test chemical x to be tested in the comprehensive runs (TCx-8) is:
   3 x $1*10^{-09}$ M = $3*10^{-09}$ M (in the well).
8. Starting with the highest concentration TCx-8 and fixed in between steps (1x, 3x, 10x, 30x, 100x, 300x, 1000x, 3000x), a complete dilution series for TCx can be prepared:
   TCx-8 = $3*10^{-09}$ M
   TCx-7 = $1*10^{-09}$ M
   TCx-6 = $3*10^{-10}$ M
   TCx-5 = $1*10^{-10}$ M
   TCx-4 = $3*10^{-11}$ M
   TCx-3 = $1*10^{-11}$ M
   TCx-2 = $3*10^{-12}$ M
   TCx-1 = $1*10^{-12}$ M

Note: The concentration range given above is the final concentration in the well. The dilution series in DMSO (stocks) have to be 100x more concentrated!

8. Dilutions passing the threshold of cytotoxicity or non-specific repression of reporter gene activity are not used in further analysis, and subsequent tests.
9. In case the test chemical does not show any agonistic or antagonistic effect, the highest, non-cytotoxic concentration tested during pre-screening has to be used as the highest concentration in the concentration range to be tested.

ANNEX B

Solubility

Agonism

compound	blind code	Solubility in medium (well) (M)		
		participant A	participant B	participant C
Norethynodrel	SPC / AEP-1	3.0E-04	3.0E-04	1.0E-03
Bisphenol A	SPC / AEP-2	1.0E-03	1.0E-03	1.0E-03
Ketoconazole	SPC / AEP-3	1.0E-04	3.0E-05	3.0E-04
meso-Hexestrol	SPC / AEP-4	3.0E-04	1.0E-04	3.0E-04
Coumestrol	SPC / AEP-5	3.0E-04	3.0E-05	1.0E-04
4-Cumylphenol	SPC / AEP-6	1.0E-03	1.0E-03	1.0E-03
Butylbenzyl phtalate	SPC / AEP-7	3.0E-04	3.0E-05	1.0E-03
Genistein	SPC / AEP-8	1.0E-04	3.0E-05	3.0E-04
p,p'-methoxychlor	SPC / AEP-9	3.0E-04	3.0E-05	1.0E-03
Diethylstilbestrol	SPC / AEP-10	1.0E-04	3.0E-05	1.0E-04
Spironolactone	SPC / AEP-11	3.0E-04	1.0E-04	3.0E-04
Reserpine	SPC / AEP-12	1.0E-04	3.0E-05	3.0E-04
Linuron	SPC / AEP-13	1.0E-04	1.0E-04	3.0E-04
Atrazine	SPC / AEP-14	1.0E-04	1.0E-04	1.0E-03
Kaempferol	SPC / AEP-15	1.0E-04	1.0E-04	1.0E-04
Corticosterone	SPC / AEP-16	3.0E-04	1.0E-03	1.0E-03
19-Nortestosterone	SPC / AEP-17	1.0E-03	1.0E-03	1.0E-03
17a-Estradiol	SPC / AEP-18	3.0E-04	3.0E-05	1.0E-03
17a-Ethinyl estradiol	SPC / AEP-19	1.0E-04	3.0E-05	1.0E-04
4-tert-Octylphenol	SPC / AEP-20	1.0E-03	3.0E-04	1.0E-03
Etyl paraben	SPC / AEP-21	1.0E-03	1.0E-03	1.0E-03
Kepone	SPC / AEP-22	1.0E-04	1.0E-04	1.0E-04

Antagonism

compound	blind code	Solubility in medium (well) (M)		
		participant A	participant B	participant C
Apigenin	SPC / AEP-51	3.0E-05	1.0E-04	1.0E-04
17a-Ethinyl-estradiol	SPC / AEP-52	1.0E-04	3.0E-04	3.0E-04
Genistein	SPC / AEP-53	1.0E-04	3.0E-04	3.0E-04
Flutamide	SPC / AEP-54	1.0E-04	3.0E-04	3.0E-04
Coumestrol	SPC / AEP-55	3.0E-05	1.0E-03	1.0E-03
Raloxifen HCl	SPC / AEP-56	3.0E-05	3.0E-04	3.0E-04
Resveratrol	SPC / AEP-57	3.0E-04	1.0E-03	1.0E-03
Tamoxifen	SPC / AEP-58	1.0E-04	3.0E-04	3.0E-04
4OH-tamoxifen	SPC / AEP-59	1.0E-04	1.0E-04	1.0E-04
Chrysin	SPC / AEP-60	3.0E-05	1.0E-03	1.0E-03
Kaempferol	SPC / AEP-61	1.0E-04	3.0E-04	3.0E-04

ANNEX C

Concentrations of test chemicals during inter-laboratory phase

Participant A

Agonism - concentration tested in well

Reference	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2
17ß-Estradiol	Norethynodrel		Bisphenol A		Ketoconazole		meso-Hexestrol		Coumestrol		4-Cumylphenol	
	AEP-1		AEP-2		AEP-3		AEP-4		AEP-5		AEP-6	
(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)
1.0E-13	1.0E-12	1.0E-12	3.0E-09	3.0E-09	3.0E-11	3.0E-11	1.0E-13	3.0E-13	3.0E-11	3.0E-11	3.0E-09	3.0E-09
3.0E-13	3.0E-12	3.0E-12	1.0E-08	1.0E-08	3.0E-10	3.0E-10	3.0E-13	1.0E-12	1.0E-10	1.0E-10	1.0E-08	1.0E-08
1.0E-12	1.0E-11	1.0E-11	3.0E-08	3.0E-08	3.0E-09	3.0E-09	1.0E-12	3.0E-12	3.0E-10	3.0E-10	3.0E-08	3.0E-08
3.0E-12	3.0E-11	3.0E-11	1.0E-07	1.0E-07	3.0E-08	3.0E-08	3.0E-12	1.0E-11	1.0E-09	1.0E-09	1.0E-07	1.0E-07
6.0E-12	1.0E-10	1.0E-10	3.0E-07	3.0E-07	3.0E-07	3.0E-07	1.0E-11	3.0E-11	3.0E-09	3.0E-09	3.0E-07	3.0E-07
1.0E-11	3.0E-10	3.0E-10	1.0E-06	1.0E-06	3.0E-06	3.0E-06	3.0E-11	1.0E-10	1.0E-08	1.0E-08	1.0E-06	1.0E-06
3.0E-11	1.0E-09	1.0E-09	3.0E-06	3.0E-06	3.0E-05	3.0E-05	1.0E-10	3.0E-10	3.0E-08	3.0E-08	3.0E-06	3.0E-06
1.0E-10	3.0E-09	3.0E-09	1.0E-05	1.0E-05	3.0E-04	3.0E-04	3.0E-10	1.0E-09	1.0E-07	1.0E-07	1.0E-05	1.0E-05

compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2
Butylbenzyl phtalate		Genistein		p,p'-Methoxychlor		Diethylstilbestrol		Spironolactone		Reserpine	
AEP-7		AEP-8		AEP-9		AEP-10		AEP-11		AEP-12	
(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)
3.0E-09	3.0E-09	1.0E-10	1.0E-10	3.0E-09	3.0E-09	3.0E-13	3.0E-13	3.0E-10	3.0E-10	3.0E-11	3.0E-11
1.0E-08	1.0E-08	3.0E-10	3.0E-10	1.0E-08	1.0E-08	1.0E-12	1.0E-12	1.0E-09	1.0E-09	3.0E-10	3.0E-10
3.0E-08	3.0E-08	1.0E-09	1.0E-09	3.0E-08	3.0E-08	3.0E-12	3.0E-12	3.0E-09	3.0E-09	3.0E-09	3.0E-09
1.0E-07	1.0E-07	3.0E-09	3.0E-09	1.0E-07	1.0E-07	1.0E-11	1.0E-11	1.0E-08	1.0E-08	3.0E-08	3.0E-08
3.0E-07	3.0E-07	1.0E-08	1.0E-08	3.0E-07	3.0E-07	3.0E-11	3.0E-11	3.0E-08	3.0E-08	3.0E-07	3.0E-07
1.0E-06	1.0E-06	3.0E-08	3.0E-08	1.0E-06	1.0E-06	1.0E-10	1.0E-10	1.0E-07	1.0E-07	3.0E-06	3.0E-06
3.0E-06	3.0E-06	1.0E-07	1.0E-07	3.0E-06	3.0E-06	3.0E-10	3.0E-10	3.0E-07	3.0E-07	3.0E-05	3.0E-05
1.0E-05	1.0E-05	3.0E-07	3.0E-07	1.0E-05	1.0E-05	1.0E-09	1.0E-09	1.0E-06	1.0E-06	3.0E-04	3.0E-04

compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2
Linuron		Atrazine		Kaempferol		Corticosterone		19-Nortestosterone		17Estradiol	
AEP-13		AEP-14		AEP-15		AEP-16		AEP-17		AEP-18	
(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)
3.0E-11	3.0E-11	1.0E-10	1.0E-10	1.0E-08	1.0E-08	1.0E-10	1.0E-10	3.0E-10	3.0E-10	1.0E-11	3.0E-11
3.0E-10	3.0E-10	1.0E-09	1.0E-09	3.0E-08	3.0E-08	1.0E-09	1.0E-09	1.0E-09	1.0E-09	3.0E-11	1.0E-10
3.0E-09	3.0E-09	1.0E-08	1.0E-08	1.0E-07	1.0E-07	1.0E-08	1.0E-08	3.0E-09	3.0E-09	1.0E-10	3.0E-10
3.0E-08	3.0E-08	1.0E-07	1.0E-07	3.0E-07	3.0E-07	1.0E-07	1.0E-07	1.0E-08	1.0E-08	3.0E-10	1.0E-09
3.0E-07	3.0E-07	1.0E-06	1.0E-06	1.0E-06	1.0E-06	1.0E-06	1.0E-06	3.0E-08	3.0E-08	1.0E-09	3.0E-09
3.0E-06	3.0E-06	1.0E-05	1.0E-05	3.0E-06	3.0E-06	1.0E-05	1.0E-05	1.0E-07	1.0E-07	3.0E-09	1.0E-08
3.0E-05	3.0E-05	1.0E-04	1.0E-04	1.0E-05	1.0E-05	1.0E-04	1.0E-04	3.0E-07	3.0E-07	1.0E-08	3.0E-08
3.0E-04	3.0E-04	1.0E-03	1.0E-03	3.0E-05	3.0E-05	1.0E-03	1.0E-03	1.0E-06	1.0E-06	3.0E-08	1.0E-07

compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2
17Ethinyl estradiol		4-tert-Octylphenol		Etyl paraben		Kepone	
AEP-19		AEP-20		AEP-21		AEP-22	
(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)
1.0E-13	1.0E-13	3.0E-10	3.0E-10	1.0E-08	1.0E-08	1.0E-09	1.0E-09
3.0E-13	3.0E-13	1.0E-09	1.0E-09	3.0E-08	3.0E-08	3.0E-09	3.0E-09
1.0E-12	1.0E-12	3.0E-09	3.0E-09	1.0E-07	1.0E-07	1.0E-08	1.0E-08
3.0E-12	3.0E-12	1.0E-08	1.0E-08	3.0E-07	3.0E-07	3.0E-08	3.0E-08
1.0E-11	1.0E-11	3.0E-08	3.0E-08	1.0E-06	1.0E-06	1.0E-07	1.0E-07
3.0E-11	3.0E-11	1.0E-07	1.0E-07	3.0E-06	3.0E-06	3.0E-07	3.0E-07
1.0E-10	1.0E-10	3.0E-07	3.0E-07	1.0E-05	1.0E-05	1.0E-06	1.0E-06
3.0E-10	3.0E-10	1.0E-06	1.0E-06	3.0E-05	3.0E-05	3.0E-06	3.0E-06

ANNEX C

Participant A

Antagonism - concentration tested in well

Reference	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2
Tamoxifen	Apigenin		17Ethinyl-estradiol		Genistein		Flutamide		Coumestrol		Raloxifen HCl	
	AEP-51		AEP-52		AEP-53		AEP-54		AEP-55		AEP-56	
(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)
3.3E-09	3.0E-11	3.0E-11	3.0E-11	3.0E-11	3.0E-10	3.0E-10	3.0E-09	3.0E-09	3.0E-09	3.0E-09	3.0E-12	3.0E-12
1.0E-08	1.0E-10	1.0E-10	1.0E-10	1.0E-10	1.0E-09	1.0E-09	1.0E-08	1.0E-08	1.0E-08	1.0E-08	1.0E-11	1.0E-11
3.3E-08	3.0E-10	3.0E-10	3.0E-10	3.0E-10	3.0E-09	3.0E-09	3.0E-08	3.0E-08	3.0E-08	3.0E-08	3.0E-11	3.0E-11
1.0E-07	1.0E-09	1.0E-09	1.0E-09	1.0E-09	1.0E-08	1.0E-08	1.0E-07	1.0E-07	1.0E-07	1.0E-07	1.0E-10	1.0E-10
3.3E-07	3.0E-09	3.0E-09	3.0E-09	3.0E-09	3.0E-08	3.0E-08	3.0E-07	3.0E-07	3.0E-07	3.0E-07	3.0E-10	3.0E-10
1.0E-06	1.0E-08	1.0E-08	1.0E-08	1.0E-08	1.0E-07	1.0E-07	1.0E-06	1.0E-06	1.0E-06	1.0E-06	1.0E-09	1.0E-09
3.3E-06	3.0E-08	3.0E-08	3.0E-08	3.0E-08	3.0E-07	3.0E-07	3.0E-06	3.0E-06	3.0E-06	3.0E-06	3.0E-09	3.0E-09
1.0E-05	1.0E-07	1.0E-07	1.0E-07	1.0E-07	1.0E-06	1.0E-06	1.0E-05	1.0E-05	1.0E-05	1.0E-05	1.0E-08	1.0E-08

compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2
Resveratrol		Tamoxifen		4OH-tamoxifen		Chrysin		Kaempferol	
AEP-57		AEP-58		AEP-59		AEP-60		AEP-61	
(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)
3.0E-09	3.0E-09	3.0E-10	3.0E-10	3.0E-12	3.0E-12	3.0E-10	3.0E-10	3.0E-10	3.3E-10
1.0E-08	1.0E-08	1.0E-09	1.0E-09	1.0E-11	1.0E-11	1.0E-09	1.0E-09	1.0E-09	1.0E-09
3.0E-08	3.0E-08	3.0E-09	3.0E-09	3.0E-11	3.0E-11	3.0E-09	3.0E-09	3.0E-09	3.3E-09
1.0E-07	1.0E-07	1.0E-08	1.0E-08	1.0E-10	1.0E-10	1.0E-08	1.0E-08	1.0E-08	1.0E-08
3.0E-07	3.0E-07	3.0E-08	3.0E-08	3.0E-10	3.0E-10	3.0E-08	3.0E-08	3.0E-08	3.3E-08
1.0E-06	1.0E-06	1.0E-07	1.0E-07	1.0E-09	1.0E-09	1.0E-07	1.0E-07	1.0E-07	1.0E-07
3.0E-06	3.0E-06	3.0E-07	3.0E-07	3.0E-09	3.0E-09	3.0E-07	3.0E-07	3.0E-07	3.3E-07
1.0E-05	1.0E-05	1.0E-06	1.0E-06	1.0E-08	1.0E-08	1.0E-06	1.0E-06	1.0E-06	1.0E-06

