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ANNEX I: DETAILED ASSAY DESCRIPTIONS IN OECD GD211 FORMAT USED WHITHIN CASE STUDY ON THE USE OF AN INTEGRATED APPROACH TO TESTING AND ASSESSMENT FOR ESTROGEN RECEPTOR ACTIVE CHEMICALS

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Environment Directorate

ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT

Paris 2019

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Annex I. Detailed assay descriptions in OECD GD211 Format

Table of Contents

ACEA_T47D_80hr_Positive

Assay Title: ACEA 80-hr T47-D Human Breast Cell Proliferation Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

One possible effect of endocrine disrupting chemicals is increased cell growth through perturbation of endocrine pathways linked to cell cycle regulation. Activation of the estrogen receptor (ER) signaling pathway, for example, is one possible mechanism that underlies cell proliferation in hormonally sensitive tissues such as mammary and endometrial tissue. The role of steroid hormones in the regulation of some mammary tumors has been well established (Russo and Russo 2006, Yager and Davidson 2006) and has motivated the development of estrogen pathway-based chemotherapeutics. This assay was designed to identify those chemicals in the ToxCast chemical library with the potential to affect cell growth by activating the estrogen receptor-mediated cell proliferation pathway. These impacts were observed by monitoring changes in electrical impedance on the surface of an electronic cell culture growth plate (E-plates) following 80-hour incubation with test chemicals.

1.2. Assay Definition

Assay Throughput:

The assay is conducted on 96-well plates with each plate containing positive controls for proliferation (17β-estradiol) and cytotoxicity (MG132), negative controls (assay media, RPMI 1640), and two concentrations (0.5% and 0.125%) of DMSO solvent controls. Following a 24-hour incubation period, the cells are exposed to test chemicals for 80 hours and response is monitored no less than once per hour.

Experimental System:

T-47D human breast carcinoma ductal cell line, originally derived in 1974 from pleural effusion of a 57-year-old patient, which exhibits epithelial-like morphology (Horwitz et al. 1978, Keydar et al. 1979).

Xenobiotic Biotransformation Potential:

T-47D cells contain specific high affinity receptors for estradiol, progesterone, glucocorticoid and androgen (Horwitz et al. 1978). Some potential for P450 mediated metabolism is present, e.g. CYP1A1, CYP1A2, CYP1B1 (Angus et al. 1999, Hevir et al. 2011, MacPherson and Matthews 2010, Spink et al. 2002, Spink et al. 1998), CYP2B6 (Lo et al. 2010), CYP3A4 (Nagaoka et al. 2006) and CYP2C8 (Mitra et al. 2011), as well as some experimental evidence for the capacity to retain expression of some phase II metabolizing enzymes, e.g., UGTs (Harrington et al. 2006, Hevir et al. 2011), GSTs (Hevir et al. 2011) and sulphotransferases (e.g., SULT1A3(Miki et al. 2006), SULT1E1, SULT2B1 (Hevir et al. 2011)).

Basic Procedure:

Materials

	Product	Source	Cat. No.
Cells	T-47D	ATCC	HTB-133

Growth media	RPMI1640	Hyclone	SH30027FS
Growth media serum	10% FBS	Hyclone	SH3007103
Test media	RPMI 1640	Gibco.	11835030
Test media serum	10% charcoal stripped FBS	Hyclone	SH3006803HI
Positive control	17β-estradiol	Tocris	2824
Reference compound	dexamethasone	Sigma-Aldrich	D1756
Reference compound	hydrocortisone	Sigma-Aldrich	H4001
Reference compound	progesterone	Sigma-Aldrich	P8783
Reference compound	aldosterone	Sigma-Aldrich	A9477
Reference compound	T3 (3,3',5-Triiodo-L-thyronine sodium salt)	Sigma-Aldrich	T6397
Reference compound	T4 (thyroxine)	Sigma-Aldrich	T2376

Protocols

T-47D cells purchased from American Tissue Culture Collection (ATCC) were maintained in RPMI1640 media supplemented with 10% characterized fetal bovine serum (FBS) until testing. Before screening, cells were preconditioned in assay medium: phenol red-free RPMI1640 supplemented with 10% charcoal-stripped FBS. Cells were then detached and seeded in 96-well E-Plates in assay medium. After overnight monitoring of growth once every hour, chemicals were added to T-47D cells and remained in the medium until the end of the experiment. Each chemical in the ToxCast library was tested in an 8-point, 1:4 serial dilution series starting at a maximum final concentration of 100 μM and was tested in duplicate using two separate E-plates for each dilution series. A maximum starting concentration of 0.5% DMSO was present in the 100 μM chemical samples and subsequent dilutions used a final concentration of 0.125% DMSO. Positive controls (MG132 for cytotoxicity and 17β-estradiol for proliferation) and a negative control (assay media) were tested in quadruplicate on each testing plate along with 0.5% and 0.125% DMSO tested in duplicates on each plate to serve as solvent controls for the highest concentration of testing chemicals and all lower dilutions, respectively. Reference chemicals were tested with 8 concentrations with 1:5 serial dilutions. The xCELLigence system Multi-E-Plate stations employing real-time cell analysis (RTCA) were used to measure cellular responses recorded once every 5 min for the first 5 h, and once every hour for an additional 100h.

Proprietary Elements:

Assay is non-proprietary; xCELLigence RTCA software and biosensor technology are available from ACEA Biosciences, Inc. and T-47D cells are commercially available from American Type Culture Collection (ATCC HTB-133) with signed Material Transfer Agreement (MTA).

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to activate endogenous estrogenic signaling pathways, and is intended to provide information on a large number of diverse chemicals. Cell proliferation may result from both estrogenic pathways and non-estrogenic pathways so results from this assay in isolation do not ensure estrogenic activity for a test chemical. Caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of chemical selection for more resource intensive toxicity studies.

Status:

The assay is fully developed, and data are publicly available.

1.3. Assay References

Assay Source Contact Information:

ACEA Biosciences
6779 Mesa Ridge Road #100, San Diego, CA 92121 USA
Tel: +1 858-724-0928 | 1 866-308-2232
Fax: +1 858-724-0927
info@aceabio.com

Assay Publication Year:

2006

Assay Publication:

Xing, J. Z., Zhu, L., Gabos, S., & Xie, L. (2006). "Microelectronic cell sensor assay for detection of cytotoxicity and prediction of acute toxicity". Toxicol In Vitro 20(6), 995-1004. (PMID: 16481145)

Method Updates / Confirmatory Studies:

None Reported

2. Assay Component Descriptions

Assay Objectives:

The ACEA T47D 80-hour Positive assay exposed human breast carcinoma cell (T-47D) cultures to the ToxCast library of diverse environmental chemicals using an eight-point, 1:4 dilution series concentration-response format (starting at a maximum final concentration of 100µM), using MG132 (cytotoxicity) and Estradiol (E2) (proliferation) as positive controls and assay media and DMSO as a negative control and solvent control, respectively. All control chemicals were tested in quadruplicate on each plate.

The ACEA_T47D ER assay analyzed changes in cell adhesion and morphology at the electrode: solution interface (located on the bottom of 96-well E-plate culture wells) using electronic microsensors. Changes in electrical impedance were monitored in real-time at the plate surface to investigate the potential activation of the estrogen signaling pathway and subsequent increases in growth or changes in cell structure following 80-hour incubation with the test chemicals. The electrical signal produced by the experimental system can be used to detect changes in cell number, morphology and adhesion which occur in response to xenoestrogenic activation of ER-mediated pathways, and concentration-response curves were modeled for each chemical to determine half-maximal activity levels.

Scientific Principles:

Endocrine disrupting chemicals (EDCs) interfere with normal hormone biosynthesis, signaling or metabolism and impact regulatory pathways in humans and wildlife. Many EDCs interfere with normal steroidal activity by impacting estrogenic signaling pathways. The estrogen receptor mediates gene expression in response to estrogen exposure, and modulates the activity for a wide variety of physiological processes. The activity of estrogenic chemicals is generally probed *in vitro* by monitoring ligand-binding in experimental systems, however estrogenic potency is also a function of interaction with transcriptional machinery and other signaling pathways. This assay was designed to identify chemical perturbagens which can affect a cell proliferation response in human breast carcinoma cells by acting as xenoestrogenic compounds which impact estrogen signaling pathways. While cell proliferation rates can be altered via multiple pathways, growth responses in T47D cells are considered to be particularly reliable indicators of estrogenic activation.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds which potentially affect endocrine systems in exposed populations by interacting with estrogen receptor mediated signaling pathways. There is strong evidence that estrogen receptor activity in early life is a molecular initiating event (MIE) in a developing Adverse Outcome Pathways (AOP) leading to breast cancer in both animal and human

models and to endometrial carcinoma in the mouse, and ER agonism is the leading to reproductive dysfunction in oviparous vertebrates (AOPs under development), and there is some evidence that estrogen receptor activation is the MIE for putative adverse outcome pathways leading to reduced survival due to renal failure and leading to skewed sex ratios due to altered sexual differentiation in males (under development). ER antagonism has strong evidence as the MIE for an AOP describing reduction of vitellogenin synthesis in liver (under review), which can lead to reduced cumulative fecundity in repeat-spawning fish species. Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive *in vivo* studies to further investigate the involvement of ER interference in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Xiao, C., Lachance, B., Sunahara, G., & Luong, J. H. (2002). "Assessment of cytotoxicity using electric cell-substrate impedance sensing: concentration and time response function approach". Analytical Chemistry 74(22), 5748-5753. (PMID: 12463358)

Assay Quality Statistics:

Neutral control well median response value, by plate:	1.66
Neutral control median absolute deviation, by plate:	0.11
Positive control well median response value, by plate:	3.00
Positive control well median absolute deviation, by plate:	0.19
Negative control well median, by plate:	0.03
Negative control well median absolute deviation value, by plate:	0.06
Z' (median across all plates, using positive control wells):	0.31
Z' (median across all plates, using negative control wells):	0.69
SSMD (median across all plates, using positive control wells):	6.00
SSMD (median across all plates, using negative control wells):	-13.00
Signal-to-noise (median across all plates, using positive control wells):	12.94
Signal-to-noise (median across all plates, using negative control wells):	-15.00
Signal-to-background (median across all plates, using positive control wells):	1.82
Signal-to-background (median across all plates, using negative control wells):	0.02
CV (median across all plates):	0.07

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Increase cell proliferation in response to xenoestrogenic interference with ER-mediated pathways as measured by monitoring electrical impedance at the cell-plate interface.

Analytical Elements:

Data were collected from the xCELLigence system which converts raw impedance values into the Cell Index (CI) value; this is a measure of adhesion where CI = (impedance at time point n – impedance in the absence of cells)/nominal impedance value. These data were then converted to a Normalized Cell Index according to the equation NCI(Ti) = [CI(Ti)]/[CI(Tk)], {i = 1,2,3,….N where CI(Tk) is the last time point before chemical addition, CI(Ti) is the cell index at the i-th measured time point, and N is the total number of time points. Data were grouped by chemical and smoothed to combine replicates using a simple moving average (as the replicates were assessed in duplicate on separate plates so the time points were not identical). DMSO controls were considered as baseline for activity, and 17β-Estradiol was used as a positive control and 100% activity for all the test chemicals on that plate. If a chemical sample was run on two different plates, then the maximum NCI values for the positive and negative controls were averaged. Concentration response curves were generated using smoothed NCI values and all statistical analyses were conducted using R programming language, employing*tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant

function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Estrogen receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC50 (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ACEA_T47D_80hr_Positive
ATG_ERE_CIS_up
ATG_ERa_TRANS_up
NVS_NR_bER
NVS_NR_hER
NVS_NR_mERa
OT_ER_ERaERa_0480
OT_ER_ERaERa_1440
OT_ER_ERaERb_0480
OT_ER_ERaERb_1440
OT_ERa_ERELUC_AG_1440
OT_ERa_ERELUC_ANT_1440
OT_ERa_EREGFP_0120
OT_ERa_EREGFP_0480
Tox21_ERa_BLA_Agonist_ratio
Tox21_ERa_BLA_Antagonist_ratio
Tox21_ERa_LUC_BG1_Agonist
Tox21_ERa_LUC_BG1_Antagonist

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations: 8
Target (nominal) number of replicates: 2
Standard minimum concentration tested: 0.39 µM
Standard maximum concentration tested: 200 µM
Baseline median absolute deviation for the assay - based on the response values at the 2 lowest tested concentrations (bmad): 4.77
The response cutoff used to derive the hit calls (e.g., 5*bmad, 10*bmad): 23.86

