DB-ALM Method Summary n° 455 : Transactivation Assays to Detect Estrogen Receptor Agonists and Antagonists *In Vitro* with Stably Transfected Human Cell Lines

Effects on Endocrine System, Effects on the female fertility

A family of mechanistically and functionally similar test methods were developed and two were validated for the identification of endocrine active substances: estrogen receptor agonists and antagonists. The assays are based on the methodology of Stably Transfected Estrogen Receptor Transactivation (ERTA) in human cancer cell lines (*The Performance-Based OECD Test Guideline 455*).

1. General Information

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Proprietary and/or Confidentiality Issues:

The cell lines on which the assays are based, were developed and commercialised by different investigators and their use requires a material transfer agreement. Further details are provided under the **Experimental system** section.

Assay Throughput:

The standard assays are in the 96-well plate format. For each agonist or antagonist assay one plate is used per maximum of two- or two plates for four compounds, tested over a range of concentrations. The complete test (including a preliminary range finder and the final test) takes approximately two working weeks.

The **BG1Luc Estrogen Receptor Transactivation (BG1Luc ERTA)** assay was recently adopted for high throughput testing using 96- and 1536-well format (e.g. Milcamps et al., 2012; Ceger et al., 2015). In this adoption wide concentration ranges and multiple replicates can be tested in one run and the preliminary range finder step is not necessary.

Status

Known Laboratory Use:

Bayer Schering Pharma AG (Germany)
Cryprotex (UK)
EURL ECVAM (IT)
Flemish Institute for Technological Research (VITO, Belgium)
Hiyoshi Corporation (JP)
National Center for Advancing Translational Sciences (NCATS; USA)
National Institute of Food and Drug Safety Evaluation (Korea)
RTC (IT)
Xenobiotic Detection Systems (USA)

Participation in Evaluation Studies:

The **Stably Transfected Transactivation Assay (STTA)** uses the human ERα-HeLa-9903 cell line, derived from a human cervical tumor. It was initially established and evaluated in Japan for detecting estrogenic activity (Takeyoshi, 2006).

BG1Luc ERTA assay uses the BG1Luc-4E2 cell line, derived from a human ovarian adenocarcinoma. It has been first developed and evaluated in the National Food Safety and Toxicology Center, Center for Integrative Toxicology, Michigan State University (USA), as a part of the US EPA Endocrine Disruptor-Screening Program (EDSP), mandated by the Food Quality Protection Act of 1996 (Hecker and Hollert, 2011).

The **ERα-CALUX** and **MELN ERTA assays** were evaluated (among a number of gene reporter assays) in a feasibility study of *in vitro* approach to detection of reproductive toxicants, conducted within the 6th European Framework Programme ReProTect project (Schenk et al., 2010; van der Burg et al., 2010; Witters et al., 2010).

Participation in Validation Studies:

Two ERTA's: **STTA** and the **BG1Luc ERTA** participated in validation studies, which demonstrated their relevance and reliability for their intended purpose (e.g. Takeyoshi, 2006; ICCVAM, 2012).

Performance Standards (PS) for luminescence-based BG1Luc ERTA assay were defined to demonstrate the sufficient accuracy and reliability of the method for its specific purpose and to facilitate the development and validation of similar methods in the future. They are included in ICCVAM Test Method Evaluation Report "The LUMI-CELL® ER Test Method: An *In Vitro* Assay for Identifying Human Estrogen Receptor Agonist and Antagonist Activity of Chemicals" (ICCVAM, 2012). These performance standards have been subsequently modified to be applicable to both the **STTA** and **BG1Luc ERTA** test methods (OECD, 2015). They also serve as benchmark in the ongoing validation of "me-too tests" **ERα-CALUX** and **MELN ERTA**.

Regulatory Acceptance:

The "Estrogen Receptor Transcriptional Activation (Human Cell Line HeLa-9903)" for detection of ER agonists with **STTA** was adopted in 2009 by the US EPA as Endocrine Disruptor Screening Program Test Guideline **OPPTS 890.1300** (US EPA, 2016) and by the OECD as the first version of the **Test Guideline No. 455** *"The Stably Transfected Human Estrogen Receptor-alpha Transcriptional Activation Assay for Detection of Estrogenic Agonist-Activity of Chemicals*" (OECD, 2015).

The **BG1Luc ERTA** was added to the OECD Test Guideline No. 455 in 2012. The detection of the Estrogen Antagonists by **BG1Luc ERTA** was formerly addressed by OECD Test Guideline No. 457: "BG1Luc Estrogen Receptor Transactivation Test Method for Identifying Estrogen Receptor Agonists and Antagonists" (now obsolete).

In 2015 TG No. 455 has been thoroughly revised and became the **Performance-Based Test Guideline for Stably Transfected Transactivation In Vitro Assays to Detect Estrogen Receptor Agonists and Antagonists**, which refers to both **STTA** and **BG1Luc ERTA** (OECD, 2015).

2. Method Definition

Purpose of the Method

TYPE OF TESTING	:	Screening, Part of an integrated testing strategy, Part of a test battery
LEVEL OF TOXICITY ASSESSMENT	:	Toxic potency, Risk assessment
PURPOSE OF TESTING	:	Screen or priority setting, Mechanistic studies, Ranking, Drug evaluation

Context of use

The **Performance-Based OECD Test Guideline (PBTG) No. 455** comprises mechanistically and functionally similar test methods for the identification of estrogen receptor agonists and antagonists and should facilitate the development of new similar or modified test methods for disruption of estrogen-regulated systems (OECD, 2015).

The two reference test methods that provide the basis for this PBTG are: the **Stably Transfected Transactivation Assay (STTA)** using the (h) ERα-HeLa-9903 cell line, derived from a human cervical tumor, and the **BG1Luc ER Transactivation assay (BG1Luc ERTA)** using the BG1Luc-4E2 cell line, derived from a human ovarian adenocarcinoma. The cell lines used in these assays express estrogene receptor (ER) and have been stably transfected with an ER responsive luciferase reporter gene. The **Experimental System** section (*page 4*) provides further details on each construct. They are collectively refrerred to as **ERTA** (Estrogen Receptor Transactivation) **assays**.

The interaction of estrogens with ERs can affect transcription of estrogen-controlled genes, which can lead to the induction or inhibition of cellular processes, including those necessary for cell proliferation, normal fetal development, and reproductive function (Cavaillès, 2002). Perturbation of normal estrogenic systems may have the potential to trigger adverse effects on normal development (ontogenesis), reproductive health and the integrity of the reproductive system (e.g. Naz et al., 1999).

ERTA assays are being proposed for screening and prioritisation purposes, but also provide mechanistic information that can be used in a weight of evidence approaches to evaluated the effects on endocrine system (reviewed in depth in the Guidance Document no. 178; OECD, 2012). However, ERTA only addresses the effects on transcription caused by chemical binding to the ER in an *in vitro* system. Thus, the results should not be directly extrapolated to the complex signaling and regulation of the intact endocrine system *in vivo* (OECD, 2015).

TA mediated by the ERs is considered one of the important mechanisms of endocrine disruption (ED), although there are other mechanisms through which ED can occur, including *(i)* interactions with other receptors and enzymatic systems within the endocrine system, *(ii)* hormone synthesis, *(iii)* metabolic activation and/or inactivation of hormones, *(iv)* distribution of hormones to target tissues, and (v) clearance of hormones from the body (OECD, 2012). None of the test methods under the PBTG No. 455 addresses these modes of action (OECD, 2015).

The ERTA assays described in the PBTG No. 455 are designed to identify estrogen receptor agonists and antagonists and correspond to the level 2 of the OECD Conceptual Framework for Screening and Testing of Endocrine Disrupting Chemicals: "*In vitro* assays providing data about selected endocrine mechanism(s)/pathway(s)"(OECD, 2012). An example of the methods' application in relation to the biological organisation/pathway is shown in the **TABLE I** below.

TABLE I: Example of the ERTA assay application in relation to the biological organisation/pathway.

Biological level of organisation	**Biological object**	**Type of effect addressed**	**Test method**
System	Endocrine/Reproductive	Modulation/perturbation	Test system: **ER Transactivation reporter systems in cancer cell lines derived from human female reproductive organs**
Organ	Ovaries		
Tissue	Ovarian tissue		
Cellular	Ovarian cells		
Molecular	Estrogen Receptor	Receptor Function: - activation (agonist) - inhibition (antagonist)	**Estrogen-responsive transactivation assays**

Scientific Principle of the Method

In vitro TA assays study an interaction of a chemical with a specific receptor that regulates the transcription of a reporter gene product. Such assays have been used extensively to evaluate gene expression regulated by specific nuclear receptors, such as ERs (e.g. Escande et al., 2006) and have been proposed for the detection of estrogenic transactivation (e.g. EDSTAC, 1998).