ANNEX C

Participant B

Agonism - concentration tested in well

Reference	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2
17ß-estradiol	Norethynodrel		Bisphenol A		Ketoconazole		meso-Hexestrol		Coumestrol		4-Cumylphenol	
	AEP-1		AEP-2		AEP-3		AEP-4		AEP-5		AEP-6	
(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)
1.0E-13	3.0E-11	3.0E-11	3.0E-09	3.0E-09	1.0E-07	1.0E-07	1.0E-12	1.0E-12	3.0E-10	3.0E-10	3.0E-09	3.0E-09
3.0E-13	1.0E-10	1.0E-10	1.0E-08	1.0E-08	3.0E-07	3.0E-07	3.0E-12	3.0E-12	1.0E-09	1.0E-09	1.0E-08	1.0E-08
1.0E-12	3.0E-10	3.0E-10	3.0E-08	3.0E-08	1.0E-06	1.0E-06	1.0E-11	1.0E-11	3.0E-09	3.0E-09	3.0E-08	3.0E-08
3.0E-12	1.0E-09	1.0E-09	1.0E-07	1.0E-07	3.0E-06	3.0E-06	3.0E-11	3.0E-11	1.0E-08	1.0E-08	1.0E-07	1.0E-07
6.0E-12	3.0E-09	3.0E-09	3.0E-07	3.0E-07	1.0E-05	1.0E-05	1.0E-10	1.0E-10	3.0E-08	3.0E-08	3.0E-07	3.0E-07
1.0E-11	1.0E-08	1.0E-08	1.0E-06	1.0E-06	3.0E-05	3.0E-05	3.0E-10	3.0E-10	1.0E-07	1.0E-07	1.0E-06	1.0E-06
3.0E-11	3.0E-08	3.0E-08	3.0E-06	3.0E-06	1.0E-04	1.0E-04	1.0E-09	1.0E-09	3.0E-07	3.0E-07	3.0E-06	3.0E-06
1.0E-10	1.0E-07	1.0E-07	1.0E-05	1.0E-05	3.0E-04	3.0E-04	3.0E-09	3.0E-09	1.0E-06	1.0E-06	1.0E-05	1.0E-05

compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2
Butylbenzyl phtalate		Genistein		p,p'-Methoxychlor		Diethylstilbestrol		Spironolactone		Reserpine	
AEP-7		AEP-8		AEP-9		AEP-10		AEP-11		AEP-12	
(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)
3.0E-09	3.0E-09	3.0E-09	3.0E-09	3.0E-09	3.0E-09	3.0E-13	3.0E-13	1.0E-07	1.0E-07	1.0E-08	1.0E-08
1.0E-08	1.0E-08	1.0E-08	1.0E-08	1.0E-08	1.0E-08	1.0E-12	1.0E-12	3.0E-07	3.0E-07	3.0E-08	3.0E-08
3.0E-08	3.0E-08	3.0E-08	3.0E-08	3.0E-08	3.0E-08	3.0E-12	3.0E-12	1.0E-06	1.0E-06	1.0E-07	1.0E-07
1.0E-07	1.0E-07	1.0E-07	1.0E-07	1.0E-07	1.0E-07	1.0E-11	1.0E-11	3.0E-06	3.0E-06	3.0E-07	3.0E-07
3.0E-07	3.0E-07	3.0E-07	3.0E-07	3.0E-07	3.0E-07	3.0E-11	3.0E-11	1.0E-05	1.0E-05	1.0E-06	1.0E-06
1.0E-06	1.0E-06	1.0E-06	1.0E-06	1.0E-06	1.0E-06	1.0E-10	1.0E-10	3.0E-05	3.0E-05	3.0E-06	3.0E-06
3.0E-06	3.0E-06	3.0E-06	3.0E-06	3.0E-06	3.0E-06	3.0E-10	3.0E-10	1.0E-04	1.0E-04	1.0E-05	1.0E-05
1.0E-05	1.0E-05	1.0E-05	1.0E-05	1.0E-05	1.0E-05	1.0E-09	1.0E-09	3.0E-04	3.0E-04	3.0E-05	3.0E-05

compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2
Linuron		Atrazine		Kaempferol		Corticosterone		19-Nortestosterone		17Estradiol	
AEP-13		AEP-14		AEP-15		AEP-16		AEP-17		AEP-18	
(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)
1.0E-07	1.0E-07	3.0E-07	3.0E-07	3.0E-09	3.0E-09	3.0E-07	3.0E-07	1.0E-08	1.0E-08	3.0E-11	1.0E-11
3.0E-07	3.0E-07	1.0E-06	1.0E-06	1.0E-08	1.0E-08	1.0E-06	1.0E-06	3.0E-08	3.0E-08	1.0E-10	3.0E-11
1.0E-06	1.0E-06	3.0E-06	3.0E-06	3.0E-08	3.0E-08	3.0E-06	3.0E-06	1.0E-07	1.0E-07	3.0E-10	1.0E-10
3.0E-06	3.0E-06	1.0E-05	1.0E-05	1.0E-07	1.0E-07	1.0E-05	1.0E-05	3.0E-07	3.0E-07	1.0E-09	3.0E-10
1.0E-05	1.0E-05	3.0E-05	3.0E-05	3.0E-07	3.0E-07	3.0E-05	3.0E-05	1.0E-06	1.0E-06	3.0E-09	1.0E-09
3.0E-05	3.0E-05	1.0E-04	1.0E-04	1.0E-06	1.0E-06	1.0E-04	1.0E-04	3.0E-06	3.0E-06	1.0E-08	3.0E-09
1.0E-04	1.0E-04	3.0E-04	3.0E-04	3.0E-06	3.0E-06	3.0E-04	3.0E-04	1.0E-05	1.0E-05	3.0E-08	1.0E-08
3.0E-04	3.0E-04	1.0E-03	1.0E-03	1.0E-05	1.0E-05	1.0E-03	1.0E-03	3.0E-05	3.0E-05	1.0E-07	3.0E-08

compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2
17Ethinyl estradiol		4-tert-Octylphenol		Etyl paraben		Kepone	
AEP-19		AEP-20		AEP-21		AEP-22	
(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)
3.0E-13	3.0E-13	3.0E-10	3.0E-10	3.0E-08	3.0E-08	3.0E-09	3.0E-09
1.0E-12	1.0E-12	1.0E-09	1.0E-09	1.0E-07	1.0E-07	1.0E-08	1.0E-08
3.0E-12	3.0E-12	3.0E-09	3.0E-09	3.0E-07	3.0E-07	3.0E-08	3.0E-08
1.0E-11	1.0E-11	1.0E-08	1.0E-08	1.0E-06	1.0E-06	1.0E-07	1.0E-07
3.0E-11	3.0E-11	3.0E-08	3.0E-08	3.0E-06	3.0E-06	3.0E-07	3.0E-07
1.0E-10	1.0E-10	1.0E-07	1.0E-07	1.0E-05	1.0E-05	1.0E-06	1.0E-06
3.0E-10	3.0E-10	3.0E-07	3.0E-07	3.0E-05	3.0E-05	3.0E-06	3.0E-06
1.0E-09	1.0E-09	1.0E-06	1.0E-06	1.0E-04	1.0E-04	1.0E-05	1.0E-05

.

ANNEX C

Participant B

Antagonism - concentration tested in well

Reference	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2
Tamoxifen	Apigenin		17Ethinyl-estradiol		Genistein		Flutamide		Coumestrol		Raloxifen HCl	
	AEP-51		AEP-52		AEP-53		AEP-54		AEP-55		AEP-56	
(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)
3.3E-09	3.3E-09	3.3E-09	1.0E-07	1.0E-07	1.0E-08	1.0E-08	1.0E-07	1.0E-07	3.3E-08	3.3E-08	1.0E-12	1.0E-12
1.0E-08	1.0E-08	1.0E-08	3.0E-07	3.0E-07	3.0E-08	3.0E-08	3.0E-07	3.0E-07	1.0E-07	1.0E-07	3.0E-12	3.0E-12
3.3E-08	3.3E-08	3.3E-08	1.0E-06	1.0E-06	1.0E-07	1.0E-07	1.0E-06	1.0E-06	3.3E-07	3.3E-07	1.0E-11	1.0E-11
1.0E-07	1.0E-07	1.0E-07	3.0E-06	3.0E-06	3.0E-07	3.0E-07	3.0E-06	3.0E-06	1.0E-06	1.0E-06	3.0E-11	3.0E-11
3.3E-07	3.3E-07	3.3E-07	1.0E-05	1.0E-05	1.0E-06	1.0E-06	1.0E-05	1.0E-05	3.3E-06	3.3E-06	1.0E-10	1.0E-10
1.0E-06	1.0E-06	1.0E-06	3.0E-05	3.0E-05	3.0E-06	3.0E-06	3.0E-05	3.0E-05	1.0E-05	1.0E-05	3.0E-10	3.0E-10
3.3E-06	3.3E-06	3.3E-06	1.0E-04	1.0E-04	1.0E-05	1.0E-05	1.0E-04	1.0E-04	3.3E-05	3.3E-05	1.0E-09	1.0E-09
1.0E-05	1.0E-05	1.0E-05	3.0E-04	3.0E-04	3.0E-05	3.0E-05	3.0E-04	3.0E-04	1.0E-04	1.0E-04	3.0E-09	3.0E-09

compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2
Resveratrol		Tamoxifen		4OH-tamoxifen		Chrysin		Kaempferol	
AEP-57		AEP-58		AEP-59		AEP-60		AEP-61	
(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)
3.3E-08	3.3E-08	1.0E-09	1.0E-09	3.3E-12	3.3E-12	1.0E-08	1.0E-08	1.0E-08	1.0E-08
1.0E-07	1.0E-07	3.0E-09	3.0E-09	1.0E-11	1.0E-11	3.0E-08	3.0E-08	3.0E-08	3.0E-08
3.3E-07	3.3E-07	1.0E-08	1.0E-08	3.3E-11	3.3E-11	1.0E-07	1.0E-07	1.0E-07	1.0E-07
1.0E-06	1.0E-06	3.0E-08	3.0E-08	1.0E-10	1.0E-10	3.0E-07	3.0E-07	3.0E-07	3.0E-07
3.3E-06	3.3E-06	1.0E-07	1.0E-07	3.3E-10	3.3E-10	1.0E-06	1.0E-06	1.0E-06	1.0E-06
1.0E-05	1.0E-05	3.0E-07	3.0E-07	1.0E-09	1.0E-09	3.0E-06	3.0E-06	3.0E-06	3.0E-06
3.3E-05	3.3E-05	1.0E-06	1.0E-06	3.3E-09	3.3E-09	1.0E-05	1.0E-05	1.0E-05	1.0E-05
1.0E-04	1.0E-04	3.0E-06	3.0E-06	1.0E-08	1.0E-08	3.0E-05	3.0E-05	3.0E-05	3.0E-05

ANNEX C

Participant C

Agonism - concentration tested in well

Reference	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2
17ß-estradiol	Norethynodrel		Bisphenol A		Ketoconazole		meso-Hexestrol		Coumestrol		4-Cumylphenol	
	AEP-1		AEP-2		AEP-3		AEP-4		AEP-5		AEP-6	
(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)
1.0E-13	1.0E-11	3.0E-11	1.0E-08	3.0E-09	1.0E-07	1.0E-08	3.0E-12	1.0E-13	3.0E-09	1.0E-09	3.0E-09	3.0E-09
3.0E-13	3.0E-11	1.0E-10	3.0E-08	1.0E-08	3.0E-07	3.0E-08	1.0E-11	3.0E-13	1.0E-08	3.0E-09	1.0E-08	1.0E-08
1.0E-12	1.0E-10	3.0E-10	1.0E-07	3.0E-08	1.0E-06	1.0E-07	3.0E-11	1.0E-12	3.0E-08	1.0E-08	3.0E-08	3.0E-08
3.0E-12	3.0E-10	1.0E-09	3.0E-07	1.0E-07	3.0E-06	3.0E-07	1.0E-10	3.0E-12	1.0E-07	3.0E-08	1.0E-07	1.0E-07
6.0E-12	1.0E-09	3.0E-09	1.0E-06	3.0E-07	1.0E-05	1.0E-06	3.0E-10	1.0E-11	3.0E-07	1.0E-07	3.0E-07	3.0E-07
1.0E-11	3.0E-09	1.0E-08	3.0E-06	1.0E-06	3.0E-05	3.0E-06	1.0E-09	3.0E-11	1.0E-06	3.0E-07	1.0E-06	1.0E-06
3.0E-11	1.0E-08	3.0E-08	1.0E-05	3.0E-06	1.0E-04	1.0E-05	3.0E-09	1.0E-10	3.0E-06	1.0E-06	3.0E-06	3.0E-06
1.0E-10	3.0E-08	1.0E-07	3.0E-05	1.0E-05	3.0E-04	3.0E-05	1.0E-08	3.0E-10	1.0E-05	3.0E-06	1.0E-05	1.0E-05

compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2
Butylbenzyl phtalate		Genistein		p,p'-Methoxychlor		Diethylstilbestrol		Spironolactone		Reserpine	
AEP-7		AEP-8		AEP-9		AEP-10		AEP-11		AEP-12	
(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)
3.0E-08	1.0E-07	3.0E-09	3.0E-09	3.0E-08	3.0E-08	1.0E-12	3.0E-13	3.0E-08	3.0E-07	3.0E-10	1.0E-08
1.0E-07	3.0E-07	1.0E-08	1.0E-08	1.0E-07	1.0E-07	3.0E-12	1.0E-12	1.0E-07	1.0E-06	1.0E-09	3.0E-08
3.0E-07	1.0E-06	3.0E-08	3.0E-08	3.0E-07	3.0E-07	1.0E-11	3.0E-12	3.0E-07	3.0E-06	3.0E-09	1.0E-07
1.0E-06	3.0E-06	1.0E-07	1.0E-07	1.0E-06	1.0E-06	3.0E-11	1.0E-11	1.0E-06	1.0E-05	1.0E-08	3.0E-07
3.0E-06	1.0E-05	3.0E-07	3.0E-07	3.0E-06	3.0E-06	1.0E-10	3.0E-11	3.0E-06	3.0E-05	3.0E-08	1.0E-06
1.0E-05	3.0E-05	1.0E-06	1.0E-06	1.0E-05	1.0E-05	3.0E-10	1.0E-10	1.0E-05	1.0E-04	1.0E-07	3.0E-06
3.0E-05	1.0E-04	3.0E-06	3.0E-06	3.0E-05	3.0E-05	1.0E-09	3.0E-10	3.0E-05	3.0E-04	3.0E-07	1.0E-05
1.0E-04	3.0E-04	1.0E-05	1.0E-05	1.0E-04	1.0E-04	3.0E-09	1.0E-09	1.0E-04	1.0E-03	1.0E-06	3.0E-05

compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2
Linuron		Atrazine		Kaempferol		Corticosterone		19-Nortestosterone		17Estradiol	
AEP-13		AEP-14		AEP-15		AEP-16		AEP-17		AEP-18	
(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)
1.0E-08	1.0E-08	3.0E-08	3.0E-08	1.0E-07	3.0E-08	1.0E-07	1.0E-07	3.0E-09	3.0E-09	3.0E-11	1.0E-11
3.0E-08	3.0E-08	1.0E-07	1.0E-07	3.0E-07	1.0E-07	3.0E-07	3.0E-07	1.0E-08	1.0E-08	1.0E-10	3.0E-11
1.0E-07	1.0E-07	3.0E-07	3.0E-07	1.0E-06	3.0E-07	1.0E-06	1.0E-06	3.0E-08	3.0E-08	3.0E-10	1.0E-10
3.0E-07	3.0E-07	1.0E-06	1.0E-06	3.0E-06	1.0E-06	3.0E-06	3.0E-06	1.0E-07	1.0E-07	1.0E-09	3.0E-10
1.0E-06	1.0E-06	3.0E-06	3.0E-06	1.0E-05	3.0E-06	1.0E-05	1.0E-05	3.0E-07	3.0E-07	3.0E-09	1.0E-09
3.0E-06	3.0E-06	1.0E-05	1.0E-05	3.0E-05	1.0E-05	3.0E-05	3.0E-05	1.0E-06	1.0E-06	1.0E-08	3.0E-09
1.0E-05	1.0E-05	3.0E-05	3.0E-05	1.0E-04	3.0E-05	1.0E-04	1.0E-04	3.0E-06	3.0E-06	3.0E-08	1.0E-08
3.0E-05	3.0E-05	1.0E-04	1.0E-04	3.0E-04	1.0E-04	3.0E-04	3.0E-04	1.0E-05	1.0E-05	1.0E-07	3.0E-08

compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2
17Ethinyl estradiol		4-tert-Octylphenol		Etyl paraben		Kepone	
AEP-19		AEP-20		AEP-21		AEP-22	
(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)
3.0E-14	1.0E-14	1.0E-09	3.0E-10	1.0E-07	1.0E-07	3.0E-08	3.0E-08
1.0E-13	3.0E-14	3.0E-09	1.0E-09	3.0E-07	3.0E-07	1.0E-07	1.0E-07
3.0E-13	1.0E-13	1.0E-08	3.0E-09	1.0E-06	1.0E-06	3.0E-07	3.0E-07
1.0E-12	3.0E-13	3.0E-08	1.0E-08	3.0E-06	3.0E-06	1.0E-06	1.0E-06
3.0E-12	1.0E-12	1.0E-07	3.0E-08	1.0E-05	1.0E-05	3.0E-06	3.0E-06
1.0E-11	3.0E-12	3.0E-07	1.0E-07	3.0E-05	3.0E-05	1.0E-05	1.0E-05
3.0E-11	1.0E-11	1.0E-06	3.0E-07	1.0E-04	1.0E-04	3.0E-05	3.0E-05
1.0E-10	3.0E-11	3.0E-06	1.0E-06	3.0E-04	3.0E-04	1.0E-04	1.0E-04