Reference Chemicals / Predictive Capacity:

ER reference chemicals (list adapted from OECD Test Guideline No. 457, as according to methods described by Judson et al. 2015 (Judson et al. 2015)):

CASRN	Chemical Name	Agonist Potency	Antagonist Potency	Activity in Assay
57-63-6	17alpha-Ethinyl estradiol	Strong	Inactive	Yes
50-28-2	17beta-Estradiol	Strong	NA	Yes

56-53-1	Diethylstilbestrol (DES)	Strong	Inactive	Yes
84-16-2	meso-Hexestrol	Strong	NA	Yes
57-91-0	17alpha-Estradiol	Moderate	NA	Yes
140-66-9	4-tert-Octylphenol	Moderate	NA	Yes
53-16-7	Estrone	Moderate	NA	Yes
599-64-4	4-Cumylphenol	Weak	NA	Yes
521-18-6	5alpha-Dihydrotestosterone	Weak	Inactive	Yes
80-05-7	Bisphenol A	Weak	Inactive	Yes
77-40-7	Bisphenol B	Weak	NA	Yes
486-66-8	Daidzein	Weak	NA	Yes
446-72-0	Genistein	Weak	Inactive	Yes
143-50-0	Kepone	Weak	Inactive	No
789-02-6	o,p'-DDT	Weak	NA	Yes
58-18-4	17alpha-Methyltestosterone	Very Weak	NA	Yes
520-36-5	Apigenin	Very Weak	Inactive	Yes
85-68-7	Butylbenzyl phthalate	Very Weak	Inactive	Yes
480-40-0	Chrysin	Very Weak	Inactive	No
115-32-2	Dicofol	Very Weak	Inactive	No
117-81-7	Diethylhexyl phthalate	Very Weak	Inactive	No
84-74-2	Di-n-butyl phthalate	Very Weak	Inactive	No
120-47-8	Ethylparaben	Very Weak	NA	Yes
60168-88-9	Fenarimol	Very Weak	NA	No
520-18-3	Kaempferol	Very Weak	NA	Yes
72-43-5	Methoxychlor	Very Weak	NA	Yes
72-55-9	p,p’-DDE	Very Weak	Inactive	No
104-40-5	p-n-Nonylphenol	Very Weak	NA	Yes
1912-24-9	Atrazine	Inactive	NA	No
50-22-6	Corticosterone	Inactive	NA	Yes
66-81-9	Cycloheximide	Inactive	NA	No
13311-84-7	Flutamide	Inactive	NA	Yes
52-86-8	Haloperidol	Inactive	NA	No
52806-53-8	Hydroxyflutamide	Inactive	NA	Yes
65277-42-1	Ketoconazole	Inactive	NA	Yes
330-55-2	Linuron	Inactive	NA	No
57-30-7	Phenobarbital Sodium	Inactive	NA	No
32809-16-8	Procymidone	Inactive	NA	Yes
50-55-5	Reserpine	Inactive	NA	No
52-01-7	Spironolactone	Inactive	NA	Yes
68392-35-8	4-Hydroxytamoxifen (E/Z)	NA	Active	Yes
82640-04-8	Raloxifene	NA	Active	Yes
10540-29-1	Tamoxifen	NA	Active	Yes
54965-24-1	Tamoxifen citrate	NA	Active	Yes
57-83-0	Progesterone	NA	Inactive	Yes

Agonist Activity	ToxCast Active	ToxCast Inactive
Active	21	7
Inactive	6	6

Antagonist Activity	ToxCast Active	ToxCast Inactive
Active	4	0
Inactive	8	6

Agonist Sensitivity = 75%
Agonist Specificity = 50%
Balanced Accuracy = 62.5%

Antagonist Sensitivity = 100%
Antagonist Specificity = 42.9%
Balanced Accuracy = 71.4%

Overall balanced accuracy for assay = 62.1%

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [16].

4. Assay Documentation

4.1. References

[1] Russo, J. and I. H. Russo (2006). J Ster Biochem Mol Biol 102(1): 89-96. (PMID: 17113977)
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4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
ATCC, American Tissue Culture Collection
CV, Coefficient of Variation
CYP, Cytochrome P450s
DMSO, Dimethyl Sulfoxide
EDC, Endocrine disrupting chemicals
ER, Estrogen Receptor
FBS, Fetal Bovine Serum
GST, Glutathione S-Transferase
MIE, Molecular Initiating Event
NCI, Normalized Cell Index
NR, Nuclear Receptor
RTCA, Real-Time Cell Analysis
SSMD, Strictly Standardized Mean Difference
SULT, Sulfotransferases
UGT, UDP-glucuronosyltransferase

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

2 May 2016

Date of Revisions:

15 November 2016

Author of Revisions:

EPA NCCT

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:

Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;

Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation

Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;

Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. Supporting Information (existing annotations):

ATG_ERa_TRANS_up

Assay Title: Attagene TRANS-FACTORIAL HepG2 Human Estrogen Receptor Alpha Activation Assay

7. Assay Descriptions

7.1. Overview

Assay Summary:

The Attagene TRANS assay tracks changes in transcription factor (TF) activity in response to chemical perturbations by chimeric GAL4-NR transcription factors utilizing a library of multiple reporter transcription unit (MRTU) constructs regulated by the yeast GAL4 DNA-binding domain. This family of Attagene assays employ a recently developed profiling technology (Factorial™) which consists of multiple RTU construct sequences that are identical with the exception of processing tag sequences assigned to each TF which create a unique restriction enzyme cleavage site for individual RTUs, and allow for specific determination of NR activity. A specific MRTU paired with a specific chimeric GAL4-NR protein are transfected into an in-house clone of human liver hepatoma cell line HepG2 (variant HG19) and, 24 hr after transfection, transfected cells for all 25 nuclear receptors and MRTU's are mixed and plated. Nuclear receptor binding by exogenous compounds alters the transactivation function of Gal4-NR and modulates reporter transcription. The chemical-NR activity is monitored by quantitating transcribed reporter RNA through cDNA synthesis, fluorescent labeling, and restriction enzyme digestion to yield specific reporter products. This trans-format Factorial™ assay was used to evaluate agonistic/antagonistic properties of the ToxCast chemical library against 25 human nuclear receptors following 24-hour incubation with cells in a 24-well plate in a single-replicate 8-point concentration series. All reporters are detected simultaneously in the same assay well and by a single reaction creating highly homogeneous detection conditions.

Assay Throughput:

Transfected HepG2 cells are aliquoted into 24-well microtiter plates and incubated with test compounds for 24 hours prior to PCR detection of total RNA transcription using capillary electrophoresis.

Experimental System:

The HepG2 cell line is a permanent cell culture isolated from the liver tumor lobectomy of a 15-yr-old Caucasian male from Argentina in 1975 [1], which has been cloned and transfected with a library of multiple reporter transcription units (– see section 2, Assay Component Descriptions, for detailed definition of MRTUs).

Xenobiotic Biotransformation Potential:

The HepG2 cells used in this assay are variant HG19, a cell line selected for enhanced xenobiotic metabolism. These cells express 2- to 13 times more cytochrome P450 activity than parental HepG2 (Attagene, personal communication). The parental HepG2 cell line has been shown by others to retain the potential for Phase I and Phase II metabolic responses to xenobiotics, e.g., expression of CYP1A1/2, 2A6, 2B6, 2C8/9, 2C19, 2D6/3A, 2E1, and 3A4/5 [2] with CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A activities reported at levels similar to human hepatocytes although variable depending on source and culture conditions [3]; some enzymes (e.g., CYP2W1) have even been observed at higher rates than in primary hepatocytes [4]. Phase II enzyme activities identified in HepG2 cells include SULTS (1A1, 1A2, 1E1 and 2A1), GSTs (mGST-1, GST μ1), NAT1, EPHX1 [5-7] and UGTs (1A1, 1A6 and 2B7) [7]. In addition, HepG2 cells can potentially express xenobiotic regulation activities via functionally active p53 protein [8] and Nrf2, a transcription factor which regulates genes containing antioxidant response element (ARE) sequences in their promoters; HepG2 cells also possess the capacity to express a number of ATP-binding cassette (ABC) xenobiotic export pumps (e.g., ABCC1, C2, C3 and G2 – membrane-bound proteins also regulated in part by Nrf2 TF DNA-binding) [9].

Basic Procedure:

Human liver HepG2 cells are transiently transfected with multiple reporter transcription units (MRTUs) in 6-well plates using FuGene 6 reagent (Roche; 3 ml FuGene/1 mg DNA). These transfected cells are aliquoted into wells of a 24-well plate, exposed to the chemical library of environmental compounds for 24 hours, and total RNA was isolated using TriZol reagent (Invitrogen). RNA was reverse-transcribed using oligo(dT) primer and Mo-MLV reverse transcriptase (Invitrogen) and treated with DNAse I (Ambion) for 30 min. Then one-tenth of the produced cDNA was amplified by PCR using Taq DNA polymerase (Invitrogen) and two reporter sequence–specific primers. The PCR products were fluorescently labeled by primer extension with 6-arboxyfluorescein (6-FAM) 5'-labeled reporter sequence–specific primer (2 min at 95 °C, 20 s at 68 °C and 10 min at 72 °C) and these products were digested with 5U of *Hpa*I (New England Biolabs) for 2h at 37 °C. The fragments were purified using Qiaquick PCR columns (Qiagen), analyzed on an ABI 3130xL genetic analyzer (Applied Biosystems) with peak positions identified by using a set of X-rhodamine (ROX)-labeled MapMarker1000 molecular weight standards (BioVentures). The raw capillary electrophoresis data was processed using Attagraph software (Attagene).

Proprietary Elements:

FACTORIAL™ is a novel pathway profiling technology trademarked and patented by Attagene, Inc.

Caveats:

The estrogen receptor used in this assay is a partial receptor consisting of the ligand-binding domain and hinge region and may not represent the physiological form of the receptor. The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote estrogen receptor mediated DNA transcription, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

7.2. Assay References

Assay Source Contact Information:

Attagene, Inc.
7020 Kit Creek Road Suite 260
Morrisville, NC 27560
Tel: (888)721-2121

Assay Publication Year:

2010

Assay Publication:

Martin, M. T., Dix, D. J., Judson, R. S., Kavlock, R. J., Reif, D. M., Richard, A. M., Rotroff, D. M., Romanov, S., Medvedev, A., Poltoratskaya, N., Gambarian, M., Moeser, M., Makarov, S. S., & Houck, K. A. (2010). Impact of environmental chemicals on key transcription regulators and correlation to toxicity end points within EPA's ToxCast program. Chem Res Toxicol, 23(3), 578-590. (PMID: 20143881)

Method Updates / Confirmatory Studies:

Attagene second generation of TRANS-FACTORIAL assays were conducted with an expanded NR platform, which covers all 48 human NRs.

8. Assay Component Descriptions

Assay Objectives:

The Attagene TRANS Estrogen Receptor alpha assay measures changes in human estrogen-receptor alpha (hERα) activation using the mammalian one-hybrid assay format, which monitored

transcriptional activation using hERα ligand-binding domain (LBD) fused with the yeast GAL4 DNA-binding domain (DBD) transiently transfected into HepG2 cells with a 5X upstream activator sequence (UAS) reporter. Each individual trans-RTU system (receptor and reporter gene) was separately transfected into the cell, followed by pooling and plating of the transfected cells prior to detection, and one replicate of each compound was screened at eight concentrations of each test compound. The presence of agonists/antagonists of ERα alters the transactivation function of Gal4-NR and modulates reporter transcription. Following 24 hour incubation with compounds from the ToxCast chemical library, TF activity was reported via cDNA synthesis and RT-PCR of the fluorescently-labeled MRTU mRNA followed by quantitation by capillary electrophoresis. Half-maximal activity (IC_{50}) was determined for each chemical using changes in transcription factor activity expressed as log2 fold induction over DMSO controls in a single replicate, 8-point concentration response series. Cytotoxicity assessments were performed concurrently using a MTT tetrazolium assay to establish maximum tolerated concentrations (MTC) (see ATG_XTT_Cytotoxicity assay description for MTT procedures).