Estrogen receptor is a nuclear protein, of which there are at least two major subtypes known: ERα and ERβ, which are encoded by distinct genes. The respective proteins have different biological functions, tissue distributions and ligand binding affinities (e.g. Harris et al., 2005). Nuclear ERα mediates the classic estrogenic response whereas the function of ERβ is less clear and seems to be related to the regulation of cell proliferation and oncogenes (e.g. Jensen and De Sombre, 1969). Therefore most models currently being developed to measure ER activation are specific to ERα.

Following ligand binding, the estrogen receptor-ligand complex translocates to the nucleus where it binds specific DNA response elements and activates the expression of target genes. The cell lines used in the OECD TG 455 carry firefly luciferase reporter genes under the control of ER- sensitive promoters. Their activation results in increased cellular expression of luciferase enzyme. Luciferin is a substrate that is transformed by the luciferase enzyme to a bioluminescence product that can be quantitatively measured with a luminometer. Luciferase activity can be evaluated quickly and inexpensively with a

number of commercially available test kits (OECD, 2015).

ER agonists (substances with estrogen-like activity) will induce luciferase activity in the test system.

ER antagonists (which upon binding to a receptor, block or dampen agonist-mediated responses) will reduce the luciferase activity in presence of an ER agonist such as E2 (17β-estradiol, a natural ER ligand).

Procedure Description

Biological Endpoint and Measurement:

The ERTA assays serve as a surrogate model of the initial steps of the process of perturbation of normal estrogenic systems, leading to potential adverse health effects. The following endpoints are addressed in these assays:

ESTROGENIC ACTIVITY (MOLECULAR INTERACTION) : assessed through transactivation gene reporter assay

ANTI-ESTROGENIC ACTIVITY (MOLECULAR INTERACTION) : assessed through transactivation gene reporter assay

CELL VIABILITY is assessed to ensure that the concentrations of the chemical tested for estrogenic or anti-estrogenic activity are not cytotoxic. For this purpose a visual assessment of cell morphology, or LDH leakage, or MTT assays are typically used.

Endpoint Value:

EC_{50} - the half maximal effective concentration of a test substance

LEC - Lowest Effective Concentration of a test compound

RPC_{Max} - maximum level of response induced by a test chemical, expressed as a percentage of the response induced by 1 nM E2 on the same plate

PC_{Max} - the concentration of a test substance inducing the RPC_{Max}

PC_{50} - the concentration of a test substance at which the measured activity in an agonist assay is 50% of the maximum activity induced by the PC (E2 at the reference concentration specified in the test method) in each plate.

Experimental System:

(h)ER-HeLa-9903 is a stably transfected cell line derived from human cervix carcinoma. The HeLa cell line carries two stably inserted constructs: *(i)* the hERα expression construct (encoding the full-length human receptor), and *(ii)* a firefly luciferase reporter construct bearing five tandem repeats of a vitellogenin Estrogen-Responsive Element (ERE) driven by a mouse metallothionein (MT) promoter TATA element. The mouse MT TATA gene construct has been shown to have the best performance, and so is commonly used. Consequently this hERα-HeLa-9903 cell line can be used to measure the ability of a test chemical to induce hERα-mediated transactivation of luciferase gene expression (Takeyoshi, 2006). It can be obtained from e.g. Japanese Collection of Research Bioresources (JCRB) Cell Bank, upon signing a material transfer agreement.

BG1Luc-4E2 cell line is derived from the ER-responsive human ovarian adenocarinoma cell line BG-1, which has been stably transfected with ER-responsive luciferase reporter gene under control of four estrogen response elements placed upstream of the mouse mammary tumor virus promoter (MMTV). The cell line predominately expresses native hERα with some contribution from hERβ (Rogers and Denison, 2000). The cell line is currently available with a technical licensing agreement from the University of California, Davis, California, USA, and from Xenobiotic Detection Systems Inc., Durham, North Carolina, USA.

Metabolic Competence:

Not evaluated in the validation studies. Can be applied in addition.

Exposure regime:

Application type : Single dose

Application volume : 50-200 µl
Dosage : concentration range (dose-response curve)
Min : 10^{-11} M
Max : 10^{-3} M
Exposure time : 19-24 h

Controls used:

TABLE II: Controls and reference chemicals typically used in the ERTA

	Agonist Assay	***Antagonist Assay***
Positive control (PC)	E2 (17β-Estradiol; strong agonist); p,p'-Methoxychlor (weak agonist)	E2 alone (baseline) E2 in combination with Tamoxifen, 4-Hydroxytamoxifen (OHT) or Digitonin (antagonists)
Negative control (VC)	Vehicle used to dissolve test chemicals	Vehicle used to dissolve test chemicals
Reference chemicals	E2 17α-Methyl testosterone (very weak agonist) Corticosterone (negative substance)	E2 in combination with Raloxifene HCl (Ral, antagonist) E2 in combination with Tamoxifen (antagonist) and Flutamide (negative substance)

Basic procedure:

Each study consists of two parts: (i) *Agonist Assay* and (ii) *Antagonist assay,* outlined in the scheme in **Figure 1**. The procedures are very similar for both parts regarding media, duration and quality criteria, with the main differences regarding the controls and reference chemicals.

Each of the two assays is typically performed in two steps:

1. *Range finder* - a preliminary test to determine the appropriate concentration range of a chemical to be tested, taking into account any possible solubility or cytotoxicity issues. Depending on the behavior of the test chemical this type of experiment might be performed once or require a number of iterations.
2. *Comprehensive (main) assay* - the definite test of a chemical over a range of concentrations selected to cover a full concentration-response curve (if possible) and remaining below the limits of solubility and cytotoxic effects.

The reference chemicals (E2, 17α-Estradiol, 17α-Methyl testosterone and Corticosterone for agonist assay and Ral or Tamoxifen and Flutamide for antagonist assay) should be tested prior to the study (to establish historical reference values) and in every run of the comprehensive assay.

The cells are maintained according to a cell line-specific protocol and cultivated in a 5% CO_2 incubator at 37±1ºC. Upon reaching 75-90% confluency the cells are subcultured in an estrogen-free medium and plated in 96-well plates for chemical exposure.

After adding the chemicals, the assay plates are returned to the CO_2 incubator and kept at 37±1ºC for 19-24 hours to induce the reporter gene products.

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

In the high throughput adaptation wide concentration ranges and multiple replicates can be tested in one run so that the preliminary range finder step is not necessary.

OECD TG No. 455 (OECD, 2015) presents the details of the two standard operating procedures for the reference test methods:

- *Annex 2* - **Stably Transfected Trans Activation (STTA) Assay** using the **(h)ER-HeLa-9903 cell line**
- *Annex 3* - **BG1Luc ER Trans Activation (TA) assay** using the **BG1Luc cell line**

Luciferase assay

A commercial luciferase assay reagent [e.g. Steady-Glo® Luciferase Assay System (Promega, E2510, Promega, E1500, or equivalents) with an appropriate lysis reagent (eg. Promega, E1531, or equivalents) can be used, following manufacturers instructions, for the endpoint measurement in the ERTA and as long as the acceptability criteria are met. The assay reagents should be selected based on the sensitivity of the available luminometer.