ANNEX C

Participant C

Antagonism - concentration tested in well

Reference	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2
Tamoxifen	Apigenin		17a-Ethinyl-estradiol		Genistein		Flutamide		Coumestrol		Raloxifen HCl	
	AEP-51		AEP-52		AEP-53		AEP-54		AEP-55		AEP-56	
(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)
3.3E-09	3.3E-09	3.3E-09	1.0E-07	1.0E-07	1.0E-08	1.0E-08	1.0E-07	1.0E-07	3.3E-08	3.3E-08	1.0E-12	1.0E-12
1.0E-08	1.0E-08	1.0E-08	3.0E-07	3.0E-07	3.0E-08	3.0E-08	3.0E-07	3.0E-07	1.0E-07	1.0E-07	3.0E-12	3.0E-12
3.3E-08	3.3E-08	3.3E-08	1.0E-06	1.0E-06	1.0E-07	1.0E-07	1.0E-06	1.0E-06	3.3E-07	3.3E-07	1.0E-11	1.0E-11
1.0E-07	1.0E-07	1.0E-07	3.0E-06	3.0E-06	3.0E-07	3.0E-07	3.0E-06	3.0E-06	1.0E-06	1.0E-06	3.0E-11	3.0E-11
3.3E-07	3.3E-07	3.3E-07	1.0E-05	1.0E-05	1.0E-06	1.0E-06	1.0E-05	1.0E-05	3.3E-06	3.3E-06	1.0E-10	1.0E-10
1.0E-06	1.0E-06	1.0E-06	3.0E-05	3.0E-05	3.0E-06	3.0E-06	3.0E-05	3.0E-05	1.0E-05	1.0E-05	3.0E-10	3.0E-10
3.3E-06	3.3E-06	3.3E-06	1.0E-04	1.0E-04	1.0E-05	1.0E-05	1.0E-04	1.0E-04	3.3E-05	3.3E-05	1.0E-09	1.0E-09
1.0E-05	1.0E-05	1.0E-05	3.0E-04	3.0E-04	3.0E-05	3.0E-05	3.0E-04	3.0E-04	1.0E-04	1.0E-04	3.0E-09	3.0E-09

compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2	compr. 1	compr. 2
Resveratrol		Tamoxifen		4OH-tamoxifen		Chrysin		Kaempferol	
AEP-57		AEP-58		AEP-59		AEP-60		AEP-61	
(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)
3.3E-08	3.3E-08	1.0E-09	1.0E-09	3.3E-12	3.3E-12	1.0E-08	1.0E-08	1.0E-08	1.0E-08
1.0E-07	1.0E-07	3.0E-09	3.0E-09	1.0E-11	1.0E-11	3.0E-08	3.0E-08	3.0E-08	3.0E-08
3.3E-07	3.3E-07	1.0E-08	1.0E-08	3.3E-11	3.3E-11	1.0E-07	1.0E-07	1.0E-07	1.0E-07
1.0E-06	1.0E-06	3.0E-08	3.0E-08	1.0E-10	1.0E-10	3.0E-07	3.0E-07	3.0E-07	3.0E-07
3.3E-06	3.3E-06	1.0E-07	1.0E-07	3.3E-10	3.3E-10	1.0E-06	1.0E-06	1.0E-06	1.0E-06
1.0E-05	1.0E-05	3.0E-07	3.0E-07	1.0E-09	1.0E-09	3.0E-06	3.0E-06	3.0E-06	3.0E-06
3.3E-05	3.3E-05	1.0E-06	1.0E-06	3.3E-09	3.3E-09	1.0E-05	1.0E-05	1.0E-05	1.0E-05
1.0E-04	1.0E-04	3.0E-06	3.0E-06	1.0E-08	1.0E-08	3.0E-05	3.0E-05	3.0E-05	3.0E-05

ANNEX D

Results intra-laboratory phase (lead laboratory) - agonism

Pre-screen study

Test item	TI max (%)	Hillslope (-)	EC10 (M)	EC50 (M)	PC10 (M)	PC50 (M)	LOG (EC10) (M)	LOG (EC50) (M)	LOG (PC10) (M)	LOG (PC50) (M)	Pos/Neg (-)
17b-estradiol (reference)	104	1.2	1.2E-12	7.5E-12	1.2E-12	7.1E-12	-11.91	-11.13	-11.91	-11.15	pos
Ethylparaben	237	1.7	9.7E-06	3.4E-05	4.8E-06	1.6E-05	-5.01	-4.46	-5.32	-4.81	pos
Kaempferol	126	4.0	1.7E-06	3.0E-06	1.5E-06	2.6E-06	-5.77	-5.53	-5.81	-5.58	pos
Butylbenzyl phtalate	134	2.4	9.3E-07	1.9E-06	6.1E-07	1.5E-06	-6.03	-5.72	-6.21	-5.82	pos
p,p'-methoxychlor	119	3.2	7.7E-07	1.5E-06	6.8E-07	1.4E-06	-6.11	-5.82	-6.17	-5.87	pos
19-Nortestosterone	93	7.0	8.1E-08	1.1E-07	7.9E-08	1.1E-07	-7.09	-6.95	-7.10	-6.95	pos
Bisphenol A	133	2.3	1.1E-07	2.8E-07	7.7E-08	2.2E-07	-6.98	-6.56	-7.11	-6.67	pos
Kepone	91	1.3	2.9E-07	1.5E-06	2.7E-07	1.7E-06	-6.54	-5.81	-6.57	-5.76	pos
4-Cumylphenol	126	1.8	1.8E-07	6.3E-07	1.6E-07	5.0E-07	-6.73	-6.20	-6.79	-6.30	pos
Genistein	229	1.1	3.3E-08	2.3E-07	1.4E-08	7.4E-08	-7.48	-6.63	-7.86	-7.13	pos
Coumestrol	108	1.0	4.7E-10	4.1E-09	4.0E-10	3.5E-09	-9.33	-8.39	-9.40	-8.45	pos
4-tert-Octylphenol	122	1.1	1.5E-09	1.2E-08	1.1E-09	8.3E-09	-8.82	-7.93	-8.94	-8.08	pos
17a-Estradiol	113	7.9	7.7E-10	1.0E-09	6.5E-10	9.8E-10	-9.11	-8.99	-9.19	-9.01	pos
Norethynodrel	92	9.7	7.6E-10	1.0E-09	7.8E-10	1.1E-09	-9.12	-8.98	-9.11	-8.98	pos
Diethylstilbestrol	142	0.8	1.1E-12	1.8E-11	6.3E-13	8.3E-12	-11.98	-10.73	-12.20	-11.08	pos
meso-Hexestrol	86	1.0	1.5E-12	1.4E-11	2.2E-12	2.0E-11	-11.81	-10.85	-11.66	-10.70	pos
17a-Ethinyl estradiol	108	1.0	7.6E-14	6.7E-13	3.7E-14	5.3E-13	-13.12	-12.18	-13.43	-12.28	pos
Atrazine	25	---	---	---	---	---	---	---	---	---	neg
Corticosterone	11	---	---	---	---	---	---	---	---	---	neg
Linuron	<10	---	---	---	---	---	---	---	---	---	neg
Spironolactone	<10	---	---	---	---	---	---	---	---	---	neg
Ketoconazole	<10	---	---	---	---	---	---	---	---	---	neg
Reserpine	<10	---	---	---	---	---	---	---	---	---	neg

Comprehensive study - 1

Test item	TI max (%)	Hillslope (-)	EC10 (M)	EC50 (M)	PC10 (M)	PC50 (M)	LOG (EC10) (M)	LOG (EC50) (M)	LOG (PC10) (M)	LOG (PC50) (M)	Pos/Neg (-)
17b-estradiol (reference)	102	1.3	8.4E-13	5.6E-12	9.0E-13	5.5E-12	-12.07	-11.25	-12.05	-11.26	pos
Ethylparaben	261	2.1	1.3E-05	3.6E-05	8.0E-06	1.8E-05	-4.89	-4.44	-5.10	-4.73	pos
Kaempferol	130	11	6.4E-06	7.9E-06	6.4E-06	7.6E-06	-5.19	-5.10	-5.19	-5.12	pos
Butylbenzyl phtalate	144	2.2	1.3E-06	3.5E-06	1.1E-06	2.6E-06	-5.90	-5.46	-5.97	-5.58	pos
p,p'-methoxychlor	142	2.0	1.5E-06	4.4E-06	1.3E-06	3.3E-06	-5.84	-5.36	-5.88	-5.48	pos
19-Nortestosterone	88	1.8	7.7E-08	2.7E-07	8.0E-08	3.1E-07	-7.11	-6.57	-7.09	-6.51	pos
Bisphenol A	188	1.5	8.6E-08	3.8E-07	5.4E-08	1.9E-07	-7.06	-6.42	-7.27	-6.72	pos
Kepone	69	2.7	6.8E-07	1.5E-06	7.9E-07	2.2E-06	-6.16	-5.81	-6.10	-5.66	pos
4-Cumylphenol	125	1.8	2.3E-07	7.9E-07	2.1E-07	6.3E-07	-6.64	-6.10	-6.69	-6.20	pos
Genistein	147	2.1	4.2E-08	1.2E-07	3.2E-08	8.8E-08	-7.37	-6.91	-7.49	-7.05	pos
Coumestrol	87	1.4	7.2E-09	3.4E-08	8.0E-09	4.2E-08	-8.14	-7.47	-8.10	-7.38	pos
4-tert-Octylphenol	109	1.9	8.7E-08	2.8E-07	9.2E-08	2.7E-07	-7.06	-6.55	-7.04	-6.57	pos
17a-Estradiol	109	1.8	3.3E-10	1.1E-09	3.1E-10	1.0E-09	-9.48	-8.95	-9.51	-8.99	pos
Norethynodrel	95	1.4	3.3E-10	1.5E-09	3.2E-10	1.6E-09	-9.48	-8.82	-9.49	-8.79	pos
Diethylstilbestrol	93	1.6	1.7E-11	6.8E-11	1.7E-11	7.4E-11	-10.77	-10.17	-10.76	-10.13	pos
meso-Hexestrol	97	1.5	9.5E-12	4.0E-11	8.4E-12	4.1E-11	-11.02	-10.40	-11.08	-10.39	pos
17a-Ethinyl estradiol	100	1.2	2.0E-12	1.3E-11	2.1E-12	1.3E-11	-11.70	-10.90	-11.67	-10.89	pos
Atrazine	<10	---	---	---	---	---	---	---	---	---	neg
Corticosterone	<10	---	---	---	---	---	---	---	---	---	neg
Linuron	<10	---	---	---	---	---	---	---	---	---	neg
Spironolactone	<10	---	---	---	---	---	---	---	---	---	neg
Ketoconazole	<10	---	---	---	---	---	---	---	---	---	neg
Reserpine	<10	---	---	---	---	---	---	---	---	---	neg

Comprehensive study - 2

Test item	TI max (%)	Hillslope (-)	EC10 (M)	EC50 (M)	PC10 (M)	PC50 (M)	LOG (EC10) (M)	LOG (EC50) (M)	LOG (PC10) (M)	LOG (PC50) (M)	Pos/Neg (-)
17b-estradiol (reference)	98	2.1	2.2E-12	6.5E-12	2.0E-12	6.5E-12	-11.66	-11.19	-11.69	-11.18	pos
Ethylparaben	206	1.6	1.3E-05	2.8E-05	9.1E-06	1.9E-05	-4.90	-4.55	-5.04	-4.73	pos
Kaempferol	97	14	6.4E-06	7.5E-06	6.4E-06	7.5E-06	-5.19	-5.12	-5.19	-5.12	pos
Butylbenzyl phtalate	98	2.2	7.4E-07	2.0E-06	7.2E-07	2.0E-06	-6.13	-5.70	-6.14	-5.69	pos
p,p'-methoxychlor	145	2.4	1.1E-06	2.7E-06	9.1E-07	2.1E-06	-5.97	-5.56	-6.04	-5.68	pos
19-Nortestosterone	96	1.7	4.1E-08	1.5E-07	4.3E-08	1.6E-07	-7.39	-6.83	-7.36	-6.80	pos
Bisphenol A	145	1.9	1.6E-07	5.3E-07	1.4E-07	3.8E-07	-6.79	-6.28	-6.86	-6.42	pos
Kepone	72	5.0	4.3E-07	6.7E-07	4.6E-07	7.9E-07	-6.36	-6.17	-6.34	-6.10	pos
4-Cumylphenol	136	1.8	1.2E-07	4.1E-07	8.4E-08	3.0E-07	-6.92	-6.38	-7.08	-6.53	pos
Genistein	231	1.1	8.0E-08	4.8E-07	4.0E-08	1.6E-07	-7.10	-6.32	-7.40	-6.79	pos
Coumestrol	88	1.7	4.0E-09	1.4E-08	4.3E-09	1.6E-08	-8.40	-7.85	-8.37	-7.79	pos
4-tert-Octylphenol	88	1.8	4.4E-08	1.5E-07	5.0E-08	1.7E-07	-7.35	-6.83	-7.30	-6.77	pos
17a-Estradiol	97	1.8	2.7E-10	9.3E-10	2.4E-10	9.4E-10	-9.57	-9.03	-9.61	-9.03	pos
Norethynodrel	99	1.7	2.5E-10	9.1E-10	2.1E-10	8.9E-10	-9.61	-9.04	-9.68	-9.05	pos
Diethylstilbestrol	110	1.8	1.1E-11	3.5E-11	4.7E-12	2.9E-11	-10.98	-10.46	-11.32	-10.54	pos
meso-Hexestrol	107	1.7	5.2E-12	1.9E-11	2.9E-12	1.6E-11	-11.29	-10.72	-11.54	-10.79	pos
17a-Ethinyl estradiol	91	1.4	1.2E-12	5.7E-12	7.4E-13	6.0E-12	-11.92	-11.25	-12.13	-11.23	pos
Atrazine	<10	---	---	---	---	---	---	---	---	---	neg
Corticosterone	<10	---	---	---	---	---	---	---	---	---	neg
Linuron	<10	---	---	---	---	---	---	---	---	---	neg
Spironolactone	<10	---	---	---	---	---	---	---	---	---	neg
Ketoconazole	<10	---	---	---	---	---	---	---	---	---	neg
Reserpine	<10	---	---	---	---	---	---	---	---	---	neg

ANNEX D

Results inter-laboratory phase (participant A) – agonism

Pre-screen study (participants A)

Test item	Blind label	TI max (%)	Hillslope (-)	EC10 (M)	EC50 (M)	PC10 (M)	PC50 (M)	LOG (EC10) (M)	LOG (EC50) (M)	LOG (PC10) (M)	LOG (PC50) (M)	Pos/Neg (-)
17ß-estradiol (avg)		101	1.5	2.3E-12	9.3E-12	1.8E-12	8.6E-12	-11.63	-11.03	-11.75	-11.06	pos
Norethynodrel	SPC-1	88	2.5	4.3E-10	1.0E-09	4.0E-10	1.1E-09	-9.37	-8.99	-9.40	-8.95	pos
Bisphenol A	SPC-2	134	1.8	1.1E-07	3.7E-07	8.1E-08	2.7E-07	-6.98	-6.44	-7.09	-6.57	pos
Ketoconazole	SPC-3	<10	---	---	---	---	---	---	---	---	---	neg
meso-Hexestrol	SPC-4	111	1.7	5.5E-12	2.0E-11	4.4E-12	1.7E-11	-11.26	-10.70	-11.35	-10.76	pos
Coumestrol	SPC-5	106	1.3	2.0E-09	1.1E-08	1.5E-09	1.0E-08	-8.70	-7.94	-8.82	-7.99	pos
4-Cumylphenol	SPC-6	120	1.8	1.2E-07	4.2E-07	1.1E-07	3.5E-07	-6.91	-6.37	-6.97	-6.46	pos
Butylbenzyl phtalate	SPC-7	102	10	8.8E-07	1.1E-06	8.9E-07	1.1E-06	-6.05	-5.95	-6.05	-5.95	pos
Genistein	SPC-8	231	0.5	3.7E-09	2.7E-07	6.1E-10	2.2E-08	-8.43	-6.56	-9.21	-7.66	pos
p,p'-methoxychlor	SPC-9	135	1.5	8.8E-07	4.0E-06	6.1E-07	2.7E-06	-6.05	-5.40	-6.22	-5.57	pos
Diethylstilbestrol	SPC-10	106	1.5	2.7E-11	1.2E-10	1.7E-11	1.1E-10	-10.56	-9.91	-10.77	-9.97	pos
Spironolactone	SPC-11	<10	---	---	---	---	---	---	---	---	---	neg
Reserpine	SPC-12	<10	---	---	---	---	---	---	---	---	---	neg
Linuron	SPC-13	<10	11	---	---	---	---	---	---	---	---	neg
Atrazine	SPC-14	<10	---	---	---	---	---	---	---	---	---	neg
Kaempferol	SPC-15	77	1.8	6.4E-07	2.0E-06	7.9E-07	2.3E-06	-6.20	-5.71	-6.10	-5.64	pos
Corticosterone	SPC-16	<10	---	---	---	---	---	---	---	---	---	neg
19-Nortestosterone	SPC-17	92	1.9	3.9E-08	1.3E-07	3.7E-08	1.4E-07	-7.41	-6.90	-7.43	-6.87	pos
17a-Estradiol	SPC-18	100	1.6	1.5E-10	6.0E-10	1.3E-10	5.8E-10	-9.81	-9.22	-9.87	-9.23	pos
17a-Ethinyl estradiol	SPC-19	102	1.5	1.9E-12	8.6E-12	1.9E-12	8.4E-12	-11.72	-11.07	-11.72	-11.08	pos
4-tert-Octylphenol	SPC-20	124	1.2	2.3E-08	1.5E-07	1.5E-08	1.0E-07	-7.64	-6.83	-7.81	-6.98	pos
Etyl paraben	SPC-21	191	2.4	7.3E-06	1.8E-05	4.3E-06	1.1E-05	-5.14	-4.74	-5.36	-4.94	pos
Kepone	SPC-22	98	7.1	7.0E-08	6.8E-08	4.8E-08	6.8E-08	-7.15	-7.17	-7.32	-7.17	pos