ATG_ERa_TRANS series assays consist of multiplexed (Factorial™) assay endpoints which assessed transcription factor activity using reporter transcription unit (RTU) quantitation in the HepG2 human liver hepatoma cell line. The multiple RTUs were constructed from essentially identical reporter gene sequences (representing a functionally inactivated fragment of human SEAP cDNA) and each tagged with unique *Hpa*I restriction cleavage sites at variable positions to allow discrimination of individual TF activities. When co-introduced into HepG2 cells, the RTUs produce reporter RNAs in amounts commensurate with the activities of the corresponding TFs present in a cell. This homogeneous construct creates a uniform environment for detecting the activity of many transcription factors simultaneously, and allowing for a more detailed assessment of xenobiotic impacts on the cellular regulatory network. ATG_ERa_TRANS is a MRTU Factorial™ assay reporting activity in exogenous, chimeric Gal4-ERα proteins that bind to a GAL4-specific upstream activation sequence promoter (5X-UAS-TATA) and regulate the transcription of an ERα-specific reporter sequence. Chemical-ERα activity can be monitored by measuring changes in fluorescence relative to DMSO controls.

Scientific Principles:

Endocrine disrupting chemicals (EDCs) interfere with normal hormone biosynthesis, signaling or metabolism and impact regulatory pathways in humans and wildlife. Many EDCs interfere with normal steroidal activity by impacting estrogen signaling pathways. The estrogen receptor mediates gene expression in response to estrogen exposure, and modulates the activity for a wide variety of physiological processes. The ATG_ERa_TRANS assay used a hepatoma cell-based platform to monitor estrogen receptor alpha transcriptional activity and this assay is designed to help identify environmental compounds with a capacity for xenoestrogenic activity. This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with estrogen receptor alpha mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that estrogen receptor agonism is the MIE leading to reproductive dysfunction in oviparous vertebrates (AOP under development), and there is some evidence that estrogen receptor activation is the MIE for putative adverse outcome pathways leading to reduced survival due to renal failure and leading to skewed sex ratios due to altered sexual differentiation in males (AOPs under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive *in vivo* studies to further investigate the involvement of ER agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Romanov, S., Medvedev, A., Gambarian, M., Poltoratskaya, N., Moeser, M., Medvedeva, L., Gambarian, M., Diatchenko, L., & Makarov, S. (2008). Homogeneous reporter system

enables quantitative functional assessment of multiple transcription factors. Nat Methods, 5(3), 253-260. (PMID: 18297081)

Assay Quality Statistics:

Neutral control well median response value, by plate:	1.77
Neutral control median absolute deviation, by plate:	0.50
Positive control well median response value, by plate:	NA
Positive control well median absolute deviation, by plate:	NA
Z' (median across all plates, using positive control wells):	NA
SSMD (median across all plates, using positive control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	NA
CV (median across all plates):	0.28

(no positive control used for this assay)

9. Assay Endpoint Descriptions

9.1. Data Interpretation

Biological Response:

Estrogen receptor agonism - nuclear steroid hormone receptor initiated pathway production of mRNA transcripts in response to ligand binding of human estrogen receptor α as measured by RT-PCR and capillary electrophoretic detection of fluorescently labeled mRNA.

Analytical Elements:

ATG_ERa_TRANS_up readout data was analyzed in the positive (gain of signal) fitting direction using log2 fold-induction over DMSO controls which provide a signal baseline. Negative and zero values are removed before analysis and raw values are log transformed. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Estrogen receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ACEA_T47D_80hr_Positive
ATG_ERE_CIS_up
NVS_NR_bER
NVS_NR_hER
NVS_NR_mERa
OT_ER_ERaERa_0480
OT_ER_ERaERa_1440
OT_ERa_ERELUC_AG_1440
OT_ERa_ERELUC_ANT_1440
OT_ERa_EREGFP_0120

OT_ERa_EREGFP_0480
Tox21_ERa_BLA_Agonist_ratio
Tox21_ERa_LUC_BG1_Agonist

9.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations: 8
Target (nominal) number of replicates: 1
Standard minimum concentration tested: 0.09 µM
Standard maximum concentration tested: 200 µM
Baseline median absolute deviation for the assay - based on the response values at the 2 lowest tested concentrations (bmad): 0.23
The response cutoff used to derive the hit calls (e.g., 5*bmad, 10*bmad): 1.13

Reference Chemicals / Predictive Capacity:

CASRN	Chemical Name	*In Vitro* Activity	*In Vivo* Activity	Activity in Assay
57-91-0	17alpha-Estradiol	Moderate	Active	Yes
57-63-6	17alpha-Ethinylestradiol	Strong	Active	Yes
50-28-2	17beta-Estradiol	Strong	Active	Yes
58-18-4	17-Methyltestosterone	Very Weak	Active	Yes
131-55-5	2,2',4,4'-Tetrahydroxybenzophenone	NA	Active	Yes
131-56-6	2,4-Dihydroxybenzophenone	NA	Active	Yes
5153-25-3	2-Ethylhexylparaben	NA	Active	Yes
140-66-9	4-(1,1,3,3-Tetramethylbutyl)phenol	Moderate	Active	No
80-46-6	4-(2-Methylbutan-2-yl)phenol	NA	Active	Yes
80-09-1	4,4'-Sulfonyldiphenol	NA	Active	Yes
599-64-4	4-Cumylphenol	Weak	Active	Yes
104-43-8	4-Dodecylphenol	NA	Active	No
99-96-7	4-Hydroxybenzoic acid	NA	Inactive	Yes
104-40-5	4-Nonylphenol	Very Weak	Active	Yes
98-54-4	4-tert-Butylphenol	NA	Active	Yes
521-18-6	5alpha-Dihydrotestosterone	Weak	Active	Yes
61-82-5	Amitrole	NA	Inactive	Yes
520-36-5	Apigenin	Very Weak	NA	Yes
85-68-7	Benzyl butyl phthalate	Very Weak	NA	Yes
103-23-1	Bis(2-ethylhexyl)hexanedioate	NA	Inactive	Yes
80-05-7	Bisphenol A	Weak	Active	Yes
1478-61-1	Bisphenol AF	NA	Active	No
77-40-7	Bisphenol B	Weak	Active	Yes
94-26-8	Butylparaben	NA	Active	No
480-40-0	Chrysin	Very Weak	NA	No
50-22-6	Corticosterone	Inactive	NA	Yes

486-66-8	Daidzein	Weak	NA	Yes
117-81-7	Di(2-ethylhexyl) phthalate	Very Weak	Inactive	No
84-74-2	Dibutyl phthalate	Very Weak	Inactive	Yes
115-32-2	Dicofol	Very Weak	NA	Yes
84-61-7	Dicyclohexyl phthalate	NA	Inactive	Yes
84-66-2	Diethyl phthalate	NA	Inactive	Yes
56-53-1	Diethylstilbestrol	Strong	Active	Yes
84-75-3	Dihexyl phthalate	NA	Inactive	Yes
474-86-2	Equilin	NA	Active	Yes
50-27-1	Estriol	NA	Active	No
53-16-7	Estrone	moderate	Active	Yes
120-47-8	Ethylparaben	Very Weak	NA	No
60168-88-9	Fenarimol	Very Weak	NA	Yes
51630-58-1	Fenvalerate	NA	Inactive	Yes
446-72-0	Genistein	Weak	Active	No
52-86-8	Haloperidol	Inactive	NA	No
520-18-3	Kaempferol	Very Weak	Inactive	Yes
143-50-0	Kepone	Weak	NA	Yes
65277-42-1	Ketoconazole	Inactive	NA	Yes
330-55-2	Linuron	Inactive	NA	Yes
84-16-2	meso-Hexestrol	Strong	NA	Yes
72-33-3	Mestranol	NA	Active	Yes
72-43-5	Methoxychlor	Very Weak	Active	Yes
68-22-4	Norethindrone	NA	Active	Yes
789-02-6	o,p'-DDT	Weak	Active	Yes
556-67-2	Octamethylcyclotetrasiloxane	NA	Active	Yes
72-55-9	p,p'-DDE	Very Weak	Weak	Yes
87-86-5	Pentachlorophenol	NA	Inactive	No
57-30-7	Phenobarbital sodium	Inactive	NA	No
32809-16-8	Procymidone	Inactive	NA	Yes
50-55-5	Reserpine	Inactive	NA	No
52-01-7	Spironolactone	Inactive	NA	No
17924-92-4	Zearalenone	NA	Active	Yes

In Vitro Activity	ToxCast Active	ToxCast Inactive
Active	26	3
Inactive	1	7

In Vivo Activity	ToxCast Active	ToxCast Inactive
Active	30	0
Inactive	5	6

In Vitro Sensitivity = 89.7%
In Vitro Specificity = 87.5%

Balanced Accuracy = 88.6%

In Vivo Sensitivity = 100%
In Vivo Specificity = 54.5%
Balanced Accuracy = 77.2%

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [10].

10. Assay Documentation

10.1. References

[1] Aden, D. P., et al. (1979). Nature 282: 615-616. (PMID: 233137)
[2] Westerink, W. M. and W. G. Schoonen (2007). Toxicol In Vitro 21(8): 1581-1591. (PMID: 17637504)
[3] Hewitt, N. and P. Hewitt (2004). Xenobiotica 34(3): 243-256. (PMID: 15204697)
[4] Guo, L., et al. (2010). Drug Metab Disposition 39(3): 528-538. (PMID: 21149542)
[5] Westerink, W. M. and W. G. Schoonen (2007). Toxicol In Vitro 21(8): 1592-1602. (PMID: 17716855)
[6] Walle, T., et al. (2000). Drug Metab Disposition 28(9): 1077-1082. (PMID: 10950852)
[7] Hart, S. N., et al. (2010). Drug Metab Disposition 38(6): 988-994. (PMID: 20228232)
[8] Boehme, K., et al. (2010). Toxicol Lett 198(2): 272-281. (PMID: 20655369)
[9] Adachi, T., et al. (2007). J Exper Therap Oncol 6(4): 335-348. (PMID: 18038766)
[10] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

10.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
ATCC, American Tissue Culture Collection
CYP, Cytochrome P450s
DMSO, Dimethyl Sulfoxide
EDC, Endocrine disrupting chemicals
ER, Estrogen Receptor
ERE, Estrogen Response Element
E2, Estradiol
GST, Glutathione S-Transferase
MIE, Molecular Initiating Event

MRTU, Multiple Reporter Transcription Unit
NR, Nuclear Receptors
RTU, Reporter Transcription Unit
SEAP, Secreted Embryonic Alkaline Phosphatase
SULT, Sulfotransferases
TF, Transcription Factor
UGT, UDP-Glucuronosyltransferase

10.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

2 May 2016

Date of Revisions:

15 November 2016

Author of Revisions:

EPA NCCT

11. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:
Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;
Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation
Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;
Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

12. Supporting Information (existing annotations):

ATG_ERE_CIS_up

Assay Title: Attagene CIS-FACTORIAL HepG2 Estrogen Response Element Activation Assay

13. Assay Descriptions

13.1. Overview

Assay Summary:

The Attagene CIS assays track changes in transcription factor (TF) activity in response to chemical perturbations by utilizing a library of multiple reporter transcription unit (MRTU) constructs regulated by individual transcription factor response elements. This family of Attagene assays employ a recently developed profiling technology (FACTORIAL™) which consists of cis-regulating element (promoter) binding by RTUs. The MRTUs are transfected into an in-house clone of human liver hepatoma cell line HepG2 (variant HG19), and the CIS- format assay measures changes in RTU expression resulting from TF binding to response element DNA-binding sites. Response to 24-hour incubation of test chemicals with cells in a 24-well plate is monitored by examining fluorescent activity produced by transcribed mRNA. All reporters are detected simultaneously in the same assay well and by single reaction creating highly homogeneous detection conditions in a single-replicate 8-point chemical concentration series.

13.2. Assay Definition

Assay Throughput:

Transfected HepG2 cells are aliquoted into 24-well microtiter plates and incubated with test compounds for 24 hours prior to PCR detection of total RNA transcription using capillary electrophoresis.

Experimental System:

The HepG2 cell line is a permanent cell culture isolated from the liver tumor lobectomy of a 15-yr-old Caucasian male from Argentina in 1975 [1], which has been cloned and transfected with a library of multiple reporter transcription units (see section 2, Assay Component Descriptions, for detailed definition of MRTUs).