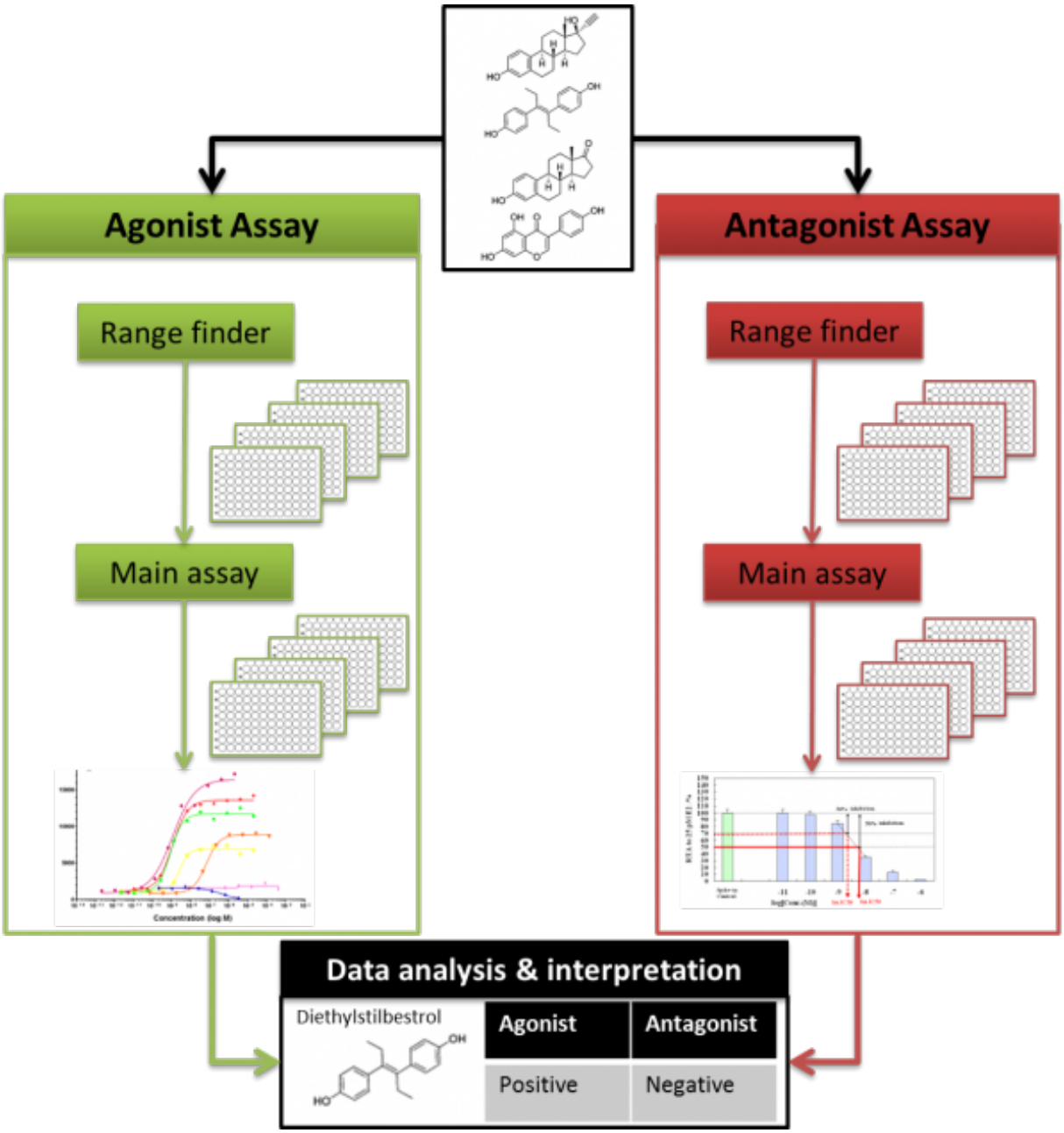


Figure 1: Schematic illustration of the main steps of the ER TA method.

Quality/Acceptance Criteria

The quality assurance procedures defined for the reference test methods: the **STTA** and the **BG1Luc ERTA assays** should be followed as described in the *Annex 2* and *Annex 3* in OECD TG 455 (OECD, 2015).

In general, the cells should be free of mycoplasma and any other contaminations, show stable and consistent ER response over time and remain within the limits of the maximal number of allowed passages.

Prior to testing any unknown chemical, a new laboratory (or any new technician in an experienced laboratory) should demonstrate a technical proficiency using the test method by testing the 14 proficiency substances listed in **Table 3** of the **OECD TG 455** for agonist assay and 10 proficiency substances in **Table 4** of the **OECD TG 455** for antagonist assay. This testing will also confirm the responsiveness of the test system. Tests with these substances should be replicated at least twice, on

different days. Proficiency is demonstrated by the correct classification (positive/negative) of each proficiency substance (OECD, 2015).

The ability to consistently conduct the test method should be demonstrated by the development and maintenance of a historical database for the reference agonists and antagonists and controls. Standard deviations (SD) or coefficients of variation (CV) for the means of reference estrogen curve fitting parameters from multiple experiments may be used as a measure of within-laboratory reproducibility.

Prior to and during any study, the responsiveness of the test system should be verified using the appropriate concentrations of the ***Reference chemicals*** for agonist and antagonist assays listed in the **TABLE III**.

TABLE III: ***Reference chemicals*** for agonist and antagonist assays

Assay	Reference Agonists	Reference Antagonists
STTA assay	strong estrogen: E2, weak estrogen: 17α-estradiol, very weak agonist: 17α-methyltestosterone, negative compound: corticosterone	Tamoxifen/E2 and Flutamide/E2 combination
BG1Luc ERTA assay	E2	Ral/E2 combination

These reference chemicals and appropriate positive and negative controls should be included with each experiment and the results should fall within the given acceptable limits, individually defined for each assay (for **STTA** see *Annex 2*, for **BG1Luc ERTA** see *Annex 3* of the OECD TG 455; OECD, 2015).

Acceptance or rejection of a test run is based on the evaluation of results obtained for the reference chemicals and controls with respect to each other as well as with the historical data. Values for the relative luminescence, concentration-response curves, PC_{10}, PC_{50}, EC_{50} or IC_{50} values for the reference chemicals should meet the acceptability criteria as provided for the selected test method, and all positive/negative controls should be correctly classified for each accepted experiment.

If this is not the case, the cause for the failure to meet the acceptability criteria should be determined (*e.g.* cell handling, and serum and antibiotics for quality and concentration) and the assay repeated.

The following principles regarding acceptability criteria should be respected:

- The concentrations tested should remain within the solubility range of the test chemical and not demonstrate cytotoxicity.
- Data should be within the standard deviation limits derived from the historical range.
- Data should be sufficient for a quantitative assessment of ER activation/inhibition (i.e. efficacy and potency).
- The mean reporter activity for the E2 (a positive control in agonist assay and a baseline in antagonist assay) should be at least the minimum (specified for the specific test method) relative to that of the vehicle control.
- The mean reporter activity for the reference concentration of the estrogen antagonists in the presence of E2 should be at least the minimum specified in the test method relative to that of the positive control to ensure adequate sensitivity.

Data Interpretation and/or Prediction Models

The data interpretation procedure should be defined for each test method for classifying a positive and negative response. There is currently no universally agreed method for further interpretation of the ERTA results, beyond the positive and negative classification. However, both qualitative (e.g. positive/negative) and/or quantitative (e.g. EC50, PC50) assessments of effect of test substance on the ER-mediated activity should be based on empirical data and sound scientific judgement. Where possible, positive results should be characterised by both the magnitude of the effect as compared to the vehicle (solvent) control or reference estrogen and the concentration at which the effect occurs (e.g. EC50, PC10, PC50, RPCMax, etc.).

Data interpretation for ERTA is based upon whether or not the maximum response level induced by a test chemical equals or exceeds a predefined criteria for the given test method (for **STTA** see paragraph

42 of the *Annex 2*, for **BG1Luc ERTA** see paragraph 41 in the *Annex 3* of the OECD TG 455; OECD, 2015).

Meeting the acceptability criteria defined for each test method indicates the assay system is operating properly, but it does not ensure that any particular test will produce accurate data (for **STTA** see paragraphs 14-20 of the *Annex 2*, for **BG1Luc ERTA** see paragraphs 11, 20, 21 and 27 in the *Annex 3* of the OECD TG 455; OECD, 2015).

Replicating the correct results of the first test is the best indication that accurate data were produced. If two tests give reproducible results (e.g., both test results indicate a substance is positive), it is not necessary to conduct a third test.

If two runs do not give reproducible results (e.g. a substance is positive in one run and negative in the other run), or if a higher degree of certainty is required regarding the outcome of this assay, at least three independent runs should be conducted.

Prediction Model (PM):

Not applicable

3. Method Performance

Robustness of the Method

Within-laboratory reproducibility:

The reproducibility of the **STTA**,**BG1Luc ERTA**, **ERα-CALUX** and **MELN** assays in individual test laboratories and the stability of the positive control response over time are documented in the respective study reports (Takeyoshi, 2006, ICCVAM, 2012; van der Burg et al., 2010; Witters et al., 2010).