Comprehensive study - 1 (participant A)

Test item	Blind label	TI max (%)	Hillslope (-)	EC10 (M)	EC50 (M)	PC10 (M)	PC50 (M)	LOG (EC10) (M)	LOG (EC50) (M)	LOG (PC10) (M)	LOG (PC50) (M)	Pos/Neg (-)
17ß-estradiol (avg)		103	1.5	2.0E-12	7.5E-12	1.3E-12	6.9E-12	-11.71	-11.12	-11.87	-11.16	pos
Norethynodrel	AEP-1	152	1.3	1.8E-10	9.3E-10	1.0E-10	5.3E-10	-9.74	-9.03	-9.99	-9.28	pos
Bisphenol A	AEP-2	148	1.4	8.4E-08	4.0E-07	5.1E-08	2.4E-07	-7.08	-6.40	-7.30	-6.62	pos
Ketoconazole	AEP-3	<10	---	---	---	---	---	---	---	---	---	neg
meso-Hexestrol	AEP-4	119	1.8	7.0E-12	2.4E-11	4.0E-12	1.9E-11	-11.16	-10.62	-11.40	-10.73	pos
Coumestrol	AEP-5	121	1.4	2.1E-09	9.9E-09	1.2E-09	7.2E-09	-8.67	-8.01	-8.93	-8.14	pos
4-Cumylphenol	AEP-6	118	1.6	9.5E-08	3.7E-07	7.5E-08	3.0E-07	-7.02	-6.43	-7.12	-6.52	pos
Butylbenzyl phtalate	AEP-7	118	1.8	5.5E-07	1.8E-06	4.3E-07	1.5E-06	-6.26	-5.74	-6.37	-5.82	pos
Genistein	AEP-8	135	1.9	9.8E-09	3.1E-08	6.3E-09	2.2E-08	-8.01	-7.51	-8.20	-7.65	pos
p,p'-methoxychlor	AEP-9	155	1.4	9.2E-07	4.4E-06	5.0E-07	2.5E-06	-6.04	-5.36	-6.30	-5.61	pos
Diethylstilbestrol	AEP-10	99	1.5	5.7E-11	2.4E-10	4.4E-11	2.3E-10	-10.24	-9.62	-10.36	-9.64	pos
Spironolactone	AEP-11	<10	---	---	---	---	---	---	---	---	---	neg
Reserpine	AEP-12	<10	---	---	---	---	---	---	---	---	---	neg
Linuron	AEP-13	14	1.3	2.4E-06	1.4E-05	2.2E-05	---	-5.62	-4.86	-4.66	---	pos
Atrazine	AEP-14	<10	---	---	---	---	---	---	---	---	---	neg
Kaempferol	AEP-15	58	30	3.0E-06	3.3E-06	3.3E-06	3.1E-06	-5.52	-5.49	-5.48	-5.52	pos
Corticosterone	AEP-16	<10	---	---	---	---	---	---	---	---	---	neg
19-Nortestosterone	AEP-17	103	1.4	2.8E-08	1.3E-07	2.2E-08	1.2E-07	-7.56	-6.89	-7.65	-6.92	pos
17a-Estradiol	AEP-18	118	1.4	2.4E-10	1.2E-09	1.5E-10	9.1E-10	-9.62	-8.92	-9.81	-9.04	pos
17a-Ethinyl estradiol	AEP-19	99	1.7	1.5E-12	5.6E-12	1.3E-12	5.5E-12	-11.81	-11.25	-11.89	-11.26	pos
4-tert-Octylphenol	AEP-20	115	1.6	3.1E-08	1.2E-07	2.6E-08	1.0E-07	-7.50	-6.91	-7.59	-6.99	pos
Etyl paraben	AEP-21	194	1.7	6.8E-06	2.4E-05	2.9E-06	1.2E-05	-5.17	-4.61	-5.53	-4.91	pos
Kepone	AEP-22	120	2.1	2.7E-07	7.8E-07	1.7E-07	6.3E-07	-6.57	-6.11	-6.76	-6.20	pos

Comprehensive study - 2 (participant A)

Test item	Blind label	TI max (%)	Hillslope (-)	EC10 (M)	EC50 (M)	PC10 (M)	PC50 (M)	LOG (EC10) (M)	LOG (EC50) (M)	LOG (PC10) (M)	LOG (PC50) (M)	Pos/Neg (-)
17ß-estradiol (avg)		104	1.4	3.2E-12	1.4E-11	2.6E-12	1.2E-11	-11.49	-10.87	-11.58	-10.90	pos
Norethynodrel	AEP-1	104	1.5	3.3E-10	1.4E-09	3.1E-10	1.3E-09	-9.48	-8.86	-9.51	-8.88	pos
Bisphenol A	AEP-2	143	1.5	1.1E-07	4.7E-07	7.2E-08	3.0E-07	-6.97	-6.33	-7.14	-6.52	pos
Ketoconazole	AEP-3	<10	---	---	---	---	---	---	---	---	---	neg
meso-Hexestrol	AEP-4	118	1.9	7.0E-12	2.2E-11	4.9E-12	1.8E-11	-11.16	-10.65	-11.31	-10.74	pos
Coumestrol	AEP-5	103	1.3	1.7E-09	9.5E-09	1.3E-09	8.7E-09	-8.77	-8.02	-8.89	-8.06	pos
4-Cumylphenol	AEP-6	115	2.1	1.2E-07	3.3E-07	8.7E-08	2.8E-07	-6.93	-6.48	-7.06	-6.55	pos
Butylbenzyl phtalate	AEP-7	121	2.1	6.8E-07	2.0E-06	5.4E-07	1.6E-06	-6.16	-5.70	-6.27	-5.79	pos
Genistein	AEP-8	160	1.9	1.2E-08	3.8E-08	7.6E-09	2.4E-08	-7.92	-7.42	-8.12	-7.62	pos
p,p'-methoxychlor	AEP-9	137	1.6	7.6E-07	2.9E-06	4.9E-07	2.0E-06	-6.12	-5.54	-6.31	-5.71	pos
Diethylstilbestrol	AEP-10	127	1.4	5.2E-11	2.6E-10	3.6E-11	1.8E-10	-10.29	-9.58	-10.44	-9.74	pos
Spironolactone	AEP-11	<10	---	---	---	---	---	---	---	---	---	neg
Reserpine	AEP-12	<10	---	---	---	---	---	---	---	---	---	neg
Linuron	AEP-13	21	1.4	6.3E-06	2.9E-05	2.5E-05	---	-5.20	-4.54	-4.60	---	pos
Atrazine	AEP-14	<10	---	---	---	---	---	---	---	---	---	neg
Kaempferol	AEP-15	48	25	1.7E-06	2.8E-06	2.0E-06	1.0E-05	-5.76	-5.56	-5.69	-5.00	pos
Corticosterone	AEP-16	<10	---	---	---	---	---	---	---	---	---	neg
19-Nortestosterone	AEP-17	109	1.4	4.2E-08	2.0E-07	3.4E-08	1.7E-07	-7.38	-6.71	-7.47	-6.76	pos
17a-Estradiol	AEP-18	77	1.7	3.7E-10	1.4E-09	4.6E-10	2.0E-09	-9.43	-8.86	-9.34	-8.69	pos
17a-Ethinyl estradiol	AEP-19	98	1.6	1.5E-12	6.2E-12	1.2E-12	6.1E-12	-11.82	-11.21	-11.90	-11.21	pos
4-tert-Octylphenol	AEP-20	116	1.8	3.3E-08	1.1E-07	2.7E-08	9.3E-08	-7.48	-6.96	-7.57	-7.03	pos
Etyl paraben	AEP-21	149	2.1	6.0E-06	1.7E-05	4.6E-06	1.2E-05	-5.22	-4.76	-5.33	-4.90	pos
Kepone	AEP-22	109	1.8	3.0E-07	1.0E-06	2.7E-07	9.2E-07	-6.53	-5.99	-6.58	-6.04	pos

ANNEX D

Results inter-laboratory phase (participant B) – agonism

Pre-screen study (participants B)

Test item	Blind label	TI max (%)	Hillslope (-)	EC10 (M)	EC50 (M)	PC10 (M)	PC50 (M)	LOG (EC10) (M)	LOG (EC50) (M)	LOG (PC10) (M)	LOG (PC50) (M)	Pos/Neg (-)
17ß-estradiol (avg)		100	3.1	2.6E-12	5.8E-12	2.3E-12	5.7E-12	-11.59	-11.24	-11.65	-11.25	pos
Norethynodrel	SPC-1	114	2.1	2.3E-10	6.4E-10	8.1E-11	5.2E-10	-9.64	-9.19	-10.09	-9.28	pos
Bisphenol A	SPC-2	185	2.6	9.2E-08	2.1E-07	4.3E-08	1.4E-07	-7.04	-6.67	-7.36	-6.86	pos
Ketoconazole	SPC-3	<10	---	---	---	---	---	---	---	---	---	neg
meso-Hexestrol	SPC-4	134	1.1	5.9E-12	4.3E-11	3.3E-12	2.6E-11	-11.23	-10.36	-11.48	-10.59	pos
Coumestrol	SPC-5	129	9.1	2.5E-09	3.3E-09	2.3E-09	3.1E-09	-8.61	-8.48	-8.63	-8.51	pos
4-Cumylphenol	SPC-6	134	1.8	7.9E-08	2.7E-07	4.4E-08	1.9E-07	-7.10	-6.57	-7.36	-6.72	pos
Butylbenzyl phtalate	SPC-7	137	1.7	2.9E-08	1.0E-07	1.7E-08	7.1E-08	-7.54	-6.98	-7.76	-7.15	pos
Genistein	SPC-8	254	0.7	5.0E-09	1.4E-07	8.4E-10	1.6E-08	-8.30	-6.85	-9.07	-7.80	pos
p,p'-methoxychlor	SPC-9	116	16	2.2E-06	2.7E-06	1.9E-06	2.7E-06	-5.66	-5.56	-5.72	-5.58	pos
Diethylstilbestrol	SPC-10	113	1.2	8.3E-12	5.0E-11	7.2E-12	4.1E-11	-11.08	-10.30	-11.14	-10.39	pos
Spironolactone	SPC-11	<10	---	---	---	---	---	---	---	---	---	neg
Reserpine	SPC-12	<10	---	---	---	---	---	---	---	---	---	neg
Linuron	SPC-13	<10	---	---	---	---	---	---	---	---	---	neg
Atrazine	SPC-14	<10	---	---	---	---	---	---	---	---	---	neg
Kaempferol	SPC-15	80	2.1	1.1E-07	3.1E-07	1.2E-07	3.9E-07	-6.97	-6.52	-6.92	-6.41	pos
Corticosterone	SPC-16	81	9.9	5.9E-05	8.8E-05	7.3E-05	9.3E-05	-4.23	-4.06	-4.14	-4.03	neg
19-Nortestosterone	SPC-17	86	9.3	8.2E-08	1.1E-07	8.6E-08	1.1E-07	-7.09	-6.96	-7.07	-6.95	pos
17a-Estradiol	SPC-18	111	1.6	1.5E-10	5.8E-10	1.3E-10	5.1E-10	-9.82	-9.23	-9.89	-9.29	pos
17a-Ethinyl estradiol	SPC-19	96	2.1	1.7E-12	4.9E-12	1.7E-12	5.0E-12	-11.77	-11.31	-11.78	-11.30	pos
4-tert-Octylphenol	SPC-20	78	9.8	3.0E-08	3.8E-08	3.2E-08	4.1E-08	-7.52	-7.42	-7.50	-7.39	pos
Etyl paraben	SPC-21	214	1.2	9.8E-06	1.2E-05	3.7E-06	1.8E-05	-5.01	-4.91	-5.44	-4.74	pos
Kepone	SPC-22	78	2.2	3.8E-07	1.0E-06	4.0E-07	1.3E-06	-6.42	-5.98	-6.40	-5.88	pos

Comprehensive study - 1 (participant B)

Test item	Blind label	TI max (%)	Hillslope (-)	EC10 (M)	EC50 (M)	PC10 (M)	PC50 (M)	LOG (EC10) (M)	LOG (EC50) (M)	LOG (PC10) (M)	LOG (PC50) (M)	Pos/Neg (-)
17ß-estradiol (avg)		107	1.4	1.5E-12	9.1E-12	1.5E-12	8.3E-12	-11.82	-11.04	-11.84	-11.08	pos
Norethynodrel	AEP-1	101	1.5	2.6E-10	1.1E-09	2.3E-10	1.1E-09	-9.58	-8.96	-9.63	-8.97	pos
Bisphenol A	AEP-2	162	1.5	1.4E-07	6.4E-07	1.0E-07	3.7E-07	-6.84	-6.19	-6.98	-6.43	pos
Ketoconazole	AEP-3	<10	---	---	---	---	---	---	---	---	---	neg
meso-Hexestrol	AEP-4	100	1.6	4.2E-12	1.6E-11	2.9E-12	1.5E-11	-11.38	-10.79	-11.54	-10.82	pos
Coumestrol	AEP-5	100	1.6	5.4E-09	2.2E-08	5.0E-09	2.1E-08	-8.26	-7.67	-8.30	-7.67	pos
4-Cumylphenol	AEP-6	116	1.8	2.3E-07	7.9E-07	2.1E-07	6.7E-07	-6.63	-6.10	-6.69	-6.17	pos
Butylbenzyl phtalate	AEP-7	114	3.2	1.3E-06	2.6E-06	1.2E-06	2.4E-06	-5.88	-5.59	-5.92	-5.62	pos
Genistein	AEP-8	214	0.9	1.5E-08	1.6E-07	1.1E-08	5.2E-08	-7.82	-6.79	-7.96	-7.29	pos
p,p'-methoxychlor	AEP-9	112	2.4	1.2E-06	3.0E-06	1.1E-06	2.8E-06	-5.92	-5.52	-5.95	-5.56	pos
Diethylstilbestrol	AEP-10	126	1.3	5.4E-12	2.8E-11	3.8E-12	2.0E-11	-11.27	-10.55	-11.42	-10.70	pos
Spironolactone	AEP-11	<10	---	---	---	---	---	---	---	---	---	neg
Reserpine	AEP-12	<10	---	---	---	---	---	---	---	---	---	neg
Linuron	AEP-13	24	2.8	1.5E-05	3.3E-05	2.8E-05	---	-4.82	-4.48	-4.55	---	pos
Atrazine	AEP-14	<10	---	---	---	---	---	---	---	---	---	neg
Kaempferol	AEP-15	120	17	2.9E-06	3.3E-06	2.9E-06	3.2E-06	-5.54	-5.48	-5.54	-5.49	pos
Corticosterone	AEP-16	<10	---	---	---	---	---	---	---	---	---	neg
19-Nortestosterone	AEP-17	84	2.1	7.2E-08	2.1E-07	6.6E-08	2.4E-07	-7.14	-6.68	-7.18	-6.61	pos
17a-Estradiol	AEP-18	110	1.7	3.3E-10	1.2E-09	3.0E-10	1.1E-09	-9.48	-8.91	-9.52	-8.96	pos
17a-Ethinyl estradiol	AEP-19	104	1.1	7.6E-13	5.9E-12	1.0E-12	5.9E-12	-12.12	-11.23	-11.99	-11.23	pos
4-tert-Octylphenol	AEP-20	151	1.5	3.9E-08	1.7E-07	2.6E-08	1.0E-07	-7.41	-6.78	-7.58	-6.99	pos
Etyl paraben	AEP-21	217	2.4	1.3E-05	3.4E-05	9.2E-06	2.0E-05	-4.88	-4.47	-5.04	-4.70	pos
Kepone	AEP-22	76	2.8	6.6E-07	1.4E-06	7.3E-07	1.8E-06	-6.18	-5.84	-6.14	-5.74	pos