Xenobiotic Biotransformation Potential:

The HepG2 cells used in this assay are variant HG19, a cell line selected for enhanced xenobiotic metabolism. These cells express 2 to 13 times more cytochrome P450 activity than parental HepG2 (Attagene, personal communication). The parental HepG2 cell line has been shown by others to retain the potential for Phase I and Phase II metabolic responses to xenobiotics, e.g., expression of CYP1A1/2, 2A6, 2B6, 2C8/9, 2C19, 2D6/3A, 2E1, and 3A4/5 [2] with CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A activities reported at levels similar to human hepatocytes although variable depending on source and culture conditions [3]; some enzymes (e.g., CYP2W1) have even been observed at higher rates than in primary hepatocytes [4]. Phase II enzyme activities identified in HepG2 cells include SULTS (1A1, 1A2, 1E1 and 2A1), GSTs (mGST-1, GST μ1), NAT1, EPHX1 [5-7] and UGTs (1A1, 1A6 and 2B7) [7]. In addition, HepG2 cells can potentially express xenobiotic regulation activities via functionally active p53 protein [8] and Nrf2, a transcription factor which regulates genes containing antioxidant response element (ARE) sequences in their promoters; HepG2 cells also possess the capacity to express a number of ATP-binding cassette (ABC) xenobiotic export pumps (e.g., ABCC1, C2, C3 and G2 – membrane-bound proteins also regulated in part by Nrf2 TF DNA-binding) [9].

Basic Procedure:

Human liver HepG2 cells are transiently transfected with multiple reporter transcription units (MRTUs) in 6-well plates using FuGene 6 reagent (Roche; 3 ml FuGene/1 mg DNA). These transfected cells are aliquoted into wells of a 24-well plate, exposed to the chemical library of environmental compounds for 24 hours, and total RNA was isolated using TriZol reagent (Invitrogen). RNA was reverse-transcribed using oligo(dT) primer and Mo-MLV reverse transcriptase (Invitrogen) and treated with DNAse I (Ambion) for 30 min. Then one-tenth of the produced cDNA was amplified by PCR using Taq DNA polymerase (Invitrogen) and two reporter

sequence–specific primers. The PCR products were fluorescently labeled by primer extension with 6-arboxyfluorescein (6-FAM) 5'-labeled reporter sequence–specific primer (2 min at 95 °C, 20 s at 68 °C and 10 min at 72 °C) and these products were digested with 5U of *Hpa*I (New England Biolabs) for 2h at 37 °C. The fragments were purified using Qiaquick PCR columns (Qiagen), analyzed on an ABI 3130xL genetic analyzer (Applied Biosystems) with peak positions identified by using a set of X-rhodamine (ROX)-labeled MapMarker1000 molecular weight standards (BioVentures). The raw capillary electrophoresis data was processed using Attagraph software (Attagene).

Proprietary Elements:

FACTORIAL™ is a novel pathway profiling technology trademarked and patented by Attagene, Inc.

Caveats:

Due to low expression levels of estrogen receptor in HepG2 cells, a full-length, human estrogen receptor α cDNA was co-transfected in to the cells together with the MRTUs. The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote androgen receptor mediated DNA transcription, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

13.3. Assay References

Assay Source Contact Information:

Attagene, Inc.
7020 Kit Creek Road Suite 260
Morrisville, NC 27560
Tel: (888)721-2121

Assay Publication Year:

2010

Assay Publication:

Martin, M. T., Dix, D. J., Judson, R. S., Kavlock, R. J., Reif, D. M., Richard, A. M., Rotroff, D. M., Romanov, S., Medvedev, A., Poltoratskaya, N., Gambarian, M., Moeser, M., Makarov, S. S., & Houck, K. A. (2010). Impact of environmental chemicals on key transcription regulators and correlation to toxicity end points within EPA's ToxCast program. Chem Res Toxicol, 23(3), 578-590. (PMID: 20143881)

Method Updates / Confirmatory Studies:

None Reported

14. Assay Component Descriptions

Assay Objectives:

The Attagene CIS estrogen response element assay measures changes in the mRNA production controlled by a cis-acting element (promoter). Multiple RTU constructs are transfected into the human liver hepatoma cell line HepG2, and the cis- format assay measures changes in RTU expression resulting from endogenous TF binding, i.e. estrogen receptor α or β, to estrogen response element (ERE) DNA-binding sites following 24 hour exposures to the ToxCast chemical library. TF activity was reported via cDNA synthesis and RT-PCR of the RTU sequences followed by quantitation by capillary electrophoresis. Half-maximal activity (IC_{50}) was determined for each chemical using changes in transcription factor activity expressed as log2 fold induction over DMSO controls in a single replicate, 8-point concentration response series. Cytotoxicity assessments were performed concurrently using a MTT tetrazolium assay to establish maximum

tolerated concentrations (MTC) (see ATG_XTT_Cytotoxicity assay description for MTT procedures).

ATG_CIS series assays consist of multiplexed (FACTORIAL™) assay endpoints which assessed transcription factor activity using reporter transcription unit (RTU) quantitation in the HepG2 human liver hepatoma cell line. The multiple RTUs were constructed from essentially identical reporter gene sequences (representing a functionally inactivated fragment of human SEAP) cDNA and each tagged with *Hpa*I restriction cleavage sites at variable positions to allow discrimination of individual TF activities. When co-introduced into HepG2 cells, the RTUs produce reporter RNAs in amounts commensurable with the activities of the corresponding TFs present in a cell. This homogeneous construct creates a uniform environment for detecting the activity of many transcription factors simultaneously, and allowing for a more detailed assessment of xenobiotic impacts on the cellular regulatory network. The ATG_ERE_CIS describes MRTU FACTORIAL™ assays reporting activity in endogenous cis-regulatory estrogen response element (ERE) constructs (which are responsive to endogenous human estrogen receptor α and β).

Scientific Principles:

Endocrine disrupting chemicals (EDCs) are compounds which interfere with normal hormone biosynthesis, signaling or metabolism and impact regulatory pathways in humans and wildlife. Many EDCs interfere with normal steroidal activity by impacting estrogen signaling pathways. The estrogen receptor mediates gene expression in response to estrogen exposure, and modulates the activity for a wide variety of physiological processes. The ATG_ERE_CIS assay used a hepatoma cell-based platform to monitor estrogen receptor transcriptional activity and this assay is designed to help identify environmental compounds with a capacity for xenoestrogenic activity.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds which potentially affect endocrine systems in exposed populations by interacting with estrogen receptor mediated signaling pathways. There is evidence that estrogen receptor activation in early life is a molecular initiating event (MIE) in a developing Adverse Outcome Pathways (AOP) leading to endometrial carcinoma in the mouse (currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive *in vivo* studies to further investigate the involvement of ER interference in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Romanov, S., Medvedev, A., Gambarian, M., Poltoratskaya, N., Moeser, M., Medvedeva, L., Gambarian, M., Diatchenko, L., & Makarov, S. (2008). Homogeneous reporter system enables quantitative functional assessment of multiple transcription factors. Nat Methods, 5(3), 253-260. (PMID: 18297081)

Assay Quality Statistics:

Neutral control well median response value, by plate:	0.88
Neutral control median absolute deviation, by plate:	0.19
Positive control well median response value, by plate:	NA
Positive control well median absolute deviation, by plate:	NA
Z' (median across all plates, using positive control wells):	NA
SSMD (median across all plates, using positive control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	NA
CV (median across all plates):	0.21

(no positive control used for this assay)

15. Assay Endpoint Descriptions

15.1. Data Interpretation

Biological Response:

Transcription factor activity; increased production of mRNA transcripts production in response to active transcription following TF interaction with ERE promoter sequences as measured by RT_PCR and capillary electrophoretic detection of fluorescently labeled mRNA.

Analytical Elements:

ATG_ERE_CIS_up readout data was analyzed in the positive (gain of signal) fitting direction using log2 fold-induction over DMSO controls which provide a baseline signal. Negative and zero values are removed before analysis and raw values are log transformed. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Estrogen receptor transactivation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ACEA_T47D_80hr_Positive
ATG_ERE_CIS_up
ATG_ERa_TRANS_up
NVS_NR_bER
NVS_NR_hER
NVS_NR_mERa
OT_ER_ERaERa_0480
OT_ER_ERaERa_1440
OT_ERa_ERELUC_AG_1440
OT_ERa_EREGFP_0120
OT_ERa_EREGFP_0480
OT_ER_ERaERb_0480
OT_ER_ERaERb_1440
OT_ER_ERbERb_0480
OT_ER_ERbERb_1440
Tox21_ERa_BLA_Agonist_ratio
Tox21_ERa_LUC_BG1_Agonist

15.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations: 8
Target (nominal) number of replicates: 1
Standard minimum concentration tested: 0.09 µM
Standard maximum concentration tested: 200 µM

Baseline median absolute deviation for the assay - based on the response values at the 2 lowest tested concentrations (bmad): 0.10
The response cutoff used to derive the hit calls (e.g., 5*bmad, 10*bmad): 0.50

Reference Chemicals / Predictive Capacity:

CASRN	Chemical Name	*In Vitro* Activity	*In Vivo* Activity	Activity in Assay
57-91-0	17alpha-Estradiol	Moderate	Active	Yes
57-63-6	17alpha-Ethinylestradiol	Strong	Active	Yes
50-28-2	17beta-Estradiol	Strong	Active	Yes
58-18-4	17-Methyltestosterone	Very Weak	Active	Yes
131-55-5	2,2',4,4'-Tetrahydroxybenzophenone	NA	Active	Yes
131-56-6	2,4-Dihydroxybenzophenone	NA	Active	Yes
5153-25-3	2-Ethylhexylparaben	NA	Active	Yes
140-66-9	4-(1,1,3,3-Tetramethylbutyl)phenol	Moderate	Active	No
80-46-6	4-(2-Methylbutan-2-yl)phenol	NA	Active	Yes
80-09-1	4,4'-Sulfonyldiphenol	NA	Active	Yes
599-64-4	4-Cumylphenol	Weak	Active	Yes
104-43-8	4-Dodecylphenol	NA	Active	No
99-96-7	4-Hydroxybenzoic acid	NA	Inactive	Yes
104-40-5	4-Nonylphenol	Very Weak	Active	Yes
98-54-4	4-tert-Butylphenol	NA	Active	Yes
521-18-6	5alpha-Dihydrotestosterone	Weak	Active	Yes
61-82-5	Amitrole	NA	Inactive	Yes
520-36-5	Apigenin	Very Weak	NA	Yes
85-68-7	Benzyl butyl phthalate	Very Weak	NA	Yes
103-23-1	Bis(2-ethylhexyl)hexanedioate	NA	Inactive	Yes
80-05-7	Bisphenol A	Weak	Active	Yes
1478-61-1	Bisphenol AF	NA	Active	No
77-40-7	Bisphenol B	Weak	Active	Yes
94-26-8	Butylparaben	NA	Active	No
480-40-0	Chrysin	Very Weak	NA	No
50-22-6	Corticosterone	Inactive	NA	Yes
486-66-8	Daidzein	Weak	NA	Yes
117-81-7	Di(2-ethylhexyl) phthalate	Very Weak	Inactive	Yes
84-74-2	Dibutyl phthalate	Very Weak	Inactive	Yes
115-32-2	Dicofol	Very Weak	NA	Yes
84-61-7	Dicyclohexyl phthalate	NA	Inactive	Yes
84-66-2	Diethyl phthalate	NA	Inactive	Yes
56-53-1	Diethylstilbestrol	Strong	Active	Yes
84-75-3	Dihexyl phthalate	NA	Inactive	Yes

474-86-2	Equilin	NA	Active	Yes
50-27-1	Estriol	NA	Active	No
53-16-7	Estrone	moderate	Active	Yes
120-47-8	Ethylparaben	Very Weak	NA	Yes
60168-88-9	Fenarimol	Very Weak	NA	Yes
51630-58-1	Fenvalerate	NA	Inactive	Yes
446-72-0	Genistein	Weak	Active	No
52-86-8	Haloperidol	Inactive	NA	No
520-18-3	Kaempferol	Very Weak	Inactive	Yes
143-50-0	Kepone	Weak	NA	Yes
65277-42-1	Ketoconazole	Inactive	NA	Yes
330-55-2	Linuron	Inactive	NA	Yes
84-16-2	meso-Hexestrol	Strong	NA	Yes
72-33-3	Mestranol	NA	Active	Yes
72-43-5	Methoxychlor	Very Weak	Active	Yes
68-22-4	Norethindrone	NA	Active	Yes
789-02-6	o,p'-DDT	Weak	Active	Yes
556-67-2	Octamethylcyclotetrasiloxane	NA	Active	Yes
72-55-9	p,p'-DDE	Very Weak	Weak	Yes
87-86-5	Pentachlorophenol	NA	Inactive	No
57-30-7	Phenobarbital sodium	Inactive	NA	No
32809-16-8	Procymidone	Inactive	NA	Yes
50-55-5	Reserpine	Inactive	NA	No
52-01-7	Spironolactone	Inactive	NA	No
17924-92-4	Zearalenone	NA	Active	Yes

In Vitro Activity	ToxCast Active	ToxCast Inactive
Active	27	2
Inactive	2	6

In Vivo Activity	ToxCast Active	ToxCast Inactive
Active	30	0
Inactive	6	5

In Vitro Sensitivity = 93.1%
In Vitro Specificity = 75.0%
Balanced Accuracy = 84.1%

In Vivo Sensitivity = 100.0%
In Vivo Specificity = 45.5%
Balanced Accuracy = 72.7%

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich

chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [10].