Between-laboratory transferability and reproducibility:

In the interlaboratory validation study **STTA** was successfully transferred among four laboratories, testing 9 coded substances and a positive control (E2) in agonist assay. The independent statistical analysis concluded that the observed level of variability of this assay seemed satisfactorily low for the intended use (Takeyoshi, 2006).

The **BG1Luc ERTA** assay was successfully transferred to three laboratories, testing 12 substances agonist and antagonist activity, followed by 36 substances tested for agonist activity and 41 for antagonist activity in the next phase of the study. The final outcome (accuracy and reliability) was considered equivalent to that of the STTA (ICCVAM, 2012).

The transferability of the **MELN Assa**y from experienced to a naive laboratory was tested with 12 substances for agonist activity and 10 for antagonist activity. The results obtained in both laboratories were in good agreement and the authors concluded that the protocol was sufficiently mature for transfer to third parties (Witters et al., 2010).

General performance measures:

Data interpretation for ERTA is primarily based upon a positive/negative classification, based on whether the response level induced by a test chemical equals or exceeds a criteria specifically defined for the given test method (OECD, 2015).

This approach accommodates the inherent variability of the quantitative ERTA results and facilitates the use of performance standards in proficiency testing and method validation. OECD TG 455 defines a list of reference substances which are expected to give negative or positive response in the correctly executed agonist and antagonist assays. The *"positive"* and *"negative"* classification of the reference substances is derived from the qualitative studies of ER binding and the *in vivo* uterotrophic assay. There is no absolute benchmark against which the quantitative results of the ERTA can be validated, save for another ERTA assay. For example the EC_{50} obtained with the **STTA** system showed high consistency with the data obtained by the **ERα-CALUX**, **HELN-ERα** and **BG1Luc ERTA** assays at R^2=0.987 (n=8), R^2=0.937 (n=7), and R^2=0.922 (n=7) respectively.

In the validation study of the **BG1Luc ERTA assay** conducted by ICCVAM, the reference chemicals used

in the evaluation of the method's performance (as illustrated in the **TABLE IV**) were classified based on a literature review for their effects on ER binding, STTA and other ERTA assays and uterotrophic response (ICCVAM, 2012).

TABLE IV: The performance of **BG1Luc ER Transactivation assay** in the ICCVAM validation study

	Agonist assay	*Antagonist assay*
Concordance	97% (34/35)	100% (25/25)
Sensitivity	96% (27/28)	100% (3/3)
Specificity	100% (7/7)	100% (22/22)
False positive rate	0% (0/7)	0% (0/22)
False negative rate	4% (1/28)	0% (0/3)

Test Compounds

In the course of the method development and validation studies numerous chemicals were tested and the results published. The **BG1Luc ERTA** assay was also performed with the 10,486 chemicals as the U.S. Tox21 Program. The results are publicly available via the PubChem BioAssay portal (**https://pubchem.ncbi.nlm.nih.gov/**) and ACTOR dashboard (**http://actor.epa.gov/dashboard**, assay name: TOX21_ERa_LUC_BG1)

The OECD TG 455 (OECD, 2015) provides the test results for the chemicals tested in both **STTA** and **BG1Luc ERTA** assays and classified as positive or negative. It covers the results of 34 chemicals tested in agonist assays (Table1 in TG 455) and 15 chemicals tested in antagonist assays (Table 2 in TG 455). There was 100% agreement between the two test methods on the classifications of all the substances except for one substance (Mifepristone) for antagonist assay, and each substance was correctly classified as an ER agonist/antagonist or negative. Supplementary information on this group of chemicals as well as additional chemicals tested in the **STTA** and **BG1Luc ERTA** test methods during the validation studies is provided in the Performance Standards for the ERTA's (OECD 2012 and 2015).

Other notable test results were published in:

- ICCVAM (2012), ICCVAM Test Method Evaluation Report, The LUMI-CELL® ER (**BG1Luc ERTA**) Test Method: An In Vitro Assay for Identifying Human Estrogen Receptor Agonist and Antagonist Activity of Chemicals, NIH Publication No. 11-7850; ***Main document*** and ***Appendix B1*** (BG1Luc ER TA – Agonist Protocol);
  Available from:
  ntp.niehs.nih.gov/pubhealth/evalatm/test-method-evaluations/endocrine-disruptors/bg1luc/
- Takeyoshi (2006), Draft Report of Pre-validation and Inter-laboratory Validation For Stably Transfected Transcriptional Activation (TA) Assay to Detect Estrogenic Activity - The Human Estrogen Receptor Alpha Mediated Reporter Gene Assay Using hER-HeLa-9903 Cell Line - Ver.2006.Oct.06
  Available from: **www.oecd.org/chemicalsafety/testing/37504278.pdf**
- Pubchem (2013) qHTS assay to identify small molecule antagonists of the estrogen receptor alpha (ER-alpha) signaling pathway using the **BG1Luc ERTA**
  Available from: **https://pubchem.ncbi.nlm.nih.gov/bioassay/743080**
- Pubchem (2013) qHTS assay to identify small molecule agonists of the estrogen receptor alpha (ER-alpha) signaling pathway using the **BG1Luc ERTA**
  Available from: **https://pubchem.ncbi.nlm.nih.gov/bioassay/743091**

Applicability:

The two ERTA assays had been validated for use with chemicals soluble vehicles such as water, ethanol or DMSO. Considering that only single substances were used during the validation, the applicability to test mixtures has not been addressed in the regulatory acceptance process.

Special considerations will need to be applied to those compounds that are highly volatile. In such cases, nearby control wells may generate false positives, and this should be considered in light of expected and historical control values. In the few cases where volatility may be of concern, the use of “plate sealers” may help to effectively isolate individual wells during testing, and is therefore recommended in such cases.

Regarding applications outside the scope of OECD TG 455, there are no technical limitations preventing the use of ER TA with mixtures, product formulations or samples with unknown composition. The only requirement is that at the tested dose the test substance can be dissolved in the culture medium and is not toxic to the cells. Some examples of such use are listed in the **Discussion**.

4. Discussion

Ethical issues and considerations for 3R's impact

The OECD TG 455 belongs to the Level 2 of the OECD Conceptual Framework (CF) for the Testing and Assessment of Endocrine Disrupters. The aim of the CF is to identify standardized test methods that can be used to evaluate chemicals for endocrine disruption. It does not rely solely on*in vivo* experimentation, but encourages the development and use of existing data and non test information (Level 1) and *in vitro* assays providing data about selected endocrine mechanism(s)/pathway(s) (Level 2) to facilitate informed decision making on requirements for further Level 3 and 4 *in vivo* assays where necessary (OECD, 2015).

Limitations of the method and potential for future development

The methods describe in the OECD TG 455 address transcriptional activation induced by chemical binding to the ERs in an *in vitro* system, as outlined in the **TABLE I**. This is a relatively simple process when compared to the complexity of the central role played by the estrogen in the development and reproduction. A perturbation of the estrogenic system may have the potential to trigger many adverse health effects that cannot be mimicked *in vitro*. Thus, the ERTA results may provide bioactivity alerts but should not be directly extrapolated to the complex signalling and regulation of the intact endocrine system *in vivo*. Rather, the ERTA's are intended as a part of a weight of evidence approach to help prioritising substances for endocrine disruption studies *in vivo* or provide input parameter for *in vitro* to *in vivo* predictions (eg. Punt et al., 2013).

Various *in vitro* assays were brought forward by the research community to tackle the complexity of the estrogen signalling with an integrated testing strategy approach: cumulative analysis of outcomes of several *in vitro* assays can be conducted towards a prediction of an *in vivo* effect, represented by eg. the results of the rodent uterotrophic assay (Wang et.al., 2014).

In parallel, Judson et al. (2015) have recently published a computational network model, integrating the results of the high throughput screening assays (including the **BG1Luc ERTA**) generated by the US EPA ToxCast program and measuring various endpoints in the estrogen signalling pathway. The authors conclude that this approach enables them to distinguish true ER receptor-mediated agonist and antagonist activity from false positive activity related to assay interference across all 1812 chemical tested and that such analysis can be extended to other assays and pathways, once more data become available (Judson et al., 2015).

Non-validated assays of similar design and their broad applications

Performance standards (PS) (OECD, 2012 and OECD 2015) are available to facilitate the development and validation of similar test methods for the same hazard endpoint and allow for timely amendment of this PBTG so that new similar test methods can be added; however, similar test methods can only be added after review and agreement that performance standards are met. The test methods included in the TG 455 can be used indiscriminately to address countries' requirements for test results on estrogen receptor transactivation while benefiting from the Mutual Acceptance of Data.