Comprehensive study - 2 (participant B)

Test item	Blind label	TI max (%)	Hillslope (-)	EC10 (M)	EC50 (M)	PC10 (M)	PC50 (M)	LOG (EC10) (M)	LOG (EC50) (M)	LOG (PC10) (M)	LOG (PC50) (M)	Pos/Neg (-)
17ß-estradiol (avg)		103	1.4	1.8E-12	8.3E-12	1.8E-12	8.0E-12	-11.74	-11.08	-11.75	-11.10	
Norethynodrel	AEP-1	104	1.5	1.8E-10	8.2E-10	1.5E-10	7.6E-10	-9.74	-9.09	-9.81	-9.12	pos
Bisphenol A	AEP-2	180	1.7	1.5E-07	5.3E-07	9.6E-08	3.0E-07	-6.82	-6.27	-7.02	-6.52	pos
Ketoconazole	AEP-3	<10	---	---	---	---	---	---	---	---	---	neg
meso-Hexestrol	AEP-4	121	1.6	5.9E-12	2.4E-11	3.6E-12	1.8E-11	-11.23	-10.62	-11.44	-10.75	pos
Coumestrol	AEP-5	114	1.3	2.7E-09	1.4E-08	2.3E-09	1.2E-08	-8.58	-7.85	-8.64	-7.93	pos
4-Cumylphenol	AEP-6	140	1.5	1.1E-07	5.0E-07	9.5E-08	3.4E-07	-6.95	-6.30	-7.02	-6.46	pos
Butylbenzyl phtalate	AEP-7	136	2.5	9.6E-07	2.3E-06	8.3E-07	1.9E-06	-6.02	-5.64	-6.08	-5.73	pos
Genistein	AEP-8	219	0.7	7.6E-09	2.1E-07	8.0E-09	4.9E-08	-8.12	-6.67	-8.10	-7.31	pos
p,p'-methoxychlor	AEP-9	120	2.7	1.0E-06	2.4E-06	9.3E-07	2.1E-06	-5.98	-5.62	-6.03	-5.68	pos
Diethylstilbestrol	AEP-10	121	1.2	1.0E-11	6.3E-11	8.2E-12	4.7E-11	-10.99	-10.20	-11.09	-10.33	pos
Spironolactone	AEP-11	<10	---	---	---	---	---	---	---	---	---	neg
Reserpine	AEP-12	<10	---	---	---	---	---	---	---	---	---	neg
Linuron	AEP-13	23	1.2	1.6E-06	2.1E-05	1.7E-05	---	-5.80	-4.68	-4.78	---	pos
Atrazine	AEP-14	<10	---	---	---	---	---	---	---	---	---	neg
Kaempferol	AEP-15	120	22	2.5E-06	2.9E-06	2.5E-06	2.8E-06	-5.61	-5.54	-5.59	-5.55	pos
Corticosterone	AEP-16	<10	---	---	---	---	---	---	---	---	---	neg
19-Nortestosterone	AEP-17	88	2.0	5.0E-08	1.5E-07	5.1E-08	1.7E-07	-7.30	-6.83	-7.29	-6.78	pos
17a-Estradiol	AEP-18	105	1.4	1.3E-10	5.9E-10	5.9E-11	5.0E-10	-9.89	-9.23	-10.23	-9.30	pos
17a-Ethinyl estradiol	AEP-19	106	1.8	2.0E-12	6.6E-12	1.4E-12	5.9E-12	-11.70	-11.18	-11.84	-11.23	pos
4-tert-Octylphenol	AEP-20	139	1.9	3.6E-08	1.1E-07	2.9E-08	8.2E-08	-7.45	-6.95	-7.54	-7.09	pos
Etyl paraben	AEP-21	224	1.0	9.0E-06	1.1E-05	8.4E-06	9.6E-06	-5.04	-4.98	-5.08	-5.02	pos
Kepone	AEP-22	97	2.0	9.7E-07	3.0E-06	7.8E-07	3.0E-06	-6.01	-5.53	-6.11	-5.53	pos

ANNEX D

Results inter-laboratory phase (participant C) – agonsim

Pre-screen study (participants C)

Test item	Blind label	TI max (%)	Hillslope (-)	EC10 (M)	EC50 (M)	PC10 (M)	PC50 (M)	LOG (EC10) (M)	LOG (EC50) (M)	LOG (PC10) (M)	LOG (PC50) (M)	Pos/Neg (-)
17ß-estradiol (avg)		105	1.6	1.3E-12	6.8E-12	1.2E-12	6.3E-12	-11.89	-11.17	-11.93	-11.20	pos
Norethynodrel	SPC-1	82	47	6.4E-11	3.7E-10	---	3.6E-10	-10.20	-9.43	---	-9.44	pos
Bisphenol A	SPC-2	185	1.4	6.8E-08	3.2E-07	4.9E-08	1.7E-07	-7.17	-6.49	-7.31	-6.78	pos
Ketoconazole	SPC-3	<10	---	---	---	---	---	---	---	---	---	neg
meso-Hexestrol	SPC-4	103	1.2	3.3E-12	2.1E-11	2.4E-12	1.9E-11	-11.48	-10.67	-11.62	-10.71	pos
Coumestrol	SPC-5	170	1.3	1.7E-09	1.5E-07	1.4E-09	3.2E-08	-8.76	-6.81	-8.86	-7.49	pos
4-Cumylphenol	SPC-6	115	2.0	1.4E-07	4.2E-07	1.4E-07	3.7E-07	-6.85	-6.38	-6.86	-6.43	pos
Butylbenzyl phtalate	SPC-7	146	8.0	6.7E-07	1.2E-06	9.0E-07	1.1E-06	-6.17	-5.93	-6.05	-5.96	pos
Genistein	SPC-8	267	0.9	1.0E-07	1.1E-06	4.5E-08	2.4E-07	-6.98	-5.97	-7.35	-6.62	pos
p,p'-methoxychlor	SPC-9	122	7.9	8.8E-07	1.2E-06	9.0E-07	1.1E-06	-6.06	-5.93	-6.05	-5.94	pos
Diethylstilbestrol	SPC-10	131	1.2	1.2E-11	7.5E-11	9.3E-12	5.0E-11	-10.92	-10.13	-11.03	-10.30	pos
Spironolactone	SPC-11	<10	---	---	---	---	---	---	---	---	---	neg
Reserpine	SPC-12	<10	---	---	---	---	---	---	---	---	---	neg
Linuron	SPC-13	19	---	1.0E-06	1.0E-05	1.2E-05	---	-5.99	-4.98	-4.92	---	neg
Atrazine	SPC-14	<10	---	---	---	---	---	---	---	---	---	neg
Kaempferol	SPC-15	66	9.8	3.1E-06	3.9E-06	3.2E-06	4.3E-06	-5.51	-5.41	-5.49	-5.36	pos
Corticosterone	SPC-16	<10	---	---	---	---	---	---	---	---	---	neg
19-Nortestosterone	SPC-17	79	1.3	7.9E-08	1.0E-07	8.5E-08	1.1E-07	-7.10	-6.98	-7.07	-6.97	pos
17a-Estradiol	SPC-18	108	1.7	1.3E-10	4.8E-10	8.6E-11	4.2E-10	-9.87	-9.32	-10.07	-9.38	pos
17a-Ethinyl estradiol	SPC-19	97	1.5	5.6E-13	2.5E-12	5.3E-13	2.5E-12	-12.25	-11.61	-12.28	-11.60	pos
4-tert-Octylphenol	SPC-20	137	1.1	6.4E-09	4.5E-08	3.6E-09	2.6E-08	-8.20	-7.35	-8.44	-7.58	pos
Etyl paraben	SPC-21	316	1.1	1.7E-06	1.8E-05	4.8E-07	3.7E-06	-5.76	-4.75	-6.32	-5.43	pos
Kepone	SPC-22	98	1.1	2.6E-07	6.1E-07	2.3E-07	6.0E-07	-6.59	-6.22	-6.63	-6.22	pos

Comprehensive study - 1 (participant C)

Test item	Blind label	TI max (%)	Hillslope (-)	EC10 (M)	EC50 (M)	PC10 (M)	PC50 (M)	LOG (EC10) (M)	LOG (EC50) (M)	LOG (PC10) (M)	LOG (PC50) (M)	Pos/Neg (-)
17ß-estradiol (avg)		108	1.6	1.6E-12	8.4E-12	1.7E-12	7.7E-12	-11.79	-11.08	-11.78	-11.12	pos
Norethynodrel	AEP-1	109	1.4	1.8E-10	9.2E-10	1.3E-10	7.7E-10	-9.74	-9.04	-9.89	-9.11	pos
Bisphenol A	AEP-2	186	1.3	3.2E-08	1.7E-07	2.4E-08	8.3E-08	-7.49	-6.78	-7.62	-7.08	pos
Ketoconazole	AEP-3	<10	---	---	---	---	---	---	---	---	---	neg
meso-Hexestrol	AEP-4	122	1.6	1.5E-11	5.7E-11	6.6E-12	4.1E-11	-10.84	-10.25	-11.18	-10.38	pos
Coumestrol	AEP-5	108	1.2	9.1E-09	5.7E-08	5.2E-09	4.7E-08	-8.04	-7.24	-8.28	-7.33	pos
4-Cumylphenol	AEP-6	134	1.3	1.0E-07	5.0E-07	6.2E-08	3.3E-07	-6.99	-6.30	-7.21	-6.49	pos
Butylbenzyl phtalate	AEP-7	108	1.4	1.6E-06	8.4E-06	1.4E-06	7.4E-06	-5.78	-5.08	-5.86	-5.13	pos
Genistein	AEP-8	190	1.1	7.0E-08	5.2E-07	2.7E-08	1.9E-07	-7.15	-6.28	-7.57	-6.72	pos
p,p'-methoxychlor	AEP-9	151	1.6	3.0E-07	1.2E-06	1.5E-07	7.1E-07	-6.52	-5.93	-6.82	-6.15	pos
Diethylstilbestrol	AEP-10	58	1.4	2.4E-11	1.1E-10	2.8E-11	3.7E-10	-10.62	-9.96	-10.55	-9.43	pos
Spironolactone	AEP-11	<10	---	---	---	---	---	---	---	---	---	neg
Reserpine	AEP-12	<10	---	---	---	---	---	---	---	---	---	neg
Linuron	AEP-13	20	---	3.2E-06	1.5E-05	1.4E-05	---	-5.49	-4.82	-4.84	---	neg
Atrazine	AEP-14	<10	---	---	---	---	---	---	---	---	---	neg
Kaempferol	AEP-15	121	20	1.1E-05	1.2E-05	1.1E-05	1.2E-05	-4.97	-4.92	-4.97	-4.93	pos
Corticosterone	AEP-16	<10	---	---	---	---	---	---	---	---	---	neg
19-Nortestosterone	AEP-17	102	1.4	3.2E-08	1.5E-07	3.0E-08	1.4E-07	-7.49	-6.82	-7.52	-6.84	pos
17a-Estradiol	AEP-18	93	1.6	1.6E-10	6.2E-10	1.5E-10	6.7E-10	-9.81	-9.21	-9.82	-9.17	pos
17a-Ethinyl estradiol	AEP-19	114	1.4	1.1E-12	5.0E-12	1.0E-12	4.2E-12	-11.96	-11.30	-12.00	-11.38	pos
4-tert-Octylphenol	AEP-20	134	2.1	2.0E-08	5.6E-08	1.6E-08	4.3E-08	-7.70	-7.25	-7.80	-7.36	pos
Etyl paraben	AEP-21	84	1.4	2.3E-06	3.3E-06	2.2E-06	3.4E-06	-5.64	-5.49	-5.66	-5.47	pos
Kepone	AEP-22	87	2.7	1.5E-06	3.4E-06	1.5E-06	3.7E-06	-5.83	-5.47	-5.84	-5.43	pos

Comprehensive study - 2 (participant C)

Test item	Blind label	TI max (%)	Hillslope (-)	EC10 (M)	EC50 (M)	PC10 (M)	PC50 (M)	LOG (EC10) (M)	LOG (EC50) (M)	LOG (PC10) (M)	LOG (PC50) (M)	Pos/Neg (-)
17ß-estradiol (avg)		101	1.7	1.9E-12	6.2E-12	1.8E-12	6.1E-12	-11.71	-11.20	-11.74	-11.21	pos
Norethynodrel	AEP-1	97	1.1	2.8E-10	2.0E-09	2.8E-10	2.1E-09	-9.55	-8.70	-9.55	-8.68	pos
Bisphenol A	AEP-2	102	1.6	1.2E-08	4.7E-08	1.4E-08	4.8E-08	-7.94	-7.33	-7.86	-7.32	pos
Ketoconazole	AEP-3	<10	---	---	---	---	---	---	---	---	---	neg
meso-Hexestrol	AEP-4	109	1.1	2.1E-12	1.5E-11	3.8E-13	1.1E-11	-11.67	-10.83	-12.42	-10.97	pos
Coumestrol	AEP-5	99	1.5	7.2E-09	3.2E-08	2.6E-09	2.9E-08	-8.14	-7.49	-8.59	-7.53	pos
4-Cumylphenol	AEP-6	123	1.4	1.2E-07	6.2E-07	6.7E-08	4.4E-07	-6.91	-6.21	-7.17	-6.36	pos
Butylbenzyl phtalate	AEP-7	122	2.2	2.9E-07	7.9E-07	1.5E-07	6.2E-07	-6.55	-6.10	-6.82	-6.21	pos
Genistein	AEP-8	199	1.0	7.6E-08	7.5E-07	3.4E-08	2.4E-07	-7.12	-6.12	-7.47	-6.62	pos
p,p'-methoxychlor	AEP-9	128	1.6	3.0E-07	1.2E-06	1.9E-07	8.6E-07	-6.52	-5.93	-6.71	-6.07	pos
Diethylstilbestrol	AEP-10	106	1.3	2.0E-11	1.1E-10	1.1E-11	9.5E-11	-10.71	-9.95	-10.95	-10.02	pos
Spironolactone	AEP-11	<10	---	---	---	---	---	---	---	---	---	neg
Reserpine	AEP-12	<10	---	---	---	---	---	---	---	---	---	neg
Linuron	AEP-13	<10	---	---	---	---	---	---	---	---	---	neg
Atrazine	AEP-14	<10	---	---	---	---	---	---	---	---	---	neg
Kaempferol	AEP-15	116	24	1.0E-05	1.1E-05	9.8E-06	1.1E-05	-5.00	-4.96	-5.01	-4.97	pos
Corticosterone	AEP-16	<10	---	---	---	---	---	---	---	---	---	neg
19-Nortestosterone	AEP-17	76	1.9	4.1E-08	1.3E-07	4.1E-08	1.8E-07	-7.38	-6.89	-7.38	-6.75	pos
17a-Estradiol	AEP-18	92	1.6	1.6E-10	6.6E-10	1.7E-10	7.4E-10	-9.79	-9.18	-9.76	-9.13	pos
17a-Ethinyl estradiol	AEP-19	96	1.4	7.5E-13	3.6E-12	8.8E-13	4.0E-12	-12.12	-11.44	-12.05	-11.40	pos
4-tert-Octylphenol	AEP-20	106	1.9	9.2E-08	2.9E-07	3.5E-08	2.5E-07	-7.04	-6.54	-7.45	-6.61	pos
Etyl paraben	AEP-21	236	1.7	2.6E-06	9.5E-06	1.4E-06	4.3E-06	-5.59	-5.02	-5.85	-5.37	pos
Kepone	AEP-22	100	1.5	3.0E-07	1.3E-06	3.0E-07	1.3E-06	-6.52	-5.87	-6.53	-5.87	pos

ANNEX D

Results intra-laboratory phase (lead laboratory) – antagonism

Pre-screen study

Test item	TI min (%)	Hillslope (-)	IC20 (M)	IC50 (M)	PC80 (M)	PC50 (M)	LOG (IC20) (M)	LOG (IC50) (M)	LOG (PC80) (M)	LOG (PC50) (M)	Pos/Neg (-)
Tamoxifen	4	-2.6	2.6E-07	2.9E-07	2.6E-07	2.9E-07	-6.58	-6.54	-6.58	-6.54	pos
4OH-tamoxifen	-10	-1.4	9.9E-09	2.8E-08	1.9E-08	3.6E-08	-8.00	-7.56	-7.71	-7.44	pos
Raloxifen HCl	5	-2.2	9.0E-11	1.7E-10	9.3E-11	1.8E-10	-10.04	-9.77	-10.03	-9.75	pos
17a-Ethinyl-estradiol	>80	---	---	---	---	---	---	---	---	---	neg
Apigenin	>80	---	---	---	---	---	---	---	---	---	neg
Chrysin	>80	---	---	---	---	---	---	---	---	---	neg
Coumestrol	>80	---	---	---	---	---	---	---	---	---	neg
Genistein	>80	---	---	---	---	---	---	---	---	---	neg
Kaempferol	>80	---	---	---	---	---	---	---	---	---	neg
Resveratrol	>80	---	---	---	---	---	---	---	---	---	neg
Flutamide	>80	---	---	---	---	---	---	---	---	---	neg