16. Assay Documentation

16.1. References

[1] Aden, D. P., et al. (1979). Nature 282: 615-616. (PMID: 233137)
[2] Westerink, W. M. and W. G. Schoonen (2007). Toxicol In Vitro 21(8): 1581-1591. (PMID: 17637504)
[3] Hewitt, N. and P. Hewitt (2004). Xenobiotica 34(3): 243-256. (PMID: 15204697)
[4] Guo, L., et al. (2010). Drug Metab Disposition 39(3): 528-538. (PMID: 21149542)
[5] Westerink, W. M. and W. G. Schoonen (2007). Toxicol In Vitro 21(8): 1592-1602. (PMID: 17716855)
[6] Walle, T., et al. (2000). Drug Metab Disposition 28(9): 1077-1082. (PMID: 10950852)
[7] Hart, S. N., et al. (2010). Drug Metab Disposition 38(6): 988-994. (PMID: 20228232)
[8] Boehme, K., et al. (2010). Toxicol Lett 198(2): 272-281. (PMID: 20655369)
[9] Adachi, T., et al. (2007). J Exper Therap Oncol 6(4): 335-348. (PMID: 18038766)
[10] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

16.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
ATCC, American Tissue Culture Collection
CYP, Cytochrome P450s
DMSO, Dimethyl Sulfoxide
EDC, Endocrine disrupting chemicals
ER, Estrogen Receptor
ERE, Estrogen Response Element
E2, Estradiol
GST, Glutathione S-Transferase
MIE, Molecular Initiating Event
MRTU, Multiple Reporter Transcription Unit
NR, Nuclear Receptors
RTU, Reporter Transcription Unit
SEAP, Secreted Embryonic Alkaline Phosphatase
SULT, Sulfotransferases
TF, Transcription Factor
UGT, UDP-Glucuronosyltransferase

16.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

2 May 2016

Date of Revisions:

25 November 2016

Author of Revisions:

EPA NCCT

17. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:

Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;

Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation

Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;

Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

18. Supporting Information (existing annotations):

NVS_NR_bER

Assay Title: NovaScreen Bovine Estrogen Receptor HTS Ligand-Binding Assay

19. Assay Descriptions

19.1. Overview

Assay Summary:

High-throughput screening of in vitro chemical-target interactions across a wide variety of compounds through a broad range of biochemical interactions will help describe the chemical-assay bioactivity space for chemicals with limited available information. There exists a large number of environmental chemicals for which there is little information about the potential for xenoestrogenic activity. This assay format allows for an efficient screening of thousands of chemicals for the ability to bind to the receptor and displace a radiolabeled ligand from the ligand-binding domain of the estrogen receptor. Biochemical high-throughput screening offers preliminary evidence for chemical targets in a cell or tissues which, when combined with information from literature or targeted in vivo studies, can indicate potential pathways for toxicity. This assay was run for a test duration of 18 hours in a 96-well plate.

19.2. Assay Definition

Assay Throughput:

This is a biochemical (cell-free) format, using 96-well plates to incubate radiolabeled ligand with estrogen receptor alpha for 18 hours to measure displacement of estradiol by test chemicals in a competitive binding assay.

Experimental System:

ERα nuclear protein, derived from bovine uterine membranes

Xenobiotic Biotransformation Potential:

None

Basic Procedure:

Materials:

Receptor Source: Bovine uterine membranes
Radioligand: [3H] Estradiol
Final ligand concentration - [0.7 nM]
Non-specific Determinant: 17β-Estradiol - [10 nM]
Positive Control: 17β-Estradiol

Methods:

Incubation Conditions: Reactions are carried out in 10 mM TRIS-HCI (pH 7.4 containing 1.5 mM EDTA, 1.0 mM DTT, and 25 mM sodium molybdate at 0-4 °C for 18 hours. The reaction is terminated by the addition of dextran-coated charcoal and incubated for 20 minutes at 0-4 °C. The reaction mixtures are centrifuged and the radioactivity bound in the supernatant is assessed and compared to control values in order to ascertain any interactions of test compound with the estradiol binding site.

Proprietary Elements:

This assay is not proprietary.

Caveats:

The NovaScreen Nuclear Receptor assays described here are run in a cell-free format, and as such lack the ability to model the protective cell membrane and biotransformation capacity expected in in vivo or cell-based systems. The potential for a particular compound to affect changes in estrogen signaling pathways is not exclusively a function of receptor-ligand binding affinity, but is also a measure of the propensity for the activated receptor-ligand complex to form dimers and recruit co-activators, of the affinity for the activated complex to bind to hormone response element DNA sequences, and of interactions with other signaling pathways.

19.3. Assay References

Assay Source Contact Information:

PerkinElmer Office
940 Winter St.
Waltham, Massachusetts 02451
United States
Tel: (781) 663-6900

Assay Publication Year:

2011

Assay Publication:

Knudsen, T. B., Houck, K. A., Sipes, N. S., Singh, A. V., Judson, R. S., Martin, M. T., Weissman, A., Kleinstreuer, N. C., Mortensen, H. M., Reif, D. M., Rabinowitz, J. R., Setzer, R. W., Richard, A. M., Dix, D. J., & Kavlock, R. J. (2011). "Activity profiles of 309 ToxCast chemicals evaluated across 292 biochemical targets". Toxicology 282(1-2), 1-15. (PMID: 21251949)

Sipes, N. S., Martin, M. T., Kothiya, P., Reif, D. M., Judson, R. S., Richard, A. M., Houck, K. A., Dix, D. J., Kavlock, R. J., & Knudsen, T. B. (2013). "Profiling 976 ToxCast chemicals across 331 enzymatic and receptor signaling assays". Chem Res Toxicol 26(6), 878-895. (PMID: 23611293

Method Updates / Confirmatory Studies:

None Reported.

20. Assay Component Descriptions

Assay Objectives:

The NovaScreen nuclear receptor bovine estrogen receptor ligand-binding assay used a biochemical (cell-free) platform in high-throughput (96-well microplate) format to screen the ToxCast chemical library for xenoestrogenic interaction with bovine nuclear receptors. An initial screening run was conducted exposing estrogen receptors to 25 μM of each chemical (in duplicate). Response to chemical perturbation was measured using radioligand detection (via liquid scintillation counting) of displacement of [^{3}H]-Estradiol. 17β-Estradiol (E2) was used as a positive control. If the response signal differed by over 30% or varied by a minimum of 3.0 baseline median absolute deviations (3BMAD) from the vehicle control (DMSO), the chemical was considered active and retested in a concentration–response format assay using 8 concentrations derived from a serial dilution series using half-log increments and a top concentration of 50 μM. This assay used a cell-free high-throughput format to probe a diverse chemical library for potential ligand-binding activity with estrogen nuclear receptor alpha (ERα) derived from bovine uterine membranes.

Scientific Principles:

Endocrine disrupting chemicals (EDCs) are compounds which interfere with normal hormone biosynthesis, signaling or metabolism and impact regulatory pathways in humans and wildlife. Many EDCs interfere with normal steroidal activity by impacting estrogen signaling pathways. The estrogen receptor mediates gene expression in response to estrogen exposure, and modulates the activity for a wide variety of physiological processes. The NovaScreen assays are modifications of pre-clinical drug development assays and are designed to examine chemical effects on a broad spectrum of biochemical targets or potential molecular initiating events in a high-throughput format. This assay is designed to help identify environmental compounds with a capacity for xenoestrogenic ligand-binding activity.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with estrogen receptor alpha mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that estrogen receptor activity in early life is a molecular initiating event (MIE)

leading to breast cancer in both animal and human models and to endometrial carcinoma in the mouse, and ER agonism is the MIE leading to reproductive dysfunction in oviparous vertebrates, and there is some evidence that estrogen receptor activation is the MIE for putative adverse outcome pathways leading to reduced survival due to renal failure and leading to skewed sex ratios due to altered sexual differentiation in males (all AOPs under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive *in vivo* studies to further investigate the involvement of ER agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Haji, M., Kato, K., Nawata, H., & Ibayashi, H. (1981). "Age-related changes in the concentrations of cytosol receptors for sex steroid hormones in the hypothalamus and pituitary gland of the rat". Brain Res 204(2), 373-386. (PMID: 6780133)

O'Keefe, J. A., & Handa, R. J. (1990). "Transient elevation of estrogen receptors in the neonatal rat hippocampus". Dev Brain Res 57(1), 119-127. (PMID: 2090365)

Assay Quality Statistics:

Neutral control well median response value, by plate:	2637.21
Neutral control median absolute deviation, by plate:	74.86
Positive control well median response value, by plate:	521.88
Positive control well median absolute deviation, by plate:	21.18
Z' (median across all plates, using positive control wells):	0.86
SSMD (median across all plates, using positive control wells):	-26
Signal-to-noise (median across all plates, using positive control wells):	-29.49
Signal-to-background (median across all plates, using positive control wells):	0.19
CV (median across all plates):	0.03

21. Assay Endpoint Descriptions

21.1. Data Interpretation

Biological Response:

Competitive radioligand binding of [3H] Estradiol (positive control) with estrogen receptor α obtained from bovine uterine membranes and measured by radiometric detection.

Analytical Elements:

The NVS_NR_bER assay results were analyzed as loss-of-signal in competitive displacement assays where the endpoint measured was inhibition of [^{3}H] 17β-estradiol binding. Raw data values were normalized to DMSO (neutral control) and reported as percent of 17-β Estradiol (positive control) binding capacity. Following initial screening of test compounds at single concentration (25 μM), if the chemical response was >30% of the solvent control (DMSO) activity or at least 2 baseline median average deviations (2BMAD), the chemical was considered active against the estrogen receptor and was tested in a concentration-response assay for ER binding using 8 concentrations with 3-fold serial dilutions generally beginning at a high concentration of 50 μM. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Estrogen receptor activity was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 30% of neutral controls or 3 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in μM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for

Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ACEA_T47D_80hr_Positive
ATG_ERE_CIS_up
ATG_ERa_TRANS_up
ATG_ERb_TRANS2_up
NVS_NR_hER
NVS_NR_mERa
OT_ER_ERaERa_0480
OT_ER_ERaERa_1440
OT_ER_ERaERb_0480
OT_ER_ERaERb_1440
OT_ER_ERbERb_0480
OT_ER_ERbERb_1440
OT_ERa_ERELUC_AG_1440
OT_ERa_ERELUC_ANT_1440
OT_ERa_EREGFP_0120
OT_ERa_EREGFP_0480
OT_ERb_ERELUC_ANT_1440
Tox21_ERa_BLA_Agonist_ratio
Tox21_ERa_BLA_Antagonist_ratio
Tox21_ERa_LUC_BG1_Agonist
Tox21_ERa_LUC_BG1_Antagonist

21.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations: 8
Target (nominal) number of replicates: 1
Standard minimum concentration tested: 0.02 µM
Standard maximum concentration tested: 50 µM
Baseline median absolute deviation for the assay - based on the response values at the 2 lowest tested concentrations (bmad): 0.4.03
The response cutoff used to derive the hit calls (e.g., 5*bmad, 10*bmad): 24.17

Reference Chemicals / Predictive Capacity:

CASRN	Chemical Name	*In Vitro* Activity	*In Vivo* Activity	Activity in Assay
57-91-0	17alpha-Estradiol	Moderate	Active	Yes
57-63-6	17alpha-Ethinylestradiol	Strong	Active	Yes
50-28-2	17beta-Estradiol	Strong	Active	Yes
58-18-4	17-Methyltestosterone	Very Weak	Active	No
131-55-5	2,2',4,4'-Tetrahydroxybenzophenone	NA	Active	Yes
131-56-6	2,4-Dihydroxybenzophenone	NA	Active	No
5153-25-3	2-Ethylhexylparaben	NA	Active	No