At present a number of assays based on the same principles as the **STTA** and **BG1Luc ERTA assays** are being evaluated and reported in the literature that are not covered by the TG 455. Different reporter responses can be used in such test methods. In luciferase-based systems, the luciferase enzyme transforms the luciferin substrate to a bioluminescent product that can be quantitatively measured with a luminometer. Other examples of common reporters are the fluorescent proteins and the *LacZ* gene, which encodes β-galactosidase, an enzyme that can transform the colourless substrate X-gal (5-bromo-4-chloro-indolyl-galactopyranoside) into a blue product that can be quantified with a spectrophotometer. These reporters can be evaluated quickly and inexpensively with commercially available test kits. Selected ERTA examples (methods evaluated by Takeyoshi (2006), Schenk et al. (2010) and Judson et al. (2015)):

- **MELN** cells are estrogen-sensitive human breast cancer cells (MCF-7) stably transfected with the

estrogen-responsive reporter gene (ERE-βGlo-Luc-SVNeo) carried by integrated plasmids (Balaguer et al., 1999; Berckmans et al, 2007). The MELN entered validation, intended for its future inclusion in the PBTG 455 (Witters et al, 2010). In comparison with BG1Luc-4E2, MELN cells show lower sensitivity towards E2, bisphenol A and bisphenol S (Grignard et al., 2012). The MELN test was also used to evaluate estrogenic disrupting potency in aquatic environments and urban wastewaters in France (Balaguer et al., 1999; Miège et al., 2009) and Australia (Leusch et al., 2010).

- **U2-OS ERα-CALUX** cell line is a human U2-OS line stably co-transfected with an expression construct for the human ER (pSG5-neo-hER) and a pGL3 (Promega)-based reporter construct containing 3 EREs in front of a TATA box (pGL3-3xERE*tata*Luc). Initial studies showed a good within and between laboratory reproducibility (van der Burg et al., 2010) and good correlation between estrogenic effects *in vitro* and endocrine effects *in vivo* (Lewin et al., 2015). The ERα-CALUX assay was included in a large-scale study of organic micropollutants in ambient water samples ranging from effluent, recycled water to drinking water (Escher et al., 2013).
- **T47D ERα-CALUX** is based on the T47D breast cancer cell line, expressing endogenous ER, stably transfected with pERE*tata*-Luc construct (Legler et al., 1999). This assay has been included in the ToxCast HTS programm (Judson et al., 2015); used for testing estrogenic activity in different water sources (groundwater, raw sewage, treated sewage, and river water) (Leusch., 2010) and particulate matter in diesel exhaust (Wenger et al., 2008).
- **HELN-ERα** cells are derived from HeLa cells, stably transfected with the ERE-βGlobin-Luc-SVNeo plasmid and a second transfection of the corresponding pSG5-puro plasmids (pSG5-ERα-puro) (Escande et al., 2006). They were used in studies on the molecular mechanism of action of bisphenols (Delfosse et al., 2012) and cosmetic ingredients (Gomez et al., 2005).
- **HEK293T ER BLA** is based on the human embryonic kidney cells HEK293T that constitutively co-express fusion protein composed of the ligand binding domain of ER coupled to the DNA-binding domain of the yeast transcription factor GAL and carry a stably integrated beta-lactamase reporter gene under the control of an Upstream Activator Sequence (UAS). This assay has been included in the ToxCast HTS programm to detect interferences across ER TA assays caused by interactions not related to the ligand binding domain of ER (Judson et al., 2015).
- **T47D-KBluc (ATCC # CRL-2865)** cells naturally express both ERα and ERβ and are stably transfected with a triplet estrogen-responsive element promoter-luciferase reporter gene construct (Wison et al., 2004). Reported applications of this assay include effluent testing (Wehmas et al., 2011) and efficacy testing of phytoestrogens (Kim and Park, 2013).

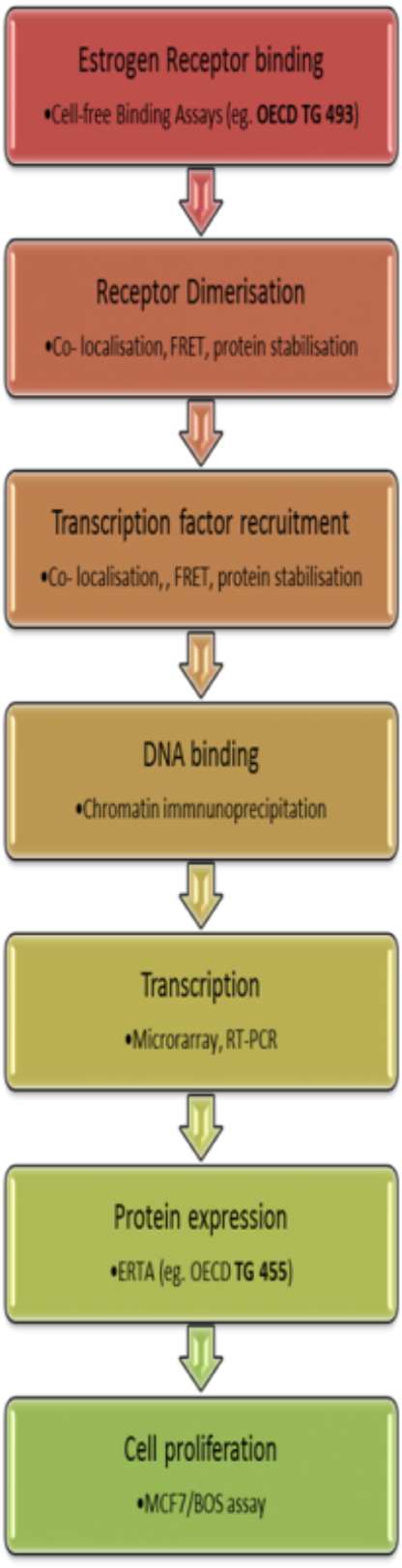
In addition to the ERTA's based on the human cell lines, there are also recombinant yeast systems avalable, expressing human ER and gene reporters. They might be considered less relevant for human physiology than the human cell-based assays. However, the host cells are easy and inexpansive to culture and are popular screening tools for estrogenicity.

- **LYES** (the Lyticase Yeast Estrogen Screen) is one of the oldest ERTA test sytems. It was developed in yeast by stable integration of the hER in the main chromosome and expression plasmids carrying the reporter gene lac-Z (encoding the enzyme b-galactosidase), which is used to measure the receptors' activity (Routledge and Sumpter, 1996). It was originally intended as accessible and affordable screening test for estrogenic activity of xenoestrogens: phytoestrogens and mycoestrogens, pesticides and herbicides, polychlorinated biphenyls (PCB), combustion pollutants, plasticizers, effluents and break- down products of surfactants. The use of the test in context of human health effects has been superseded by more mechanisticly relevant human cell-based assays. LYES remains a common method for assessment of estrogen potency of sewage waters (eg. in hospital effluents in Switzerland and in UK) (Fent et al., 2006; Maletz et al., 2013).
- **Yeast Estrogen Bioassay** is based on recombinant yeast cells expressing the full length human estrogen receptor α (ERα) and yeast Enhanced Green Fluorescence Protein (yEGFP) as a reporter (Bovee et al., 2004). It has been used for studies of estrogenicity in complex samples of animal origin: calf urine (Bovee at al., 2005), hair samples (Becue et al., 2011) or milk (Stypuła-Trębas et al., 2015). Interestingly, this assay also showed lower senstivity but the best correlation with the uterotrophic assay when compared with U2-OS ER-CALUX, T47D ER-CALUX, BG1Luc ER, MC7/BOS proliferation assay and ER binding assay (Wang et al., 2014).

The above-listed assay are only a selection of the multitude of transactivation assays for estrogen receptor developed in the recent years for the purpose of mechanistic exploration of estrogenic activity. ERTA assays applications are not limited to chemical safety testing and environmental monitoring. Many of the pharmaceutical products target estrogen signalling (reviewed in depth by eg. Wallace et al., 2003). Selective Estrogen-Receptor Modulators (SERM) are used in anti-hormone therapy against breast cancer

(Biswas et al., 1998), hormone replacement therapy and dietary supplements in postmenopausal women (De Naeyer et al., 2004) or in treatment of ovulatory dysfunction (Jordan et.al., 2014). Exploratory studies and screening for novel therapeutic ER-ligands are often based on the ERTA (eg. Peekhaus et al., 2001; Liao et al., 2014).