Comprehensive study - 1

Test item	TI min (%)	Hillslope (-)	IC20 (M)	IC50 (M)	PC80 (M)	PC50 (M)	LOG (IC20) (M)	LOG (IC50) (M)	LOG (PC80) (M)	LOG (PC50) (M)	Pos/Neg (-)
Tamoxifen	1	-4.4	3.6E-08	7.1E-08	4.2E-08	7.7E-08	-7.44	-7.15	-7.38	-7.12	pos
4OH-tamoxifen	-6	-1.8	1.1E-10	2.4E-10	1.8E-10	2.9E-10	-9.96	-9.62	-9.75	-9.53	pos
Raloxifen HCl	0	-2.0	5.8E-11	1.1E-10	5.8E-11	1.1E-10	-10.24	-9.94	-10.24	-9.94	pos
17a-Ethinyl-estradiol	>80	---	---	---	---	---	---	---	---	---	neg
Apigenin	>80	---	---	---	---	---	---	---	---	---	neg
Chrysin	>80	---	---	---	---	---	---	---	---	---	neg
Coumestrol	>80	---	---	---	---	---	---	---	---	---	neg
Genistein	>80	---	---	---	---	---	---	---	---	---	neg
Kaempferol	>80	---	---	---	---	---	---	---	---	---	neg
Resveratrol	>80	---	---	---	---	---	---	---	---	---	neg
Flutamide	>80	---	---	---	---	---	---	---	---	---	neg

Comprehensive study - 2

Test item	TI min (%)	Hillslope (-)	IC20 (M)	IC50 (M)	PC80 (M)	PC50 (M)	LOG (IC20) (M)	LOG (IC50) (M)	LOG (PC80) (M)	LOG (PC50) (M)	Pos/Neg (-)
Tamoxifen	0	-2.1	5.7E-08	1.2E-07	5.5E-08	1.2E-07	-7.25	-6.92	-7.26	-6.93	pos
4OH-tamoxifen	-5	-2.1	1.7E-10	3.3E-10	1.8E-10	3.2E-10	-9.78	-9.48	-9.75	-9.49	pos
Raloxifen HCl	-9	-2.1	1.0E-11	1.1E-10	7.0E-12	6.4E-11	-10.99	-9.94	-11.15	-10.19	pos
17a-Ethinyl-estradiol	>80	---	---	---	---	---	---	---	---	---	neg
Apigenin	>80	---	---	---	---	---	---	---	---	---	neg
Chrysin	>80	---	---	---	---	---	---	---	---	---	neg
Coumestrol	>80	---	---	---	---	---	---	---	---	---	neg
Genistein	>80	---	---	---	---	---	---	---	---	---	neg
Kaempferol	>80	---	---	---	---	---	---	---	---	---	neg
Resveratrol	>80	---	---	---	---	---	---	---	---	---	neg
Flutamide	>80	---	---	---	---	---	---	---	---	---	neg

ANNEX D

Results inter-laboratory phase (participant A) – antagonism

Pre-screen study (participants A)

Test item	Blind label	TI min (%)	Hillslope (-)	IC20 (M)	IC50 (M)	PC80 (M)	PC50 (M)	LOG (IC20) (M)	LOG (IC50) (M)	LOG (PC80) (M)	LOG (PC50) (M)	Pos/Neg (-)
Tamoxifen (avg)		3	-2.1	3.3E-08	6.3E-08	3.3E-08	6.4E-08	-7.49	-7.20	-7.48	-7.19	pos
Apigenin	SPC-51	>80	---	---	---	---	---	---	---	---	---	neg
17a-Ethinyl-estradiol	SPC-52	>80	---	---	---	---	---	---	---	---	---	neg
Genistein	SPC-53	>80	---	---	---	---	---	---	---	---	---	neg
Flutamide	SPC-54	>80	---	---	---	---	---	---	---	---	---	neg
Coumestrol	SPC-55	>80	---	---	---	---	---	---	---	---	---	neg
Raloxifen HCl	SPC-56	<0	-2.7	4.1E-11	6.9E-11	4.2E-11	7.0E-11	-10.39	-10.16	-10.37	-10.16	pos
Resveratrol	SPC-57		---									neg
Tamoxifen	SPC-58	<0	-1.7	7.5E-08	8.6E-08	7.6E-08	8.5E-08	-7.12	-7.06	-7.12	-7.07	pos
4OH-tamoxifen	SPC-59	0	-0.9	1.3E-11	5.9E-11	1.9E-11	7.1E-11	-10.90	-10.23	-10.72	-10.15	pos
Chrysin	SPC-60	>80	---	---	---	---	---	---	---	---	---	neg
Kaempferol	SPC-61	>80	---	---	---	---	---	---	---	---	---	neg

Comprehensive study - 1 (participant A)

Test item	Blind label	TI min (%)	Hillslope (-)	IC20 (M)	IC50 (M)	PC80 (M)	PC50 (M)	LOG (IC20) (M)	LOG (IC50) (M)	LOG (PC80) (M)	LOG (PC50) (M)	Pos/Neg (-)
Tamoxifen (avg)		3	-1.8	1.5E-08	3.2E-08	1.5E-08	3.3E-08	-7.81	-7.49	-7.82	-7.49	pos
Apigenin	AEP-51	>80	---	---	---	---	---	---	---	---	---	neg
17a-Ethinyl-estradiol	AEP-52	>80	---	---	---	---	---	---	---	---	---	neg
Genistein	AEP-53	>80	---	---	---	---	---	---	---	---	---	neg
Flutamide	AEP-54	>80	---	---	---	---	---	---	---	---	---	neg
Coumestrol	AEP-55	>80	---	---	---	---	---	---	---	---	---	neg
Raloxifen HCl	AEP-56	1	-1.4	1.7E-11	4.6E-11	1.7E-11	4.7E-11	-10.77	-10.34	-10.76	-10.33	pos
Resveratrol	AEP-57	>80	---	---	---	---	---	---	---	---	---	neg
Tamoxifen	AEP-58	-2	-1.9	2.6E-08	5.2E-08	2.4E-08	5.0E-08	-7.59	-7.28	-7.62	-7.30	pos
4OH-tamoxifen	AEP-59	-10	-1.3	5.3E-11	1.6E-10	4.3E-11	1.3E-10	-10.28	-9.80	-10.37	-9.88	pos
Chrysin	AEP-60	>80	---	---	---	---	---	---	---	---	---	neg
Kaempferol	AEP-61	>80	---	---	---	---	---	---	---	---	---	neg

Comprehensive study - 2 (participant A)

Test item	Blind label	TI min (%)	Hillslope (-)	IC20 (M)	IC50 (M)	PC80 (M)	PC50 (M)	LOG (IC20) (M)	LOG (IC50) (M)	LOG (PC80) (M)	LOG (PC50) (M)	Pos/Neg (-)
Tamoxifen (ref)		3	-2.3	1.9E-08	3.6E-08	2.1E-08	3.8E-08	-7.71	-7.44	-7.69	-7.42	pos
Apigenin	AEP-51	>80	---	---	---	---	---	---	---	---	---	neg
17a-Ethinyl-estradiol	AEP-52	>80	---	---	---	---	---	---	---	---	---	neg
Genistein	AEP-53	>80	---	---	---	---	---	---	---	---	---	neg
Flutamide	AEP-54	>80	---	---	---	---	---	---	---	---	---	neg
Coumestrol	AEP-55	>80	---	---	---	---	---	---	---	---	---	neg
Raloxifen HCl	AEP-56	4	-1.6	2.3E-11	5.4E-11	2.4E-11	5.6E-11	-10.63	-10.27	-10.62	-10.25	pos
Resveratrol	AEP-57	>80	---	---	---	---	---	---	---	---	---	neg
Tamoxifen	AEP-58	1	-3.1	3.9E-08	6.1E-08	4.2E-08	6.3E-08	-7.41	-7.22	-7.38	-7.20	pos
4OH-tamoxifen	AEP-59	-4	-1.5	5.3E-11	1.3E-10	5.3E-11	1.3E-10	-10.28	-9.87	-10.27	-9.89	pos
Chrysin	AEP-60	>80	---	---	---	---	---	---	---	---	---	neg
Kaempferol	AEP-61	>80	---	---	---	---	---	---	---	---	---	neg

ANNEX D

Results inter-laboratory phase (participant B) – antagonism

Pre-screen study (participants B)

Test item	Blind label	TI min (%)	Hillslope (-)	IC20 (M)	IC50 (M)	PC80 (M)	PC50 (M)	LOG (IC20) (M)	LOG (IC50) (M)	LOG (PC80) (M)	LOG (PC50) (M)	Pos/Neg (-)
Tamoxifen (avg)		3	-2.3	3.2E-08	6.1E-08	3.3E-08	6.3E-08	-7.49	-7.21	-7.48	-7.20	pos
Apigenin	SPC-51	>80	---	---	---	---	---	---	---	---	---	neg
17a-Ethinyl-estradiol	SPC-52	>80	---	---	---	---	---	---	---	---	---	neg
Genistein	SPC-53	>80	---	---	---	---	---	---	---	---	---	neg
Flutamide	SPC-54	>80	---	---	---	---	---	---	---	---	---	neg
Coumestrol	SPC-55	>80	---	---	---	---	---	---	---	---	---	neg
Raloxifen HCl	SPC-56	0	-1.5	2.1E-12	5.3E-12	4.1E-12	7.7E-12	-11.69	-11.27	-11.39	-11.12	pos
Resveratrol	SPC-57	>80	---	---	---	---	---	---	---	---	---	neg
Tamoxifen	SPC-58	9	-2.4	9.3E-09	1.7E-08	1.1E-08	1.9E-08	-8.03	-7.78	-7.96	-7.72	pos
4OH-tamoxifen	SPC-59	-3	-1.5	3.6E-11	9.1E-11	4.5E-11	9.8E-11	-10.44	-10.04	-10.35	-10.01	pos
Chrysin	SPC-60	>80	---	---	---	---	---	---	---	---	---	neg
Kaempferol	SPC-61	>80	---	---	---	---	---	---	---	---	---	neg

Comprehensive study - 1 (participant B)

Test item	Blind label	TI min (%)	Hillslope (-)	IC20 (M)	IC50 (M)	PC80 (M)	PC50 (M)	LOG (IC20) (M)	LOG (IC50) (M)	LOG (PC80) (M)	LOG (PC50) (M)	Pos/Neg (-)
Tamoxifen (avg)		0	-2.2	4.0E-08	8.1E-08	4.0E-08	8.1E-08	-7.40	-7.09	-7.40	-7.09	pos
Apigenin	AEP-51	>80	---	---	---	---	---	---	---	---	---	neg
17a-Ethinyl-estradiol	AEP-52	>80	---	---	---	---	---	---	---	---	---	neg
Genistein	AEP-53	>80	---	---	---	---	---	---	---	---	---	neg
Flutamide	AEP-54	>80	---	---	---	---	---	---	---	---	---	neg
Coumestrol	AEP-55	>80	---	---	---	---	---	---	---	---	---	neg
Raloxifen HCl	AEP-56	3	-1.1	1.8E-11	6.4E-11	5.8E-11	1.3E-10	-10.75	-10.19	-10.23	-9.89	pos
Resveratrol	AEP-57	>80	---	---	---	---	---	---	---	---	---	neg
Tamoxifen	AEP-58	-4	-2.4	1.5E-08	2.7E-08	2.1E-08	3.0E-08	-7.82	-7.58	-7.69	-7.52	pos
4OH-tamoxifen	AEP-59	-1	-1.3	6.7E-11	2.0E-10	1.3E-10	2.8E-10	-10.17	-9.70	-9.87	-9.55	pos
Chrysin	AEP-60	>80	---	---	---	---	---	---	---	---	---	neg
Kaempferol	AEP-61	>80	---	---	---	---	---	---	---	---	---	neg

Comprehensive study - 2 (participant B)

Test item	Blind label	TI min (%)	Hillslope (-)	IC20 (M)	IC50 (M)	PC80 (M)	PC50 (M)	LOG (IC20) (M)	LOG (IC50) (M)	LOG (PC80) (M)	LOG (PC50) (M)	Pos/Neg (-)
Tamoxifen (avg)		9	-3.9	3.8E-08	5.7E-08	4.4E-08	6.3E-08	-7.42	-7.25	-7.36	-7.20	pos
Apigenin	AEP-51	>80	---	---	---	---	---	---	---	---	---	neg
17a-Ethinyl-estradiol	AEP-52	>80	---	---	---	---	---	---	---	---	---	neg
Genistein	AEP-53	>80	---	---	---	---	---	---	---	---	---	neg
Flutamide	AEP-54	>80	---	---	---	---	---	---	---	---	---	neg
Coumestrol	AEP-55	>80	---	---	---	---	---	---	---	---	---	neg
Raloxifen HCl	AEP-56	9	-1.1	2.1E-11	7.4E-11	7.8E-11	1.7E-10	-10.67	-10.13	-10.11	-9.77	pos
Resveratrol	AEP-57	>80	---	---	---	---	---	---	---	---	---	neg
Tamoxifen	AEP-58	12	-1.7	6.1E-09	1.4E-08	1.4E-08	2.5E-08	-8.21	-7.86	-7.86	-7.61	pos
4OH-tamoxifen	AEP-59	3	-1.4	6.0E-11	2.2E-10	1.5E-10	3.7E-10	-10.23	-9.65	-9.84	-9.43	pos
Chrysin	AEP-60	>80	---	---	---	---	---	---	---	---	---	neg
Kaempferol	AEP-61	>80	---	---	---	---	---	---	---	---	---	neg

ANNEX D

Results inter-laboratory phase (participant C) – antagonism

Pre-screen study (participants C)

Test item	Blind label	TI min (%)	Hillslope (-)	IC20 (M)	IC50 (M)	PC80 (M)	PC50 (M)	LOG (IC20) (M)	LOG (IC50) (M)	LOG (PC80) (M)	LOG (PC50) (M)	Pos/Neg (-)
Tamoxifen (avg)		3	-2.1	3.6E-08	8.5E-08	3.8E-08	8.9E-08	-7.45	-7.07	-7.42	-7.05	pos
Apigenin	SPC-51	>80	---	---	---	---	---	---	---	---	---	neg
17a-Ethinyl-estradiol	SPC-52	>80	---	---	---	---	---	---	---	---	---	neg
Genistein	SPC-53	>80	---	---	---	---	---	---	---	---	---	neg
Flutamide	SPC-54	>80	---	---	---	---	---	---	---	---	---	neg
Coumestrol	SPC-55	>80	---	---	---	---	---	---	---	---	---	neg
Raloxifen HCl	SPC-56	-1	-1.2	1.7E-11	5.4E-11	2.8E-11	6.8E-11	-10.77	-10.27	-10.55	-10.17	pos
Resveratrol	SPC-57	>80	---	---	---	---	---	---	---	---	---	neg
Tamoxifen	SPC-58	0	-2.1	2.6E-08	5.0E-08	3.3E-08	5.6E-08	-7.59	-7.30	-7.48	-7.25	pos
4OH-tamoxifen	SPC-59	-7	-0.7	2.5E-12	2.0E-11	3.9E-12	2.1E-11	-11.59	-10.70	-11.41	-10.68	pos
Chrysin	SPC-60	>80	---	---	---	---	---	---	---	---	---	neg
Kaempferol	SPC-61	>80	---	---	---	---	---	---	---	---	---	neg

Comprehensive study - 1 (participant C)