140-66-9	4-(1,1,3,3-Tetramethylbutyl)phenol	Moderate	Active	Yes
80-46-6	4-(2-Methylbutan-2-yl)phenol	NA	Active	Yes
80-09-1	4,4'-Sulfonyldiphenol	NA	Active	No
599-64-4	4-Cumylphenol	Weak	Active	Yes
104-43-8	4-Dodecylphenol	NA	Active	Yes
99-96-7	4-Hydroxybenzoic acid	NA	Inactive	Yes
104-40-5	4-Nonylphenol	Very Weak	Active	Yes
98-54-4	4-tert-Butylphenol	NA	Active	No
521-18-6	5alpha-Dihydrotestosterone	Weak	Active	Yes
61-82-5	Amitrole	NA	Inactive	No
520-36-5	Apigenin	Very Weak	NA	No
85-68-7	Benzyl butyl phthalate	Very Weak	NA	Yes
103-23-1	Bis(2-ethylhexyl)hexanedioate	NA	Inactive	No
80-05-7	Bisphenol A	Weak	Active	No
1478-61-1	Bisphenol AF	NA	Active	No
77-40-7	Bisphenol B	Weak	Active	Yes
94-26-8	Butylparaben	NA	Active	No
480-40-0	Chrysin	Very Weak	NA	Yes
50-22-6	Corticosterone	Inactive	NA	Yes
486-66-8	Daidzein	Weak	NA	Yes
117-81-7	Di(2-ethylhexyl) phthalate	Very Weak	Inactive	Yes
84-74-2	Dibutyl phthalate	Very Weak	Inactive	Yes
115-32-2	Dicofol	Very Weak	NA	No
84-61-7	Dicyclohexyl phthalate	NA	Inactive	No
84-66-2	Diethyl phthalate	NA	Inactive	Yes
56-53-1	Diethylstilbestrol	Strong	Active	No
84-75-3	Dihexyl phthalate	NA	Inactive	No
474-86-2	Equilin	NA	Active	No
50-27-1	Estriol	NA	Active	No
53-16-7	Estrone	Moderate	Active	Yes
120-47-8	Ethylparaben	Very Weak	NA	Yes
60168-88-9	Fenarimol	Very Weak	NA	No
51630-58-1	Fenvalerate	NA	Inactive	No
446-72-0	Genistein	Weak	Active	No
52-86-8	Haloperidol	Inactive	NA	Yes
520-18-3	Kaempferol	Very Weak	Inactive	No
143-50-0	Kepone	Weak	NA	No
65277-42-1	Ketoconazole	Inactive	NA	No
330-55-2	Linuron	Inactive	NA	Yes
84-16-2	meso-Hexestrol	Strong	NA	No

72-33-3	Mestranol	NA	Active	No
72-43-5	Methoxychlor	Very Weak	Active	No
68-22-4	Norethindrone	NA	Active	No
789-02-6	o,p'-DDT	Weak	Active	No
556-67-2	Octamethylcyclotetrasiloxane	NA	Active	Yes
72-55-9	p,p'-DDE	Very Weak	Weak	Yes
87-86-5	Pentachlorophenol	NA	Inactive	Yes
57-30-7	Phenobarbital sodium	Inactive	NA	Yes
32809-16-8	Procymidone	Inactive	NA	Yes
50-55-5	Reserpine	Inactive	NA	No
52-01-7	Spironolactone	Inactive	NA	Yes
17924-92-4	Zearalenone	NA	Active	No

In Vitro Activity	ToxCast Active	ToxCast Inactive
Active	12	16
Inactive	0	6

In Vivo Activity	ToxCast Active	ToxCast Inactive
Active	22	8
Inactive	0	6

In Vitro Sensitivity = 42.9%
In Vitro Specificity = 100.0%
Balanced Accuracy = 71.4%

In Vivo Sensitivity = 73.3%
In Vivo Specificity = 100.0%
Balanced Accuracy = 86.7%

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [1].

22. Assay Documentation

22.1. References

[1] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

22.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
DMSO, Dimethyl Sulfoxide
EDC, Endocrine disrupting chemicals
ER, Estrogen Receptor
E2, Estradiol
MIE, Molecular Initiating Event
NR, Nuclear Receptor
NVS, NovaScreen

22.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

2 May 2016

Date of Revisions:

25 November 2016

Author of Revisions:

EPA NCCT

23. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:

Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;

Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation

Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;

Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

24. Supporting Information (existing annotations):

NVS_NR_hER

Assay Title: NovaScreen Human Estrogen Receptor HTS Ligand-Binding Assay

25. Assay Descriptions

25.1. Overview

Assay Summary:

High-throughput screening of in vitro chemical-target interactions across a wide variety of compounds through a broad range of biochemical interactions will help describe the chemical-assay bioactivity space for chemicals with limited available information. There exists a large number of environmental chemicals for which there is little information about the potential for xenoestrogenic activity. This assay format allows for an efficient screening of thousands of chemicals for the ability to bind to the receptor and displace a radiolabeled ligand from the ligand-binding domain of the estrogen receptor. Biochemical high-throughput screening offers preliminary evidence for chemical targets in a cell or tissues which, when combined with information from literature or targeted in vivo studies, can indicate potential pathways for toxicity. This assay was run for a test duration of 18 hours in a 96-well plate.

25.2. Assay Definition

Assay Throughput:

Human ERα nuclear protein incubated in 96-well microtiter plates for 18 hours prior to measuring displacement of radiolabeled 17β-Estradiol by test compounds.

Experimental System:

ERα nuclear protein, derived from human breast adenocarcinoma (MCF-7) cell line.

Xenobiotic Biotransformation Potential:

None

Basic Procedure:

Materials:

Receptor Source: MCF-7 cells
Radioligand: [3H] Estradiol
Final Ligand Concentration – [0.1 nM]
Non-specific Determinant: 17β-Estradiol - [300 nM]
Reference Compound: 17β-Estradiol
Positive Control: 17β-Estradiol

Methods:

Incubation Conditions: Reactions are carried out in 10 mM TRIS-HCI (pH 7.4) containing 1.5 mM EDTA, 1.0 mM DTT, and 25 mM sodium molybdate at 0-4 °C for 18 hours. The reaction is terminated by the addition of dextran-coated charcoal and incubated for 20 minutes at 0-4°C. The reaction mixtures are centrifuged and the radioactivity bound in the supernatant is assessed and compared to control values in order to ascertain any interactions of test compound with the estradiol binding site.

Proprietary Elements:

This assay is not proprietary.

Caveats:

The NovaScreen Nuclear Receptor assays described here are run in a cell-free format, and as such lack the ability to model the protective cell membrane and biotransformation capacity expected in in vivo and cell-based systems. The potential for a particular compound to affect changes in estrogen signaling pathways is not exclusively a function of receptor-ligand binding affinity, but is also a measure of the propensity for the activated receptor-ligand complex to form dimers and recruit co- activators, of the affinity for the activated complex to bind to hormone response element DNA sequences, and of interactions with other signaling pathways.

25.3. Assay References

Assay Source Contact Information:

PerkinElmer Office
940 Winter St.
Waltham, Massachusetts 02451
United States
Tel: (781) 663-6900

Assay Publication Year:

2011

Assay Publication:

Knudsen, T. B., Houck, K. A., Sipes, N. S., Singh, A. V., Judson, R. S., Martin, M. T., Weissman, A., Kleinstreuer, N. C., Mortensen, H. M., Reif, D. M., Rabinowitz, J. R., Setzer, R. W., Richard, A. M., Dix, D. J., & Kavlock, R. J. (2011). "Activity profiles of 309 ToxCast chemicals evaluated across 292 biochemical targets". Toxicology 282(1-2), 1-15. (PMID: 21251949)

Sipes, N. S., Martin, M. T., Kothiya, P., Reif, D. M., Judson, R. S., Richard, A. M., Houck, K. A., Dix, D. J., Kavlock, R. J., & Knudsen, T. B. (2013). "Profiling 976 ToxCast chemicals across 331 enzymatic and receptor signaling assays". Chem Res Toxicol 26(6), 878-895. (PMID: 23611293

Method Updates / Confirmatory Studies:

None Reported.

26. Assay Component Descriptions

Assay Objectives:

The NovaScreen nuclear receptor human estrogen receptor ligand-binding assay used a biochemical (cell-free) platform in high-throughput (96-well microplate) format to screen the ToxCast chemical library for xenoestrogenic interaction with estrogen receptors. An initial screening run was conducted exposing human estrogen receptors to 25 μM of each chemical (in duplicate). Response to chemical perturbation was measured using radioligand detection (via liquid scintillation counting) of displacement of [^{3}H]-Estradiol. 17β-Estradiol (E2) was used as a positive control. If the response signal differed by over 30% or varied by a minimum of 3.0 baseline median absolute deviations (3BMAD) from the vehicle control (DMSO), the chemical was considered active and retested in a concentration–response format assay using 8 concentrations derived from a serial dilution series using half-log increments and a top concentration of 50 μM. This assay used a cell-free high-throughput format to probe a diverse chemical library for potential ligand-binding activity with estrogen nuclear receptor alpha (ERα) derived from MCF-7 human breast adenocarcinoma lysate.

Scientific Principles:

Endocrine disrupting chemicals (EDCs) are compounds which interfere with normal hormone biosynthesis, signaling or metabolism and impact regulatory pathways in humans and wildlife. Many EDCs interfere with normal steroidal activity by impacting estrogen signaling pathways. The estrogen receptor mediates gene expression in response to estrogen exposure, and modulates the activity for a wide variety of physiological processes. The NovaScreen assays are modifications of pre-clinical drug development assays and are designed to examine chemical effects on a broad spectrum of biochemical targets or potential molecular initiating events in a high-throughput format. This assay is designed to help identify environmental compounds with a capacity for xenoestrogenic ligand-binding activity.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with estrogen receptor alpha mediated pathways and potentially affect endocrine systems in exposed populations. There is

strong evidence that estrogen receptor activity in early life is a molecular initiating event (MIE) leading to breast cancer in both animal and human models and to endometrial carcinoma in the mouse, and ER agonism is the MIE leading to reproductive dysfunction in oviparous vertebrates, and there is some evidence that estrogen receptor activation is the MIE for putative adverse outcome pathways leading to reduced survival due to renal failure and leading to skewed sex ratios due to altered sexual differentiation in males (all AOPs under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive in vivo studies to further investigate the involvement of ER agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Haji, M., Kato, K., Nawata, H., & Ibayashi, H. (1981). "Age-related changes in the concentrations of cytosol receptors for sex steroid hormones in the hypothalamus and pituitary gland of the rat". Brain Res 204(2), 373-386. (PMID: 6780133)

O'Keefe, J. A., & Handa, R. J. (1990). "Transient elevation of estrogen receptors in the neonatal rat hippocampus". Dev Brain Res 57(1), 119-127. (PMID: 2090365)

Assay Quality Statistics:

Neutral control well median response value, by plate:	4467.25
Neutral control median absolute deviation, by plate:	93.76
Positive control well median response value, by plate:	293.52
Positive control well median absolute deviation, by plate:	20.89
Z' (median across all plates, using positive control wells):	0.9
SSMD (median across all plates, using positive control wells):	-36
Signal-to-noise (median across all plates, using positive control wells):	-40.33
Signal-to-background (median across all plates, using positive control wells):	0.08
CV (median across all plates):	0.02

27. Assay Endpoint Descriptions

27.1. Data Interpretation

Biological Response:

Competitive displacement of [3H] Estradiol (positive control) with estrogen receptor α obtained from human breast adenocarcinoma cell line (MCF-7) as measured by detection of radioligand.