Other *in vitro* methods for studying estrogen pathway



Ligand binding to estrogen receptor is the prerequisite for any transactivation to occur and the molecular initiating event for any adverse outcome pathway related to estrogenicity. The first steps in the cascade of events are outlined in the adjacent **Figure 2.**

ER binding can be studied by many experimental approaches, including peptide microarrays (Aarts et al, 2013), time-resolved fluorescence energy transfer (Hilal et al., 2010) or competitive binding assays (OECD, 2015).

A recently adopted OECD TG No. 493 **Performance-Based Test Guideline for Human Recombinant Estrogen Receptor (hrER) In Vitro Assays to Detect Chemicals with ER Binding Affinity** describes two in vitro assays which measure the ability of a radiolabeled ligand ([3H]17β-estradiol) to bind with the ER in the presence of increasing concentrations of a test chemical (i.e. competitor) (OECD, 2015).

Lee et al. (2015) demonstrated, using a selected set of chemicals, a very good correlation between the ER binding assay and **STTA**. However, receptor binding alone is not awlays a sufficient trigger for downstream events and neither is the activation or inhibition of the gene expression downstream of the estrogen response element in the gene promoter.

Unlike LC-MS based detection methods, *in vitro* bioassays have the advantage that they can potentially detect (anti-)estrogenic activity of substances where the chemical structure is not known in advance (Becue et al., 2011). However, extensive and comparative evaluation of *in vitro* and *in vivo* data demonstrates that even similar assays may produce contradicting results (eg. Wang et al., 2014; Judson et al., 2015) and no *in vitro* assay is 100% concordant with the *in vivo* data. Instead they all have their advantages and disadvantages (Wang et al., 2014).

An *in vitro* assay which seems to have the best correlation with the uterotophic assay (considered as *in vivo* reference test for estrogenic activity) is the **MCF-7/ Bos proliferation assay (or E-SCREEN)** (Wang,et al., 2012). E-SCREEN measures estrogen-induced increase of the number of human breast MCF-7 cells. This downstream event depends on the transcription regulation by ER and is recognized as biologically equivalent to the increase of mitotic activity in the rodent endometrium by estrogen (Soto et al., 1995).

Further improvement of the ITS will depend on the development of adverse outcome pathways for estrogen agonism and antagonism and additional data on steroid hormone metabolism (eg. H295R Stereoidogensis assay, OEDC TG 456; 2011), interactions between endocrine pathways and accurate models for uptake, bioavailability and metabolism of substances under investigation (Wang et al., 2014)

Figure 2: Schematic representation of the initial molecular events of the estrogen signaling pathway and experimental techniques commonly used in their evaluation (based on Judson et al. (2015)).

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6. Abbreviations & Definitions

Acceptability criteria: Minimum standards. All acceptability criteria should be met for an experiment to be considered valid.

Accuracy (concordance): The closeness of agreement between test method results and an accepted reference values. It is a measure of test method performance and one aspect of relevance. The term is often used interchangeably with “concordance” to mean the proportion of correct outcomes of a test method (OECD, 2005).

Agonist: A substance that produces a response, e.g., transcription, when it binds to a specific receptor

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

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

BG-1: An immortalized adenocarcinoma cell that endogenously express estrogen receptor.

BG-1Luc4E2: The BG-1Luc4E2 cell line was derived from BG-1 immortalized human-derived adenocarcinoma cells that endogenously express both forms of the estrogen receptor (ERα and ERβ) and have been stably transfected with the plasmid pGudLuc7.ERE. This plasmid contains four copies of a synthetic oligonucleotide containing the estrogen response element upstream of the mouse mammary tumor viral (MMTV) promoter and the firefly luciferase gene.

CF: The OECD Conceptual Framework for the Testing and Evaluation of Endocrine Disrupters.

Cytotoxicity: Harmful effects to cell structure or function that can ultimately cause cell death and can be reflected by a reduction in the number of cells present in the well at the end of the exposure period or a reduction of the capacity for a measure of cellular function when compared to the concurrent vehicle control.

CV: Coefficient of variation

DMSO: Dimethyl sulfoxide

E2: 17β-estradiol

EC_{50}: The half maximal effective concentration of a test substance.

ED: Endocrine disruption

hER: Human estrogen receptor alpha

hERß: Human estrogen receptor beta

ER: Estrogen receptor

ERE: Estrogen response element

ERTA: Estrogen receptor transactivation

Estrogenic activity: The capability of a chemical to mimic 17β-estradiol in its ability to bind to and activate estrogen receptors. hERα-mediated estrogenic activity can be detected with this PBTG.

FBS: Fetal bovine serum

HeLa: An immortal human cervical cell line

HeLa9903: A HeLa cell subclone into which hERα and a luciferase reporter gene have been stably transfected

IC_{50}: The half maximal effective concentration of an inhibitory test substance.

ICCVAM: The Interagency Coordinating Committee on the Validation of Alternative Methods.

Inter-laboratory reproducibility: A measure of the extent to which different qualified laboratories, using the same protocol and testing the same substances, can produce qualitatively and quantitatively similar results. Interlaboratory reproducibility is determined during the prevalidation and validation processes, and indicates the extent to which a test method can be successfully transferred between laboratories, also referred to as between-laboratory reproducibility (OECD, 2005).

Intra-laboratory reproducibility: A determination of the extent that qualified people within the same laboratory can successfully replicate results using a specific protocol at different times. Also referred to as “within-laboratory reproducibility” (OECD, 2005).

LEC: Lowest effective concentration is the lowest concentration of test substance that produces a response (*i.e.* the lowest test substance concentration at which the fold induction is statistically different from the concurrent vehicle control).

Me-too test: A colloquial expression for a test method that is structurally and functionally similar to a validated and accepted reference test method. Interchangeably used with similar test method

MT: Metallothionein

MMTV: Mouse Mammary Tumor Virus

OHT: 4-Hydroxytamoxifen

PBTG: Performance-Based Test Guideline

PC (Positive control)**:** a strongly active substance, preferably 17ß-estradiol, that is included in all tests to help ensure proper functioning of the assay.

PC10**:** the concentration of a test substance at which the measured activity in an agonist assay is 10% of the maximum activity induced by the PC (E2 at 1nM for the STTA assay) in each plate.

PC50**:** the concentration of a test substance at which the measured activity in an agonist assay is 50% of the maximum activity induced by the PC (E2 at the reference concentration specified in the test method) in each plate.

PCMax**:** the concentration of a test substance inducing the RPCMax

Performance standards: Standards, based on a validated test method, that provide a basis for evaluating the comparability of a proposed test method that is mechanistically and functionally similar. Included are (1) essential test method components; (2) a minimum list of reference chemicals selected from among the chemicals used to demonstrate the acceptable performance of the validated test method; and (3) the comparable levels of accuracy and reliability, based on what was obtained for the validated test method, that the proposed test method should demonstrate when evaluated using the minimum list of reference chemicals (OECD, 2005).

Proficiency chemicals (substances): A subset of the Reference Chemicals included in the Performance Standards that can be used by laboratories to demonstrate technical competence with a standardized test method. Selection criteria for these substances typically include that they represent the range of responses, are commercially available, and have high quality reference data available.

Proficiency: The demonstrated ability to properly conduct a test method prior to testing unknown substances.

Reference estrogen (Positive control, PC): 17ß-estradiol (E2, CAS 50-28-2).

Reference standard: a reference substance used to demonstrate the adequacy of a test method. 17β-estradiol is the reference standard for the STTA and BG1Luc ER TA assays.

Reference test methods: The test methods upon which this PBTG is based.

Relevance: Description of relationship of the test to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the test correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of the accuracy (concordance) of a test method (OECD, 2005).

Reliability: Measure of the extent that a test method can be performed reproducibly within and between laboratories over time, when performed using the same protocol. It is assessed by calculating intra- and inter-laboratory reproducibility.