Test item	Blind label	TI min (%)	Hillslope (-)	IC20 (M)	IC50 (M)	PC80 (M)	PC50 (M)	LOG (IC20) (M)	LOG (IC50) (M)	LOG (PC80) (M)	LOG (PC50) (M)	Pos/Neg (-)
Tamoxifen (avg)		2	-3.1	4.8E-08	7.3E-08	5.1E-08	7.5E-08	-7.32	-7.14	-7.29	-7.12	pos
Apigenin	AEP-51	>80	---	---	---	---	---	---	---	---	---	neg
17a-Ethinyl-estradiol	AEP-52	>80	---	---	---	---	---	---	---	---	---	neg
Genistein	AEP-53	>80	---	---	---	---	---	---	---	---	---	neg
Flutamide	AEP-54	>80	---	---	---	---	---	---	---	---	---	neg
Coumestrol	AEP-55	>80	---	---	---	---	---	---	---	---	---	neg
Raloxifen HCl	AEP-56	5	-1.3	1.9E-11	5.6E-11	2.8E-11	7.0E-11	-10.72	-10.26	-10.55	-10.15	pos
Resveratrol	AEP-57	>80	---	---	---	---	---	---	---	---	---	neg
Tamoxifen	AEP-58	-4	-1.7	1.4E-08	3.2E-08	3.0E-08	4.8E-08	-7.84	-7.49	-7.52	-7.32	pos
4OH-tamoxifen	AEP-59	9	-1.7	7.2E-11	1.6E-10	9.6E-11	2.0E-10	-10.14	-9.79	-10.02	-9.70	pos
Chrysin	AEP-60	>80	---	---	---	---	---	---	---	---	---	neg
Kaempferol	AEP-61	>80	---	---	---	---	---	---	---	---	---	neg

Comprehensive study - 2 (participant C)

Test item	Blind label	TI min (%)	Hillslope (-)	IC20 (M)	IC50 (M)	PC80 (M)	PC50 (M)	LOG (IC20) (M)	LOG (IC50) (M)	LOG (PC80) (M)	LOG (PC50) (M)	Pos/Neg (-)
Tamoxifen (avg)		3	-1.9	4.0E-08	8.5E-08	5.0E-08	9.6E-08	-7.40	-7.07	-7.30	-7.02	pos
Apigenin	AEP-51	>80	---	---	---	---	---	---	---	---	---	neg
17a-Ethinyl-estradiol	AEP-52	>80	---	---	---	---	---	---	---	---	---	neg
Genistein	AEP-53	>80	---	---	---	---	---	---	---	---	---	neg
Flutamide	AEP-54	>80	---	---	---	---	---	---	---	---	---	neg
Coumestrol	AEP-55	>80	---	---	---	---	---	---	---	---	---	neg
Raloxifen HCl	AEP-56	10	-1.4	2.9E-11	8.0E-11	1.1E-10	2.0E-10	-10.54	-10.10	-9.96	-9.70	pos
Resveratrol	AEP-57	>80	---	---	---	---	---	---	---	---	---	neg
Tamoxifen	AEP-58	15	-2.5	3.4E-08	6.0E-08	6.4E-08	9.4E-08	-7.47	-7.22	-7.19	-7.02	pos
4OH-tamoxifen	AEP-59	0	-1.8	1.4E-10	3.0E-10	2.3E-10	3.9E-10	-9.85	-9.52	-9.63	-9.41	pos
Chrysin	AEP-60	>80	---	---	---	---	---	---	---	---	---	neg
Kaempferol	AEP-61	>80	---	---	---	---	---	---	---	---	---	neg

ANNEX E

Intra-laboratory phase

Intra-laboratory comparison

Agonistic and antagonistic classification of selected test chemicals (3, 4) used for the determination of intra-laboratory reproducibility, based on three independent consecutive test runs.

Agonism

Compound	Positive/Negative classification		
	run 1	run 2	run 3
Ketoconazole	neg	neg	neg
Butylbenzyl phtalate	pos	pos	pos
Genistein	pos	pos	pos
p,p'-methoxychlor	pos	pos	pos
Spironolactone	neg	neg	neg
Atrazine	neg	neg	neg
Kaempferol	pos	pos	pos
Etyl paraben	pos	pos	pos

Antagonism

Compound	Positive/Negative classification		
	run 1	run 2	run 3
Apigenin	neg	neg	neg
17a-Ethinyl-e	neg	neg	neg
Genistein	neg	neg	neg
Coumestrol	neg	neg	neg
Raloxifen HCl	pos	pos	pos
Resveratrol	neg	neg	neg
Tamoxifen	pos	pos	pos
4OH-tamoxife	pos	pos	pos
Chrysin	neg	neg	neg
Kaempferol	neg	neg	neg

The agonistic intra-laboratory coefficient of variance (%CV) (per test chemical) - based on log(EC_{50}) and log(PC_{10}) from all test chemicals determined in both comprehensive runs during the intra-laboratory phase

(LOG [EC50])

Compound	X1	X2	avg	sd	%CV
17b-estradiol	-11.25	-11.19	-11.22	0.05	0.4
Ethylparaben	-4.44	-4.55	-4.49	0.08	1.7
Kaempferol	-5.10	-5.12	-5.11	0.01	0.3
Butylbenzyl phtalate	-5.46	-5.79	-5.58	0.17	3.1
p,p'-methoxychlor	-5.36	-5.56	-5.46	0.14	2.6
19-Nortestosterone	-6.57	-6.83	-6.70	0.18	2.7
Bisphenol A	-6.42	-6.28	-6.35	0.10	1.6
Kepone	-5.81	-6.17	-5.99	0.25	4.2
4-Cumylphenol	-6.10	-6.38	-6.24	0.20	3.2
Genistein	-6.91	-6.32	-6.61	0.41	6.3
Coumestrol	-7.47	-7.85	-7.66	0.27	3.6
4-tert-Octylphenol	-6.55	-6.83	-6.69	0.20	3.0
17a-Estradiol	-8.95	-9.03	-8.99	0.06	0.6
Norethynodrel	-8.82	-9.04	-8.93	0.16	1.8
Diethylstilbestrol	-10.17	-10.46	-10.31	0.20	2.0
meso-Hexestrol	-10.40	-10.72	-10.56	0.23	2.2
17a-Ethinyl estradiol	-10.90	-11.25	-11.07	0.25	2.2
avg					2.4

(LOG [PC10])

Compound	X1	X2	avg	sd	%CV
17b-estradiol	-12.05	-11.69	-11.87	0.25	2.1
Ethylparaben	-5.10	-5.04	-5.07	0.04	0.8
Kaempferol	-5.19	-5.19	-5.19	0.00	0.0
Butylbenzyl phtalate	-5.97	-6.14	-6.05	0.12	2.0
p,p'-methoxychlor	-5.80	-6.04	-5.96	0.12	2.0
19-Nortestosterone	-7.09	-7.36	-7.23	0.19	2.6
Bisphenol A	-7.27	-6.86	-7.07	0.28	4.0
Kepone	-6.10	-6.34	-6.22	0.17	2.7
4-Cumylphenol	-6.69	-7.08	-6.88	0.27	4.0
Genistein	-7.40	-7.40	-7.45	0.06	0.9
Coumestrol	-8.10	-8.37	-8.23	0.19	2.3
4-tert-Octylphenol	-7.04	-7.30	-7.17	0.18	2.6
17a-Estradiol	-9.51	-9.61	-9.56	0.07	0.7
Norethynodrel	-9.49	-9.68	-9.58	0.13	1.4
Diethylstilbestrol	-10.76	-11.32	-11.04	0.40	3.6
meso-Hexestrol	-11.08	-11.54	-11.31	0.33	2.9
17a-Ethinyl estradiol	-11.67	-12.13	-11.90	0.33	2.7
avg					2.2

The antagonistic intra-laboratory coefficient of variance (%CV) (per test chemical) - based on log(IC_{50}) and log(PC_{80}) from all test chemicals determined in both comprehensive runs during the intra-laboratory phase

(LOG [IC50])

Compound	X1	X2	avg	sd	%CV
Raloxifen HCl	-7.15	-6.92	-7.03	0.16	2.3
Tamoxifen	-9.62	-9.48	-9.55	0.10	1.0
4OH-tamoxifen	-9.94	-9.94	-9.94	0.00	0.0
avg					1.1

(LOG [PC80])

Compound	X1	X2	avg	sd	%CV
Raloxifen HCl	-7.38	-7.25	-7.32	0.08	1.1
Tamoxifen	-9.75	-9.75	-9.75	0.00	0.0
4OH-tamoxifen	-10.24	-11.15	-10.70	0.65	6.1
avg					2.4

ANNEX F

Inter-laboratory phase

Intra-laboratory comparison

Agonistic and antagonistic classification of selected test chemicals (3, 4) used for the determination of intra-laboratory reproducibility, based on three independent consecutive test runs.

Agonism

Participant A

Compound	Positive/Negative classification		
	run 1	run 2	run 3
Ketoconazole	neg	neg	neg
Butylbenzyl phtalate	pos	pos	pos
Genistein	pos	pos	pos
p,p'-methoxychlor	pos	pos	pos
Spironolactone	neg	neg	neg
Atrazine	neg	neg	neg
Kaempferol	pos	pos	pos
Etyl paraben	pos	pos	pos

Paticipant B

Compound	Positive/Negative classification		
	run 1	run 2	run 3
Ketoconazole	neg	neg	neg
Butylbenzyl phtalate	pos	pos	pos
Genistein	pos	pos	pos
p,p'-methoxychlor	pos	pos	pos
Spironolactone	neg	neg	neg
Atrazine	neg	neg	neg
Kaempferol	pos	pos	pos
Etyl paraben	pos	pos	pos

Participant C

Compound	Positive/Negative classification		
	run 1	run 2	run 3
Ketoconazole	neg	neg	neg
Butylbenzyl phtalate	pos	pos	pos
Genistein	pos	pos	pos
p,p'-methoxychlor	pos	pos	pos
Spironolactone	neg	neg	neg
Atrazine	neg	neg	neg
Kaempferol	pos	pos	pos
Etyl paraben	pos	pos	pos

Antagonism

Participant A

Compound	Positive/Negative classification		
	run 1	run 2	run 3
Apigenin	neg	neg	neg
17a-Ethinyl-estradiol	neg	neg	neg
Genistein	neg	neg	neg
Coumestrol	neg	neg	neg
Raloxifen HCl	pos	pos	pos
Resveratrol	neg	neg	neg
Tamoxifen	pos	pos	pos
4OH-tamoxifen	pos	pos	pos
Chrysin	neg	neg	neg
Kaempferol	neg	neg	neg

Paticipant B

Compound	Positive/Negative classification		
	run 1	run 2	run 3
Apigenin	neg	neg	neg
17a-Ethinyl-estradiol	neg	neg	neg
Genistein	neg	neg	neg
Coumestrol	neg	neg	neg
Raloxifen HCl	pos	pos	pos
Resveratrol	neg	neg	neg
Tamoxifen	pos	pos	pos
4OH-tamoxifen	pos	pos	pos
Chrysin	neg	neg	neg
Kaempferol	neg	neg	neg

Participant C

Compound	Positive/Negative classification		
	run 1	run 2	run 3
Apigenin	neg	neg	neg
17a-Ethinyl-estradiol	neg	neg	neg
Genistein	neg	neg	neg
Coumestrol	neg	neg	neg
Raloxifen HCl	pos	pos	pos
Resveratrol	neg	neg	neg
Tamoxifen	pos	pos	pos
4OH-tamoxifen	pos	pos	pos
Chrysin	neg	neg	neg
Kaempferol	neg	neg	neg

ANNEX F

The intra-laboratory coefficient of variance (%CV) - based on log(EC_{50}) and log(IC_{50}) from reference standards 17β-estradiol and tamoxifen

Agonism	log(EC_{50})		
	participant A	participant B	participant C
	-11.1	-11.3	-11.3
	-11.1	-11.3	-11.1
	-11.1	-11.1	-11.0
	-11.1	-11.1	-10.9
	-10.9	-11.3	-11.4
	-11.1	-10.9	-11.2
	-11.1	-10.9	-10.9
	-11.1	-10.9	-11.1
	-11.1	-11.3	-11.1
	-11.1	-11.1	-11.2
	-10.8	-11.0	-11.2
	-10.9	-11.0	-11.2
	-10.8	-10.9	-11.3
	-11.0	-11.3	-11.2
Avg	-11.0	-11.1	-11.1
SD	0.12	0.16	0.15
%CV	1.1	1.5	1.3

Antagonism	log(IC_{50})		
	participant A	participant B	participant C
	-7.1	-7.2	-7.2
	-7.1	-7.2	-6.9
	-7.3	-7.0	-7.2
	-7.7	-7.1	-7.2
	-7.4	-7.2	-6.9
	-7.4	-7.3	-7.2
	-7.5		
	-7.5		
Avg	-7.4	-7.2	-7.1
SD	0.19	0.08	0.14
%CV	2.6	1.1	2.0

Note: The intra-laboratory coefficient of variance was calculated using the log[EC50] (agonism) or log[IC50] (antagonism) derived from all available reference standards dose-response curve-fits.

The agonistic intra-laboratory coefficient of variance (%CV) (per test chemical) - based on log(EC_{50}) and log(PC_{10}) from all test chemicals determined in both comprehensive runs during the inter-laboratory phase

Compound	participant A (LOG [EC50])					participant B (LOG [EC50])					participant C (LOG [EC50])				
	X1	X2	avg	sd	%CV	X1	X2	avg	sd	%CV	X1	X2	avg	sd	%CV
17β-estradiol	-11.12	-10.87	-11.0	0.18	1.6	-11.04	-11.08	-11.1	0.03	0.3	-11.08	-11.20	-11.1	0.09	0.8
Norethynodrel	-9.03	-8.86	-8.9	0.12	1.4	-8.96	-9.09	-9.0	0.09	1.0	-9.04	-8.70	-8.9	0.24	2.7
Diethylstilbestrol	-9.62	-9.58	-9.6	0.03	0.3	-10.55	-10.20	-10.4	0.25	2.4	-9.96	-9.95	-10.0	0.01	0.1
Linuron	-4.86	-4.54	-4.7	0.23	4.8	-4.48	-4.68	-4.6	0.14	3.1	---	---	---	---	---
Kaempferol	-5.49	-5.56	-5.5	0.05	1.0	-5.48	-5.54	-5.5	0.04	0.8	-4.92	-4.96	-4.9	0.03	0.5
19-Nortestosterone	-6.89	-6.71	-6.8	0.13	1.9	-6.68	-6.83	-6.8	0.10	1.5	-6.82	-6.89	-6.9	0.05	0.7
17a-Estradiol	-8.92	-8.86	-8.9	0.04	0.5	-8.91	-9.23	-9.1	0.22	2.5	-9.21	-9.18	-9.2	0.02	0.2
17a-Ethinyl estradiol	-11.25	-11.21	-11.2	0.03	0.3	-11.23	-11.18	-11.2	0.03	0.3	-11.30	-11.44	-11.4	0.09	0.8
Bisphenol A	-6.40	-6.33	-6.4	0.05	0.8	-6.19	-6.27	-6.2	0.06	0.9	-6.78	-7.33	-7.1	0.39	5.5
4-tert-Octylphenol	-6.91	-6.96	-6.9	0.04	0.5	-6.78	-6.95	-6.9	0.13	1.8	-7.25	-6.54	-6.9	0.50	7.3
Etyl paraben	-4.61	-4.76	-4.7	0.10	2.2	-4.47	-4.98	-4.7	0.36	7.6	-5.49	-5.02	-5.3	0.33	6.2
Kepone	-6.11	-5.99	-6.0	0.08	1.4	-5.84	-5.53	-5.7	0.22	3.9	-5.47	-5.87	-5.7	0.28	5.0
meso-Hexestrol	-10.62	-10.65	-10.6	0.02	0.2	-10.79	-10.62	-10.7	0.12	1.1	-10.25	-10.83	-10.5	0.41	3.9
Coumestrol	-8.01	-8.02	-8.0	0.01	0.2	-7.67	-7.85	-7.8	0.13	1.6	-7.24	-7.49	-7.4	0.17	2.4
4-Cumylphenol	-6.43	-6.48	-6.5	0.03	0.5	-6.10	-6.30	-6.2	0.14	2.2	-6.30	-6.21	-6.3	0.07	1.1
Butylbenzyl phtalate	-5.74	-5.70	-5.7	0.03	0.5	-5.59	-5.64	-5.6	0.04	0.7	-5.08	-6.10	-5.6	0.73	13.0
Genistein	-7.51	-7.42	-7.5	0.06	0.9	-6.79	-6.67	-6.7	0.09	1.3	-6.28	-6.12	-6.2	0.11	1.8
p,p'-methoxychlor	-5.36	-5.54	-5.5	0.13	2.4	-5.52	-5.62	-5.6	0.08	1.4	-5.93	-5.93	-5.9	0.00	0.1
Avg					1.2					1.9					3.1