Analytical Elements:

The NVS_NR_hER assay results were analyzed as loss-of-signal in competitive displacement assays where the endpoint measured was inhibition of [^{3}H] 17β-estradiol binding. Raw data values were normalized as percent of 17-β Estradiol (positive control) binding capacity. If the chemical interaction was >30% of the solvent control (DMSO) or if the signal varied by more than 3.0 median average deviations (3MAD), the chemical was considered active against the estrogen receptor and was tested in a concentration-response assay for ER binding using 8 concentrations with 3-fold serial dilutions generally beginning at a high concentration of 50 µM. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Estrogen receptor activity was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 30% of neutral controls or 3 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for

Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ACEA_T47D_80hr_Positive
ATG_ERE_CIS_up
ATG_ERa_TRANS_up
ATG_ERb_TRANS2_up
NVS_NR_bER
NVS_NR_mERa
OT_ER_ERaERa_0480
OT_ER_ERaERa_1440
OT_ER_ERaERb_0480
OT_ER_ERaERb_1440
OT_ER_ERbERb_0480
OT_ER_ERbERb_1440
OT_ERa_ERELUC_AG_1440
OT_ERa_ERELUC_ANT_1440
OT_ERa_EREGFP_0120
OT_ERa_EREGFP_0480
OT_ERb_ERELUC_ANT_1440
Tox21_ERa_BLA_Agonist_ratio
Tox21_ERa_BLA_Antagonist_ratio
Tox21_ERa_LUC_BG1_Agonist
Tox21_ERa_LUC_BG1_Antagonist

27.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations: 8
Target (nominal) number of replicates: 1
Standard minimum concentration tested: 0.02 µM
Standard maximum concentration tested: 50 µM
Baseline median absolute deviation for the assay - based on the response values at the 2 lowest tested concentrations (bmad): 4.07
The response cutoff used to derive the hit calls (e.g., 5*bmad, 10*bmad): 24.43

Reference Chemicals / Predictive Capacity:

CASRN	Chemical Name	*In Vitro* Activity	*In Vivo* Activity	Activity in Assay
57-91-0	17alpha-Estradiol	Moderate	Active	Yes
57-63-6	17alpha-Ethinylestradiol	Strong	Active	Yes
50-28-2	17beta-Estradiol	Strong	Active	Yes
58-18-4	17-Methyltestosterone	Very Weak	Active	Yes
131-55-5	2,2',4,4'-Tetrahydroxybenzophenone	NA	Active	Yes
131-56-6	2,4-Dihydroxybenzophenone	NA	Active	Yes
5153-25-3	2-Ethylhexylparaben	NA	Active	Yes

140-66-9	4-(1,1,3,3-Tetramethylbutyl)phenol	Moderate	Active	Yes
80-46-6	4-(2-Methylbutan-2-yl)phenol	NA	Active	Yes
80-09-1	4,4'-Sulfonyldiphenol	NA	Active	Yes
599-64-4	4-Cumylphenol	Weak	Active	Yes
104-43-8	4-Dodecylphenol	NA	Active	Yes
99-96-7	4-Hydroxybenzoic acid	NA	Inactive	Yes
104-40-5	4-Nonylphenol	Very Weak	Active	Yes
98-54-4	4-tert-Butylphenol	NA	Active	Yes
521-18-6	5alpha-Dihydrotestosterone	Weak	Active	No
61-82-5	Amitrole	NA	Inactive	No
520-36-5	Apigenin	Very Weak	NA	Yes
85-68-7	Benzyl butyl phthalate	Very Weak	NA	No
103-23-1	Bis(2-ethylhexyl)hexanedioate	NA	Inactive	No
80-05-7	Bisphenol A	Weak	Active	No
1478-61-1	Bisphenol AF	NA	Active	Yes
77-40-7	Bisphenol B	Weak	Active	No
94-26-8	Butylparaben	NA	Active	No
480-40-0	Chrysin	Very Weak	NA	Yes
50-22-6	Corticosterone	Inactive	NA	Yes
486-66-8	Daidzein	Weak	NA	Yes
117-81-7	Di(2-ethylhexyl) phthalate	Very Weak	Inactive	Yes
84-74-2	Dibutyl phthalate	Very Weak	Inactive	Yes
115-32-2	Dicofol	Very Weak	NA	Yes
84-61-7	Dicyclohexyl phthalate	NA	Inactive	No
84-66-2	Diethyl phthalate	NA	Inactive	Yes
56-53-1	Diethylstilbestrol	Strong	Active	No
84-75-3	Dihexyl phthalate	NA	Inactive	Yes
474-86-2	Equilin	NA	Active	Yes
50-27-1	Estriol	NA	Active	No
53-16-7	Estrone	Moderate	Active	No
120-47-8	Ethylparaben	Very Weak	NA	Yes
60168-88-9	Fenarimol	Very Weak	NA	Yes
51630-58-1	Fenvalerate	NA	Inactive	Yes
446-72-0	Genistein	Weak	Active	Yes
52-86-8	Haloperidol	Inactive	NA	No
520-18-3	Kaempferol	Very Weak	Inactive	Yes
143-50-0	Kepone	Weak	NA	No
65277-42-1	Ketoconazole	Inactive	NA	No
330-55-2	Linuron	Inactive	NA	No
84-16-2	meso-Hexestrol	Strong	NA	Yes

72-33-3	Mestranol	NA	Active	No
72-43-5	Methoxychlor	Very Weak	Active	No
68-22-4	Norethindrone	NA	Active	No
789-02-6	o,p'-DDT	Weak	Active	No
556-67-2	Octamethylcyclotetrasiloxane	NA	Active	No
72-55-9	p,p'-DDE	Very Weak	Weak	Yes
87-86-5	Pentachlorophenol	NA	Inactive	Yes
57-30-7	Phenobarbital sodium	Inactive	NA	Yes
32809-16-8	Procymidone	Inactive	NA	Yes
50-55-5	Reserpine	Inactive	NA	Yes
52-01-7	Spironolactone	Inactive	NA	Yes
17924-92-4	Zearalenone	NA	Active	Yes

In Vitro Activity	ToxCast Active	ToxCast Inactive
Active	20	8
Inactive	0	7

In Vivo Activity	ToxCast Active	ToxCast Inactive
Active	26	4
Inactive	1	5

In Vitro Sensitivity = 71.4%
In Vitro Specificity = 100.0%
Balanced Accuracy = 85.7%

In Vivo Sensitivity = 86.7%
In Vivo Specificity = 83.3%
Balanced Accuracy = 85.0%

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds

recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [1].

28. Assay Documentation

28.1. References

[1] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

28.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
DMSO, Dimethyl Sulfoxide
EDC, Endocrine disrupting chemicals
ER, Estrogen Receptor
E2, Estradiol
MIE, Molecular Initiating Event
NR, Nuclear Receptor
NVS, NovaScreen

28.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

2 May 2016

Date of Revisions:

25 November 2016

Author of Revisions:

EPA NCCT

29. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:

Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;

Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation

Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;

Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

30. Supporting Information (existing annotations):

NVS_NR_mERa

Assay Title: NovaScreen Murine Estrogen Receptor HTS Ligand-Binding Assay

31. Assay Descriptions

31.1. Overview

Assay Summary:

High-throughput screening of in vitro chemical-target interactions across a wide variety of compounds through a broad range of biochemical interactions will help describe the chemical-assay bioactivity space for chemicals with limited available information. There exists a large number of environmental chemicals for which there is little information about the potential for xenoestrogenic activity. This assay format allows for an efficient screening of thousands of chemicals for the ability to bind to the receptor and displace a radiolabeled ligand from the ligand-binding domain of the estrogen receptor. Biochemical high-throughput screening offers preliminary evidence for chemical targets in a cell or tissues which, when combined with information from literature or targeted in vivo studies, can indicate potential pathways for toxicity. This assay was run for a test duration of 18 hours in a 96-well plate.

31.2. Assay Definition

Assay Throughput:

This is a biochemical (cell-free) format, using 96-well plates to incubate radiolabeled ligand with estrogen receptor alpha for 18 hours to measure displacement of estradiol by test chemicals in a competitive binding assay.

Experimental System:

ERα nuclear protein, derived from mouse tissue

Xenobiotic Biotransformation Potential:

None

Basic Procedure:

Materials:

Receptor Source: murine ER alpha
Radioligand: [3H] Estradiol - [1 nM]
Non-specific Determinant: 17β-Estradiol - [1 μM]
Reference Compound: 17β-Estradiol
Positive Control: 17β-Estradiol

Methods:

Incubation Conditions: Reactions are carried out in 25 mM TRIS-HCI (pH 7.4) containing 1.5 mM EDTA, 1 mM DTT, and 25 mM sodium molybdate at 0-4 °C for 18 hours. The reaction is terminated by rapid vacuum filtration onto glass fiber filters soaked in 0.5% PEI. Filters were washed with cold 50 mM NaCl. Radioactivity trapped onto the filters is determined and compared to control values in order to ascertain any interactions of test compound with the estrogen receptor binding site.

Proprietary Elements:

This assay is not proprietary.

Caveats:

The NovaScreen Nuclear Receptor assays described here are run in a cell-free format, and as such lack the ability to model the protective cell membrane and biotransformation capacity expected in in vivo and cell-based systems. The potential for a particular compound to affect changes in estrogen signaling pathways is not exclusively a function of receptor-ligand binding affinity, but is also a measure of the propensity for the activated receptor-ligand complex to form dimers and recruit co-activators, of the affinity for the activated complex to bind to hormone response element DNA sequences, and of interactions with other signaling pathways.

31.3. Assay References

Assay Source Contact Information:

PerkinElmer Office
940 Winter St.
Waltham, Massachusetts 02451
United States
Tel: (781) 663-6900

Assay Publication Year:

2011

Assay Publication:

Knudsen, T. B., Houck, K. A., Sipes, N. S., Singh, A. V., Judson, R. S., Martin, M. T., Weissman, A., Kleinstreuer, N. C., Mortensen, H. M., Reif, D. M., Rabinowitz, J. R., Setzer, R. W., Richard, A. M., Dix, D. J., & Kavlock, R. J. (2011). "Activity profiles of 309 ToxCast chemicals evaluated across 292 biochemical targets". Toxicology 282(1-2), 1-15. (PMID: 21251949)

Sipes, N. S., Martin, M. T., Kothiya, P., Reif, D. M., Judson, R. S., Richard, A. M., Houck, K. A., Dix, D. J., Kavlock, R. J., & Knudsen, T. B. (2013). "Profiling 976 ToxCast chemicals across 331 enzymatic and receptor signaling assays". Chem Res Toxicol 26(6), 878-895. (PMID: 23611293

Method Updates / Confirmatory Studies:

None Reported.

32. Assay Component Descriptions

Assay Objectives:

The NovaScreen nuclear receptor murine estrogen receptor ligand-binding assay used a biochemical (cell-free) platform in high-throughput (96-well microplate) format to screen the ToxCast chemical library for xenoestrogenic interaction with murine estrogen receptors. An initial screening run was conducted exposing estrogen receptors to 25 μM of each chemical (in duplicate). Response to chemical perturbation was measured using radioligand detection (via liquid scintillation counting) of displacement of [^{3}H]-Estradiol. 17β-Estradiol (E2) was used as a positive control. If the response signal differed by over 30% from the solvent control (DMSO) or if the signal varied by more than 2.0 median average deviations (2MAD), the chemical was considered active and retested in a concentration response format assay using 8 concentrations derived from a serial dilution series using half-log increments and a top concentration of 50 μM. This assay used a cell-free high-throughput format to probe a diverse chemical library for potential ligand-binding activity with estrogen nuclear receptor alpha (ERα) derived from murine tissues.

Scientific Principles:

Endocrine disrupting chemicals (EDCs) are compounds which interfere with normal hormone biosynthesis, signaling or metabolism and impact regulatory pathways in humans and wildlife. Many EDCs interfere with normal steroidal activity by impacting estrogen signaling pathways. The estrogen receptor mediates gene expression in response to estrogen exposure, and modulates the activity for a wide variety of physiological processes. The NovaScreen assays are modifications of pre-clinical drug development assays and are designed to examine chemical effects on a broad spectrum of biochemical targets or potential molecular initiating events in a high-throughput format. This assay is designed to help identify environmental compounds with a capacity for xenoestrogenic ligand-binding activity.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with estrogen receptor alpha mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that estrogen receptor activity in early life is a molecular initiating event (MIE)

leading to breast cancer in both animal and human models and to endometrial carcinoma in the mouse, and ER agonism is the MIE leading to reproductive dysfunction in oviparous vertebrates, and there is some evidence that estrogen receptor activation is the MIE for putative adverse outcome pathways leading to reduced survival due to renal failure and leading to skewed sex ratios due to altered sexual differentiation in males (all AOPs under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive in vivo studies to further investigate the involvement of ER agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Haji, M., Kato, K., Nawata, H., & Ibayashi, H. (1981). "Age-related changes in the concentrations of cytosol receptors for sex steroid hormones in the hypothalamus and pituitary gland of the rat". Brain Res 204(2), 373-386. (PMID: 6780133)

O'Keefe, J. A., & Handa, R. J. (1990). "Transient elevation of estrogen receptors in the neonatal rat hippocampus". Dev Brain Res 57(1), 119-127. (PMID: 2090365)

Assay Quality Statistics:

Neutral control well median response value, by plate:	29473
Neutral control median absolute deviation, by plate:	1040.79
Positive control well median response value, by plate:	601
Positive control well median absolute deviation, by plate:	35.58
Z' (median across all plates, using positive control wells):	0.83
SSMD (median across all plates, using positive control wells):	-19
Signal-to-noise (median across all plates, using positive control wells):	-18.65
Signal-to-background (median across all plates, using positive control wells):	0.02
CV (median across all plates):	0.05

33. Assay Endpoint Descriptions

33.1. Data Interpretation

Biological Response:

Competitive radioligand binding of [3H] Estradiol (positive control) with estrogen receptor α obtained from murine tissue source as measured by radiometric detection.