RPCMax**:** maximum level of response induced by a test chemical, expressed as a percentage of the response induced by 1 nM E2 on the same plate

7. Bibliography

Method development:

- ICCVAM (2012)
  ICCVAM Test Method Evaluation Report on the LUMI-CELL® ER (BG1Luc ER TA) Test Method, An In Vitro Method for Identifying ER Agonists and Antagonists.
- OECD (2015)
  OECD Test Guideline No. 455: Performance-Based Test Guideline for Stably Transfected Transactivation In Vitro Assays to Detect Estrogen Receptor Agonists. First adopted on the 2nd of October 2012. Last update adopted on the 28th of July 2015. **Link to document** (last access 22.01.2016)
  OECD Guidelines for the Testing of Chemicals, Section 4, Health Effects
- OECD (2012)
  OECD Series on Testing and Assessment No. 173: Performance Standards For Stably Transfected Transactivation In Vitro Assay to Detect Estrogen Receptor Agonists (for TG 455)

OECD Environmental Health and Safety Publications

- Takeyoshi, M. (2006)
  Draft Report of Pre-validation and Inter-laboratory Validation For Stably Transfected Transcriptional Activation (TA) Assay to Detect Estrogenic Activity - The Human Estrogen Receptor Alpha Mediated Reporter Gene Assay Using hER-HeLa-9903 Cell Line.

General references:

- Aarts, J.M.M.J.G., Wang, S., Houtman, R., van Beuningen, R.M.G.J., Westerink, W.M.A., Van De Waart, B.J., Rietjens, I.M.C.M., and Bovee, T.F.H. (2013)
  Robust array-based coregulator binding assay predicting ERα-agonist potency and generating binding profiles reflecting ligand structure.
  Chemical Research in Toxicology 26, 336-346
- Balaguer, P., François, F., Comunale, F., Fenet, H., Boussioux, A.-M., Pons, M., Nicolas, J.-C., and Casellas, C. (1999)
  Reporter cell lines to study the estrogenic effects of xenoestrogens.
  Science of the Total Environment 233, 47-56
- Becue, I., Bovee, T.F.H., Van Poucke, C., Groot, M.J., Nielen, M.W.F., and Van Peteghem, C. (2011)
  Applicability of a yeast bioassay in the detection of steroid esters in hair.
  Analytical and Bioanalytical Chemistry 399, 1031-1039
- Berckmans P., Leppens H., Vangenechten C., and Witters H. (2007)
  Screening of endocrine disrupting chemicals with MELN cells, an ER-transactivation assay combined with cytotoxicity assessment.
  Toxicology In Vitro 21(7), 1262-1267
- Biswas, D.K., Averboukh, L., Sheng, S., Martin, K., Ewaniuk, D.S., Jawde, T.F., Wang, F., and Pardee, A.B. (1998)
  Classification of breast cancer cells on the basis of a functional assay for estrogen receptor.
  Molecular Medicine 4, 454-467
- Bovee, T.F.H., Helsdingen, R.J.R., Koks, P.D., Kuiper, H.A., Hoogenboom, R.L.A.P., and Keijer, J. (2004)
  Development of a rapid yeast estrogen bioassay, based on the expression of green fluorescent protein.
  Gene 325, 187–200
- Bovee, T.F.H., Heskamp, H.H., Hamers, A.R.M., Hoogenboom, R.L.A.P., and Nielen, M.W.F. (2005)
  Validation of a rapid yeast estrogen bioassay, based on the expression of green fluorescent protein, for the screening of estrogenic activity in calf urine.
  .Analytica Chimica Acta 529, 57–64
- Cavaillès, V (2002)
  Estrogens and receptors: an evolving concept.
  Climacteric: The Journal of the International Menopause Society 2 Suppl 2, 20-26
- Ceger, P., Allen, D., Huang, R., Xia, M., and Casey, W. (2015)
  Performance of the BG1Luc ER TA method in a qHTS format.
  Alternatives to Animal Experimentation (ALTEX) 32, 287–296
- De Naeyer, A., Vanden Berghe, W., Pocock, V., Milligan, S., Haegeman, G., and De Keukeleire, D. (2004) (2004)
  Estrogenic and anticarcinogenic properties of kurarinone, a lavandulyl flavanone from the roots of Sophora flavescens.
  J. Nat. Prod. 67, 1829–1832
- Delfosse, V., Grimaldi, M., Pons, J.-L., Boulahtouf, A., Maire, A. le, Cavailles, V., Labesse, G., Bourguet, W., and Balaguer, P. (2012)
  Structural and mechanistic insights into bisphenols action provide guidelines for risk assessment and discovery of bisphenol A substitutes.
  Proceedings of the National Academy of Sciences of the United States of America 109, 14930–14935
- EDSTAC (1998)
  Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) Final Report.
- Escande, A., Pillon, A., Servant, N., Cravedi, J.-P., Larrea, F., Muhn, P., (…) Balaguer, P. (2006)
  Evaluation of ligand selectivity using reporter cell lines stably expressing estrogen receptor alpha or beta.
  Biochemical Pharmacology 71(10), 1459–69.
- Escande, A., Pillon, A., Servant, N., Cravedi, J.-P., Larrea, F., Muhn, P., Nicolas, J.-C., Cavaillès, V., and

Balaguer, P. (2006)
Evaluation of ligand selectivity using reporter cell lines stably expressing estrogen receptor alpha or beta.
Biochemical Pharmacology 71, 1459-1469

- Escher, B.I., Allinson, M., Altenburger, R., Bain, P.A., Balaguer, P., Busch, W., Crago, J., Denslow, N.D., Dopp, E., Hilscherova, K., et al. (2014)
Benchmarking Organic Micropollutants in Wastewater, Recycled Water and Drinking Water with In Vitro Bioassays.
Environmental Science & Technology 48, 194-1956
- Fent, K., Escher, C., and Caminada, D. (2006)
Estrogenic activity of pharmaceuticals and pharmaceutical mixtures in a yeast reporter gene system.
Reproductive Toxicology 22, 175–185
- Gomez, E., Pillon, A., Fenet, H., Rosain, D., Duchesne, M.J., Nicolas, J.C., Balaguer, P., and Casellas, C. (2005)
Estrogenic Activity of Cosmetic Components in Reporter Cell Lines: Parabens, UV Screens, and Musks.
Journal of Toxicology and Environmental Health - Part A 68, 239-251
- Grignard, E., Lapenna, S., and Bremer, S. (2012)
Weak estrogenic transcriptional activities of Bisphenol A and Bisphenol S.
Toxicology In Vitro 26, 727-731
- Harris, D. M., Besselink, E., Henning, S. M., Go, V. L. W., & Heber, D. (2005)
Phytoestrogens induce differential estrogen receptor alpha- or Beta-mediated responses in transfected breast cancer cells.
Experimental Biology and Medicine 230(8), 558–68
- Hecker, M., and Hollert, H. (2011)
Endocrine disruptor screening: regulatory perspectives and needs.
Environ Sci Eur 23, 1-14
- Hilal, T., Puetter, V., Otto, C., Parczyk, K., and Bader, B. (2010)
A dual estrogen receptor TR-FRET assay for simultaneous measurement of steroid site binding and coactivator recruitment.
Journal of Biomolecular Screening 15, 268-278
- Jensen, E. V, & De Sombre, E. R. (1969)
Oestrogen-receptor interaction in target tissues.
Biochemical Journal 115(5), 28P–29P
- Jordan, V.C., McDaniel, R., Agboke, F., and Maximov, P.Y. (2014)
The Evolution of Nonsteroidal Antiestrogens to become Selective Estrogen Receptor Modulators.
Steroids 90, 3–12
- Judson, R.S., Magpantay, F.M., Chickarmane, V., Haskell, C., Tania, N., Taylor, J., Xia, M., Huang, R., Rotroff, D.M., Filer, D.L., et al. (2015)
Integrated Model of Chemical Perturbations of a Biological Pathway Using 18 In Vitro High-Throughput Screening Assays for the Estrogen Receptor.
Toxicological Sciences 148, 137-154
- Kim, S., and Park, T.I. (2013)
Naringenin: a partial agonist on estrogen receptor in T47D-KBluc breast cancer cells.
Int J Clin Exp Med 6, 890–899
- Lee, H.K., Kim, T.S., Kim, C.Y., Kang, I.H., Kim, M.G., Jung, K.K., Kim, H.S., Han, S.Y., Yoon, H.J., and Rhee, G.S. (2012)
Evaluation of in vitro screening system for estrogenicity: comparison of stably transfected human estrogen receptor-α transcriptional activation (OECD TG455) assay and estrogen receptor (ER) binding assay.
Journal of Toxicological Sciences 37, 431-437
- Legler, J., van den Brink, C.E., Brouwer, A., Murk, A.J., van der Saag, P.T., Vethaak, A.D., and 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.
Toxicological Sciences 48, 55-66
- Leusch, F.D.L., Khan, S.J., Gagnon, M.M., Quayle, P., Trinh, T., Coleman, H., Rawson, C., Chapman, H.F., Blair, P., Nice, H., et al. (2014)
Assessment of wastewater and recycled water quality: a comparison of lines of evidence from in vitro, in