Compound	participant A (LOG [PC10])					participant B (LOG [PC10])					participant C (LOG [PC10])				
	X1	X2	avg	sd	%CV	X1	X2	avg	sd	%CV	X1	X2	avg	sd	%CV
17β-estradiol	-11.87	-11.58	-11.7	0.21	1.8	-11.84	-11.75	-11.8	0.06	0.5	-11.78	-11.74	-11.8	0.03	0.3
Norethynodrel	-9.99	-9.51	-9.8	0.34	3.5	-9.63	-9.81	-9.7	0.12	1.3	-9.89	-9.55	-9.7	0.24	2.5
Diethylstilbestrol	-10.36	-10.44	-10.4	0.06	0.6	-11.42	-11.09	-11.3	0.23	2.1	-10.55	-10.95	-10.8	0.28	2.6
Linuron	-4.66	-4.60	-4.6	0.04	0.9	-4.55	-4.78	-4.7	0.16	3.5	-4.84		---	---	---
Kaempferol	-5.48	-5.69	-5.6	0.15	2.7	-5.54	-5.59	-5.6	0.04	0.7	-4.97	-5.01	-5.0	0.03	0.5
19-Nortestosterone	-7.65	-7.47	-7.6	0.13	1.8	-7.18	-7.29	-7.2	0.08	1.1	-7.52	-7.38	-7.5	0.10	1.3
17a-Estradiol	-9.81	-9.34	-9.6	0.33	3.5	-9.52	-10.23	-9.9	0.50	5.1	-9.82	-9.76	-9.8	0.04	0.4
17a-Ethinyl estradiol	-11.89	-11.90	-11.9	0.01	0.1	-11.99	-11.84	-11.9	0.10	0.9	-12.00	-12.05	-12.0	0.04	0.3
Bisphenol A	-7.30	-7.14	-7.2	0.11	1.5	-6.98	-7.02	-7.0	0.02	0.3	-7.62	-7.86	-7.7	0.17	2.1
4-tert-Octylphenol	-7.59	-7.57	-7.6	0.02	0.2	-7.58	-7.54	-7.6	0.03	0.4	-7.80	-7.45	-7.6	0.24	3.2
Etyl paraben	-5.53	-5.33	-5.4	0.14	2.6	-5.04	-5.08	-5.1	0.03	0.6	-5.66	-5.85	-5.8	0.13	2.3
Kepone	-6.76	-6.58	-6.7	0.13	2.0	-6.14	-6.11	-6.1	0.02	0.3	-5.84	-6.53	-6.2	0.49	7.9
meso-Hexestrol	-11.40	-11.31	-11.4	0.07	0.6	-11.54	-11.44	-11.5	0.07	0.6	-11.18	-12.42	-11.8	0.88	7.5
Coumestrol	-8.93	-8.89	-8.9	0.03	0.3	-8.30	-8.64	-8.5	0.24	2.9	-8.28	-8.59	-8.4	0.22	2.6
4-Cumylphenol	-7.12	-7.06	-7.1	0.05	0.7	-6.69	-7.02	-6.9	0.24	3.5	-7.21	-7.17	-7.2	0.03	0.4
Butylbenzyl phtalate	-6.37	-6.27	-6.3	0.07	1.1	-5.92	-6.08	-6.0	0.11	1.9	-5.86	-6.82	-6.3	0.68	10.7
Genistein	-8.20	-8.12	-8.2	0.06	0.7	-7.96	-8.10	-8.0	0.10	1.2	-7.57	-7.47	-7.5	0.07	1.0
p,p'-methoxychlor	-6.30	-6.31	-6.3	0.00	0.1	-5.95	-6.03	-6.0	0.06	0.9	-6.82	-6.71	-6.8	0.08	1.2
Avg					1.4					1.5					2.7

ANNEX F

The antagonistic intra-laboratory coefficient of variance (%CV) (per test chemical) - based on log(IC_{50}) and log(PC_{80}) from all test chemicals determined in both comprehensive runs during the inter-laboratory phase

Compound	participant A (LOG [IC50])					participant B (LOG [IC50])					participant C (LOG [IC50])				
	X1	X2	avg	sd	%CV	X1	X2	avg	sd	%CV	X1	X2	avg	sd	%CV
Tamoxifen	-7.49	-7.44	-7.5	0.04	0.5	-7.09	-7.25	-7.2	0.11	1.5	-7.14	-7.07	-7.1	0.05	0.7
Raloxifen HCl	-10.34	-10.27	-10.3	0.05	0.5	-10.19	-10.13	-10.2	0.04	0.4	-10.26	-10.10	-10.2	0.11	1.1
Tamoxifen	-7.28	-7.22	-7.2	0.05	0.6	-7.58	-7.86	-7.7	0.20	2.6	-7.49	-7.22	-7.4	0.19	2.6
4OH-tamoxifen	-9.80	-9.87	-9.8	0.05	0.5	-9.70	-9.65	-9.7	0.04	0.4	-9.79	-9.52	-9.7	0.19	2.0
Avg					0.5					1.2					1.6

Compound	participant A (LOG [PC80])					participant B (LOG [PC80])					participant C (LOG [PC80])				
	X1	X2	avg	sd	%CV	X1	X2	avg	sd	%CV	X1	X2	avg	sd	%CV
Tamoxifen	-7.82	-7.69	-7.8	0.10	1.2	-7.40	-7.36	-7.4	0.03	0.5	-7.29	-7.30	-7.3	0.01	0.2
Raloxifen HCl	-10.76	-10.62	-10.7	0.10	0.9	-10.23	-10.11	-10.2	0.09	0.9	-10.55	-9.96	-10.3	0.42	4.1
Tamoxifen	-7.62	-7.38	-7.5	0.17	2.3	-7.69	-7.86	-7.8	0.12	1.5	-7.52	-7.19	-7.4	0.23	3.1
4OH-tamoxifen	-10.37	-10.27	-10.3	0.07	0.7	-9.87	-9.84	-9.9	0.03	0.3	-10.02	-9.63	-9.8	0.27	2.8
Avg					1.3					0.8					2.5

ANNEX G

Inter-laboratory phase

Inter-laboratory comparison

Agonistic and antagonistic classification of selected test chemicals (3, 4) used for the determination of inter-laboratory reproducibility.

Agonism

	participant a	participant B	participant c
Norethynodrel[b]	pos	pos	pos
Bisphenol A[b]	pos	pos	pos
Ketoconazole[a]	neg	neg	neg
meso-Hexestrol[b]	pos	pos	pos
Coumestrol[b]	pos	pos	pos
4-Cumylphenol[b]	pos	pos	pos
Butylbenzyl phtalate[a]	pos	pos	pos
Genistein[a]	pos	pos	pos
p,p'-methoxychlor[a]	pos	pos	pos
Diethylstilbestrol[b]	pos	pos	pos
Spironolactone[a]	neg	neg	neg
Reserpine[b]	neg	neg	neg
Linuron[b]	pos	pos	neg
Atrazine[a]	neg	neg	neg
Kaempferol[a]	pos	pos	pos
Corticosterone[b]	neg	neg	neg
19-Nortestosterone[b]	pos	pos	pos
17a-Estradiol[b]	pos	pos	pos
17a-Ethinyl estradiol[b]	pos	pos	pos
4-tert-Octylphenol[b]	pos	pos	pos
Etyl paraben[a]	pos	pos	pos
Kepone[b]	pos	pos	pos

[a]: Positive/negative classification based on 3 consecutive test runs (pre-screen and 2 comprehensive studies) performed by each of the participating laboratories.

[b]: Positive/negative classification based on 2 consecutive test runs (2 comprehensive studies) performed by each of the participating laboratories.

Antagonism

	participant a	participant B	participant c
Apigenin[a]	neg	neg	neg
17a-Ethinyl-estradiol[a]	neg	neg	neg
Genistein[a]	neg	neg	neg
Flutamide[a]	neg	neg	neg
Coumestrol[a]	neg	neg	neg
Raloxifen HCl[a]	pos	pos	pos
Resveratrol[a]	neg	neg	neg
Tamoxifen[a]	pos	pos	pos
4OH-tamoxifen[a]	pos	pos	pos
Chrysin[a]	neg	neg	neg
Kaempferol[a]	neg	neg	neg

[a]: Positive/negative classification based on 3 consecutive test runs (pre-screen and 2 comprehensive runs) performed by each of the participating laboratories.

ANNEX G

The agonistic inter-laboratory coefficient of variance (%CV) (per test chemical) - based on log(EC_{50}) and log(PC_{10}) from all test chemicals determined in both comprehensive runs during the inter-laboratory phase

Compound	participant A	participant B	participant C			
	log(EC50) (average compr. 1 and 2 study)			Avg	SD	%CV
17β-estradiol (reference)	-11.0	-11.1	-11.1	-11.1	0.08	0.7
Norethynodrel	-8.9	-9.0	-8.8	-8.9	0.09	1.0
Bisphenol A	-6.4	-6.2	-7.0	-6.5	0.39	6.0
Ketoconazole	---	---	---	---	---	---
meso-Hexestrol	-10.6	-10.7	-10.4	-10.6	0.13	1.2
Coumestrol	-8.0	-7.7	-7.3	-7.7	0.33	4.3
4-Cumylphenol	-6.5	-6.2	-6.3	-6.3	0.14	2.2
Butylbenzyl phtalate	-5.7	-5.6	-5.3	-5.6	0.20	3.5
Genistein	-7.5	-6.7	-6.2	-6.8	0.64	9.4
p,p'-methoxychlor	-5.4	-5.6	-5.9	-5.6	0.25	4.5
Diethylstilbestrol	-9.6	-10.3	-10.0	-10.0	0.37	3.7
Spironolactone	---	---	---	---	---	---
Reserpine	---	---	---	---	---	---
Linuron	-4.7	-4.6	---	nd	nd	nd
Atrazine	---	---	---	---	---	---
Kaempferol	-5.5	-5.5	-4.9	-5.3	0.33	6.2
Corticosterone	---	---	---	---	---	---
19-Nortestosterone	-6.8	-6.7	-6.9	-6.8	0.05	0.8
17a-Estradiol	-8.9	-9.0	-9.2	-9.0	0.15	1.7
17a-Ethinyl estradiol	-11.2	-11.2	-11.4	-11.3	0.09	0.8
4-tert-Octylphenol	-6.9	-6.9	-6.8	-6.9	0.08	1.2
Etyl paraben	-4.7	-4.7	-5.2	-4.8	0.31	6.3
Kepone	-6.0	-5.7	-5.6	-5.8	0.23	4.0

Compound	participant A	participant B	participant C			
	log(PC10) (average compr. 1 and 2 study)			Avg	SD	%CV
17β-estradiol (reference)	-11.7	-11.8	-11.8	-11.8	0.04	0.4
Norethynodrel	-9.7	-9.7	-9.7	-9.7	0.01	0.1
Bisphenol A	-7.2	-7.0	-7.7	-7.3	0.37	5.1
Ketoconazole	---	---	---	---	---	---
meso-Hexestrol	-11.4	-11.5	-11.5	-11.4	0.07	0.6
Coumestrol	-8.9	-8.4	-8.4	-8.6	0.28	3.3
4-Cumylphenol	-7.1	-6.8	-7.2	-7.0	0.19	2.7
Butylbenzyl phtalate	-6.3	-6.0	-6.1	-6.1	0.16	2.7
Genistein	-8.2	-8.0	-7.5	-7.9	0.34	4.3
p,p'-methoxychlor	-6.3	-6.0	-6.8	-6.4	0.39	6.1
Diethylstilbestrol	-10.4	-11.2	-10.7	-10.8	0.42	3.9
Spironolactone	---	---	---	---	---	---
Reserpine	---	---	---	---	---	---
Linuron	-4.6	-4.6	---	nd	nd	nd
Atrazine	---	---	---	---	---	---
Kaempferol	-5.6	-5.6	-5.0	-5.4	0.33	6.2
Corticosterone	---	---	---	---	---	---
19-Nortestosterone	-7.5	-7.2	-7.4	-7.4	0.16	2.2
17a-Estradiol	-9.5	-9.7	-9.8	-9.7	0.15	1.5
17a-Ethinyl estradiol	-11.9	-11.9	-12.0	-11.9	0.07	0.6
4-tert-Octylphenol	-7.6	-7.6	-7.6	-7.6	0.02	0.2
Etyl paraben	-5.4	-5.1	-5.7	-5.4	0.35	6.4
Kepone	-6.7	-6.1	-6.1	-6.3	0.33	5.3

ANNEX G

The antagonistic inter-laboratory coefficient of variance (%CV) (per test chemical) - based on log(IC_{50}) and log(PC_{80}) from all test chemicals determined in both comprehensive runs during the interlaboratory phase

Compound	participant A	participant B	participant C	Avg	SD	%CV
	log(IC50) (average compr. 1 and 2 study)					
Tamoxifen	-7.2	-7.7	-7.4	-7.4	0.25	3.4
4OH-tamoxifen	-9.8	-9.7	-9.7	-9.7	0.06	0.6
Raloxifen HCl	-10.3	-10.2	-10.2	-10.2	0.06	0.6
17a-Ethinyl-estradiol	---	---	---	---	---	---
Apigenin	---	---	---	---	---	---
Chrysin	---	---	---	---	---	---
Coumestrol	---	---	---	---	---	---
Genistein	---	---	---	---	---	---
Kaempferol	---	---	---	---	---	---
Resveratrol	---	---	---	---	---	---
Flutamide	---	---	---	---	---	---

Compound	participant A	participant B	participant C	Avg	SD	%CV
	log(PC80) (average compr. 1 and 2 study)					
Tamoxifen	-7.5	-7.8	-7.4	-7.5	0.21	2.8
4OH-tamoxifen	-10.3	-9.9	-9.8	-10.0	0.28	2.8
Raloxifen HCl	-10.7	-10.2	-10.3	-10.4	0.28	2.7
17a-Ethinyl-estradiol	---	---	---	---	---	---
Apigenin	---	---	---	---	---	---
Chrysin	---	---	---	---	---	---
Coumestrol	---	---	---	---	---	---
Genistein	---	---	---	---	---	---
Kaempferol	---	---	---	---	---	---
Resveratrol	---	---	---	---	---	---
Flutamide	---	---	---	---	---	---

ANNEX H

Comparison between ERα CALUX inter-laboratory validation results and results from OECD-accepted stably transfected transactivation *in vitro* assays to detect estrogen receptor agonists and antagonists (Bg1Luc and STTA)

Compound	Bg1Luc LOG (EC50)	ERα CALUX LOG (EC50)	STTA LOG (PC10)	ERα CALUX LOG (PC10)
17β-estradiol (reference)	-11.2	-11.1	-11.2	-11.8
Norethynodrel	-9.0	-8.9	-9.0	-9.7
Bisphenol A	-6.3	-6.5	-6.3	-7.3
Ketoconazole	---	---	---	---
meso-Hexestrol	-10.8	-10.6	-10.8	-11.4
Coumestrol	-6.9	-7.7	-6.9	-8.6
4-Cumylphenol	-6.5	-6.3	-6.5	-7.0
Butylbenzyl phtalate	-5.7	-5.6	-5.7	-6.1
Genistein	-6.6	-6.8	-6.6	-7.9
p,p'-methoxychlor	-5.7	-5.6	-5.7	-6.4
Diethylstilbestrol	-10.5	-10.0	-10.5	-10.8
Spironolactone	---	---	---	---
Reserpine	---	---	---	---
Linuron	---	nd	---	nd
Atrazine	---	---	---	---
Kaempferol	-5.4	-5.3	-5.4	-5.4
Corticosterone	---	---	---	---
19-Nortestosterone	-5.7	-6.8	-5.7	-7.4
17a-Estradiol	-8.9	-9.0	-8.9	-9.7
17a-Ethinyl estradiol	-11.1	-11.3	-11.1	-11.9
4-tert-Octylphenol	-7.5	-6.9	-7.5	-7.6
Etyl paraben	-4.6	-4.8	-4.6	-5.4
Kepone	-6.3	-5.8	-6.3	-6.3

Compound	Bg1Luc LOG (IC50)	STTA LOG (IC50)	ERα CALUX LOG (IC50)
Tamoxifen	-6.1	-6.3	-7.4
4OH-tamoxifen	-6.7	-8.4	-9.7
Raloxifen HCl	-8.9	-9.1	-10.2
17a-Ethinyl-estradiol	---	---	---
Apigenin	---	---	---
Chrysin	---	---	---
Coumestrol	---	---	---
Genistein	---	---	---
Kaempferol	---	---	---
Resveratrol	---	---	---
Flutamide	---	---	---

Compound	Bg1Luc LOG (PC80)	STTA LOG (PC80)	ERα CALUX LOG (PC80)
Tamoxifen	not available	not available	-7.5
4OH-tamoxifen	not available	not available	-10.0
Raloxifen HCl	not available	not available	-10.4
17a-Ethinyl-estradiol	not available	not available	---
Apigenin	not available	not available	---
Chrysin	not available	not available	---
Coumestrol	not available	not available	---
Genistein	not available	not available	---
Kaempferol	not available	not available	---
Resveratrol	not available	not available	---
Flutamide	not available	not available	---