vivo and chemical analyses.
Water Res. 50, 420–431

- Lewin, G., Escher, S.E., van der Burg, B., Simetska, N., and Mangelsdorf, I. (2015)
Structural features of endocrine active chemicals – A comparison of in vivo and in vitro data.
Reproductive Toxicology 55, 81-94
- Maletz, S., Floehr, T., Beier, S., Klümper, C., Brouwer, A., Behnisch, P., Higley, E., Giesy, J.P., Hecker, M., Gebhardt, W., et al. (2013)
In vitro characterization of the effectiveness of enhanced sewage treatment processes to eliminate endocrine activity of hospital effluents.
Water Research 47, 1545-1557
- Milcamps, A., Bories, G., Poblador, J.C., Gineste, J.M., Grignard, E., Liska, R., Saavedra, L., and Whelan, M. (2012)
Measuring endocrine activity through an automated in vitro Lumicell BG1Luc4E2 assay.
Toxicology Letters 211, S107
- Miège, C., Gabet, V., Coquery, M., Karolak, S., Jugan, M.-L., Oziol, L., Levi, Y., and Chevreuil, M. (2009)
Evaluation of estrogenic disrupting potency in aquatic environments and urban wastewaters by combining chemical and biological analysis.
Trends in Analytical Chemistry 28, 186-195
- Naz, R.K. (1999)
Endocrine Disruptors: Effects on Male and Female Reproductive Systems (CRC Press).
- OECD (2015)
OECD Test Guideline No. 493: Performance-Based Test Guideline for Human Recombinant Estrogen Receptor (hrER) In Vitro Assays to Detect Chemicals with ER Binding Affinity. First adopted on the 28th of July 2015. **Link to document** (last access 22.01.2016)
OECD Guidelines for the Testing of Chemicals, Section 4, Health Effects
- OECD (2012)
Guidance Document No. 150 on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption.
OECD Environmental Health and Safety Publications
- OECD (2012)
OECD Giudance Document No. 178: Detailed review paper on the state of the science on Novel in vitro and in vivo screening and testing methods and endpoints for evaluating endocrine disruptors. (Authors: LeBlanc, G., Norris, D., and Kloas, W.)
OECD Environmental Health and Safety Publications *178*
- OECD (2012)
Performance-Based Test Guideline for Stably Transfected Transactivation *In Vitro* Assays to Detect Estrogen Receptor Agonists.
OECD Guidelines for the Testing of Chemicals, Section 4, Health Effects
- OECD (2011)
OECD Test Guideline No. 456: H295R Steroidogenesis Assay. First adopted on the 28th of July 2011. **Link to document** (last access 14.01.2016)
OECD Guidelines for the Testing of Chemicals, Section 4, Health Effects
- OECD (2005)
OECD Series on Testing and Assessment No. 34: Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment.
OECD Environmental Health and Safety Publications
- Peekhaus, N.T., Ferrer, M., Chang, T., Kornienko, O., Schneeweis, J.E., Smith, T.S., Hoffman, I., Mitnaul, L.J., Chin, J., Fischer, P.A., et al. (2001)
A β-Lactamase-Dependent Gal4-Estrogen Receptor β Transactivation Assay for the Ultra-High Throughput Screening of Estrogen Receptor β Agonists in a 3,456-Well Format.
Assay and Drug Development Technologies 1, 789–800
- Punt, A., Brand, W., Murk, A.J., van Wezel, A.P., Schriks, M., and Heringa, M.B. (2013)
Effect of combining in vitro estrogenicity data with kinetic characteristics of estrogenic compounds on the in vivo predictive value.
Toxicology In Vitro 27, 44-51
- Rogers JM and Denison MS. (2000)

Recombinant cell bioassays for endocrine disruptors: development of a stably transfected human ovarian cell line for the detection of estrogenic and anti-estrogenic chemicals.
In Vitro & Molecular Toxicology 13(1), 67-82

- Routledge, E.J., and Sumpter, J.P. (1996)
Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen.
Environmental Toxicology and Chemistry (SETAC) 15, 241-248
- Schenk, B.; Weimer, M.; Bremer, S.; van der Burg, B.; Cortvrindt, R.; Freyberger, A.; Lazzari, G.; Pellizzer, C.; Piersma, A.; Schafer, W.R.; Seiler, A.; Witters, H.; Schwarz, M. (2010)
The ReProTect Feasibility Study, a novel comprehensive *in vitro* approach to detect reproductive toxicants.
Reproductive Toxicology 30, 200-218
- Soto, A.M., Sonnenschein, C., Chung, K.L., Fernandez, M.F., Olea, N., and Serrano, F.O. (1995)
The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants.
Environmental Health Perspectives 103, 113-122
- Stypuła-Trębas, S., Minta, M., Radko, L., and Żmudzki, J. (2015)
Application of the yeast-based reporter gene bioassay for the assessment of estrogenic activity in cow's milk from Poland.
Environmental Toxicology and Pharmacology 40, 876-885
- Triaryl-Substituted Schiff Bases Are High-Affinity Subtype-Selective Ligands for the Estrogen Receptor. (2014)
Liao, Z.-Q., Dong, C., Carlson, K.E., Srinivasan, S., Nwachukwu, J.C., Chesnut, R.W., Sharma, A., Nettles, K.W., Katzenellenbogen, J.A., and Zhou, H.-B.
Journal of Medicinal Chemistry 57, 3532–3545
- US EPA (2016)
Series 890 - Endocrine Disruptor Screening Program Test Guidelines. Data and Tools. **Link to the list** (last access 20.01.2016)
- Wallace, O., Richardson, T., and Dodge, J. (2003)
Estrogen Receptor Modulators: Relationships of Ligand Structure, Receptor Affinity and Functional Activity.
Current Topics in Medicinal Chemistry 3, 1663–1680
- Wang, S., Aarts, J.M.M.J.G., de Haan, L.H.J., Argyriou, D., Peijnenburg, A.A.C.M., Rietjens, I.M.C.M., and Bovee, T.F.H. (2014)
Towards an integrated in vitro strategy for estrogenicity testing.
Journal of Applied Toxicology 34, 1031-1040
- Wang, S., Aarts, J.M.M.J.G., de Haan, L.H.J., Argyriou, D., Peijnenburg, A.A.C.M., Rietjens, I.M.C.M., and Bovee, T.F.H.0. (2014)
Towards an integrated in vitro strategy for estrogenicity testing.
Journal of Applied Toxicology 34, 1031-1040
- Wehmas, L.C., Cavallin, J.E., Durhan, E.J., Kahl, M.D., Martinovic, D., Mayasich, J., Tuominen, T., Villeneuve, D.L., and Ankley, G.T. (2011)
Screening complex effluents for estrogenic activity with the T47D-KBluc cell bioassay: assay optimization and comparison with in vivo responses in fish.
Environmental Toxicology and Chemistry (SETAC) 30, 439-455
- Wenger, D., Gerecke, A.C., Heeb, N.V., Naegeli, H., and Zenobi, R. (2008)
Catalytic diesel particulate filters reduce the in vitro estrogenic activity of diesel exhaust.
Analytical and Bioanalytical Chemistry 390, 2021-2029
- Witters, H., Freyberger, A., Smits, K., Vangenechten, C., Lofink, W., Weimer, M., Bremer, S., Ahr, P.H.-J., and Berckmans, P. (2010)
The assessment of estrogenic or anti-estrogenic activity of chemicals by the human stably transfected estrogen sensitive MELN cell line: results of test performance and transferability.
Reproductive Toxicology 30, 60–72
- van der Burg, B., Winter, R., Weimer, M., Berckmans, P., Suzuki, G., Gijsbers, L., Jonas, A., van der Linden, S., Witters, H., Aarts, J., et al. (2010)
Optimization and prevalidation of the in vitro ERalpha CALUX method to test estrogenic and antiestrogenic activity of compounds.
Reproductive Toxicology 30, 73-80

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