Analytical Elements:

The NVS_NR_mER assay results were analyzed as loss-of-signal in competitive displacement assays where the endpoint measured was inhibition of [^{3}H] 17β-estradiol binding. Raw data values were normalized to DMSO (neutral control) and reported as percent of 17-β Estradiol (positive control) binding capacity. Following initial screening of test compounds at single concentration (25 μM), if the chemical response was >30% of the solvent control (DMSO) activity or at least 2 baseline median average deviations (2BMAD), the chemical was considered active against the estrogen receptor and was tested in a concentration-response assay for ER binding using 8 concentrations with 3-fold serial dilutions generally beginning at a high concentration of 50 μM. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Estrogen receptor activity was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 30% of neutral controls or 3 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in μM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for

Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ACEA_T47D_80hr_Positive
ATG_ERE_CIS_up
ATG_ERa_TRANS_up
ATG_ERb_TRANS2_up
NVS_NR_bER
NVS_NR_mERa
OT_ER_ERaERa_0480
OT_ER_ERaERa_1440
OT_ER_ERaERb_0480
OT_ER_ERaERb_1440
OT_ER_ERbERb_0480
OT_ER_ERbERb_1440
OT_ERa_ERELUC_AG_1440
OT_ERa_ERELUC_ANT_1440
OT_ERa_EREGFP_0120
OT_ERa_EREGFP_0480
OT_ERb_ERELUC_ANT_1440
Tox21_ERa_BLA_Agonist_ratio
Tox21_ERa_BLA_Antagonist_ratio
Tox21_ERa_LUC_BG1_Agonist
Tox21_ERa_LUC_BG1_Antagonist

33.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations: 8
Target (nominal) number of replicates: 1
Standard minimum concentration tested: 0.02 µM
Standard maximum concentration tested: 50 µM
Baseline median absolute deviation for the assay - based on the response values at the 2 lowest tested concentrations (bmad): 4.33
The response cutoff used to derive the hit calls (e.g., 5*bmad, 10*bmad): 25.96

Reference Chemicals / Predictive Capacity:

CASRN	Chemical Name	*In Vitro* Activity	*In Vivo* Activity	Activity in Assay
57-91-0	17alpha-Estradiol	Moderate	Active	Yes
57-63-6	17alpha-Ethinylestradiol	Strong	Active	Yes
50-28-2	17beta-Estradiol	Strong	Active	Yes
58-18-4	17-Methyltestosterone	Very Weak	Active	Yes
131-55-5	2,2',4,4'-Tetrahydroxybenzophenone	NA	Active	No
131-56-6	2,4-Dihydroxybenzophenone	NA	Active	No
5153-25-3	2-Ethylhexylparaben	NA	Active	Yes

140-66-9	4-(1,1,3,3-Tetramethylbutyl)phenol	Moderate	Active	Yes
80-46-6	4-(2-Methylbutan-2-yl)phenol	NA	Active	Yes
80-09-1	4,4'-Sulfonyldiphenol	NA	Active	Yes
599-64-4	4-Cumylphenol	Weak	Active	Yes
104-43-8	4-Dodecylphenol	NA	Active	Yes
99-96-7	4-Hydroxybenzoic acid	NA	Inactive	No
104-40-5	4-Nonylphenol	Very Weak	Active	No
98-54-4	4-tert-Butylphenol	NA	Active	Yes
521-18-6	5alpha-Dihydrotestosterone	Weak	Active	No
61-82-5	Amitrole	NA	Inactive	No
520-36-5	Apigenin	Very Weak	NA	Yes
85-68-7	Benzyl butyl phthalate	Very Weak	NA	No
103-23-1	Bis(2-ethylhexyl)hexanedioate	NA	Inactive	No
80-05-7	Bisphenol A	Weak	Active	Yes
1478-61-1	Bisphenol AF	NA	Active	No
77-40-7	Bisphenol B	Weak	Active	No
94-26-8	Butylparaben	NA	Active	No
480-40-0	Chrysin	Very Weak	NA	Yes
50-22-6	Corticosterone	Inactive	NA	Yes
486-66-8	Daidzein	Weak	NA	Yes
117-81-7	Di(2-ethylhexyl) phthalate	Very Weak	Inactive	Yes
84-74-2	Dibutyl phthalate	Very Weak	Inactive	No
115-32-2	Dicofol	Very Weak	NA	Yes
84-61-7	Dicyclohexyl phthalate	NA	Inactive	Yes
84-66-2	Diethyl phthalate	NA	Inactive	Yes
56-53-1	Diethylstilbestrol	Strong	Active	Yes
84-75-3	Dihexyl phthalate	NA	Inactive	No
474-86-2	Equilin	NA	Active	Yes
50-27-1	Estriol	NA	Active	No
53-16-7	Estrone	Moderate	Active	No
120-47-8	Ethylparaben	Very Weak	NA	No
60168-88-9	Fenarimol	Very Weak	NA	No
51630-58-1	Fenvalerate	NA	Inactive	Yes
446-72-0	Genistein	Weak	Active	Yes
52-86-8	Haloperidol	Inactive	NA	No
520-18-3	Kaempferol	Very Weak	Inactive	No
143-50-0	Kepone	Weak	NA	Yes
65277-42-1	Ketoconazole	Inactive	NA	No
330-55-2	Linuron	Inactive	NA	No
84-16-2	meso-Hexestrol	Strong	NA	Yes

72-33-3	Mestranol	NA	Active	No
72-43-5	Methoxychlor	Very Weak	Active	No
68-22-4	Norethindrone	NA	Active	No
789-02-6	o,p'-DDT	Weak	Active	Yes
556-67-2	Octamethylcyclotetrasiloxane	NA	Active	Yes
72-55-9	p,p'-DDE	Very Weak	Weak	Yes
87-86-5	Pentachlorophenol	NA	Inactive	Yes
57-30-7	Phenobarbital sodium	Inactive	NA	Yes
32809-16-8	Procymidone	Inactive	NA	No
50-55-5	Reserpine	Inactive	NA	No
52-01-7	Spironolactone	Inactive	NA	Yes
17924-92-4	Zearalenone	NA	Active	Yes

In Vitro Activity	ToxCast Active	ToxCast Inactive
Active	16	10
Inactive	1	5

In Vivo Activity	ToxCast Active	ToxCast Inactive
Active	20	10
Inactive	1	4

In Vitro Sensitivity = 61.5%
In Vitro Specificity = 83.3%
Balanced Accuracy = 72.4%

In Vivo Sensitivity = 66.7%
In Vivo Specificity = 80.0%
Balanced Accuracy = 73.3%

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [1].

34. Assay Documentation

34.1. References

[1] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

34.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
DMSO, Dimethyl Sulfoxide
EDC, Endocrine disrupting chemicals
ER, Estrogen Receptor
E2, Estradiol
MIE, Molecular Initiating Event
NR, Nuclear Receptor
NVS, NovaScreen

34.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

2 May 2016

Date of Revisions:

25 November 2016

Author of Revisions:

EPA NCCT

35. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:
Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;
Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation
Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;
Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

36. Supporting Information (existing annotations):

OT_ER_ERaERa_0480

Assay Name: Odyssey Thera Estrogen Receptor α/α Homodimer 8-hour Protein-Complementation Assay

37. Assay Descriptions

37.1. Overview

Assay Summary:

The Odyssey Thera estrogen receptor alpha homodimer (ERα/ERα) assay used Protein-Fragment Complementation Assays (PCAs) to investigate the biochemical pathways which bring separate protein fragments into close proximity and result in functional reporter enzyme (yellow fluorescent protein, YFP) signal production when the pathway is stimulated. Changes in protein complex interactions can be impacted by a variety of biochemical events within a pathway, and this assay is designed to track xenobiotic changes at the level of cell functioning which may occur at a number of points along the estrogen signaling pathway following 8-hour incubation of test chemical with transformed HEK293T cells in 384-well plates.

37.2. Assay Definition

Assay Throughput:

HEK-293T cells are seeded into 384-well microtiter plates and allowed to adhere for 24 hours prior to 8 hour chemical exposures.

Experimental System:

This assay monitors the ligand binding domain (LBD) of the wild type human ERα (stably expressed in HEK293T) for xenoestrogenic activation. The HEK-293 cell line are human embryonic epithelial kidney cells (of unknown parentage) transformed with sheared adenovirus 5 DNA by Frank Graham in 1973 [1]. The transformation incorporated approximately 4.5 kilobases from the viral genome into human chromosome 19 of the HEK cells, and subsequent cytogenetic characterization established that the 293 line is pseudotriploid [2]. HEK-293T cells are derived from the HEK293 cell line by the addition of the SV40 large T antigen that has been shown to increase vector production of some viral vectors. HEK293T are reported to have relatively high transfection efficiencies when compared to other cell lines (COS-7 and HepG2) [3] and it is among the most frequently utilized cell lines for in small-scale protein production and in viral vector propagation using the transient transfection method [4].

Xenobiotic Biotransformation Potential:

Constitutive expression of Phase I/II enzymes has not been well characterized in this cell line and metabolic activation/detoxification of test compounds is potentially limited.

Basic Procedure:

The OT ERα / ERα assay is a homodimer PCA of the ligand binding domain (amino acids 310-547) of human ERα stably expressed in HEK293T cells. Cells are seeded into optical quality 384-well poly-D-lysine coated plates in phenol red-free medium supplemented with 10% dextran-treated FBS and allowed to adhere for 24 hours prior to treatment with compounds of interest or controls for 8 hours. Cells are fixed in 4% formaldehyde and stained with Draq5 (BioStatus) to identify cells prior to signal detection. Fluorescent signal in the basal state of the assay is very low and is primarily punctuate and cytoplasmic. Modulation of this assay is quantified as an increase in mean fluorescence intensity in the nucleus (for agonists) or in the nucleus and cytoplasm (antagonists) relative to vehicle controls, and can be quantified on a high content imaging device or a laser scanning cytometer. The latter instrument is favored for this assay because of the rapid mode of data acquisition, the ability to acquire data from the entire cell population in a well (as opposed to a fraction of the well on the high content devices) and the larger dynamic range typically achieved with this instrument. To assess sensitivity of the ERα/ERα LBD assay to ligand, cells were treated with the ER agonist 17-β-estradiol (E2) in 10-point concentration- response format for 8 hours and monitored response on a laser scanning plate cytometer (Acumen eX3; TTP Lab Tech).

Proprietary Elements:

Odyssey Thera assay described here used patented PCA technology.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote estrogen receptor homodimerization, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

37.3. Assay References

Assay Source Contact Information:

Odyssey Thera Inc.
4550 Norris Canyon Road, Suite 140
San Ramon, CA 94583
Tel: (925) 242-5000

Assay Publication Year:

2003

Assay Publication:

MacDonald, M. L., Lamerdin, J., Owens, S., Keon, B. H., Bilter, G. K., Shang, Z., Huang, Z., Yu, H., Dias, J., Minami, T., Michnick, S. W., & Westwick, J. K. (2006). "Identifying off-target effects and hidden phenotypes of drugs in human cells". Nat Chem Biol 2(6), 329-337. (PMID: 16680159)

Yu, H., West, M., Keon, B. H., Bilter, G. K., Owens, S., Lamerdin, J., & Westwick, J. K. (2003). "Measuring drug action in the cellular context using protein-fragment complementation assays". Assay Drug Dev Technol 1(6), 811-822. (PMID: 15090227)

Method Updates / Confirmatory Studies:

None Reported.

38. Assay Component Descriptions

Assay Objectives:

The Odyssey Thera ERα/ERα LBD assay utilized the ability of the ERα to homodimerize upon ligand-binding with estrogenic compounds [5]. This activity is monitored via Protein-Fragment Complementation Assays (PCAs) which investigate the biochemical pathways capable of bringing separate protein fragments into close proximity. Each ERα protein contains a rationally dissected fragment of a yellow-fluorescent protein (YFP) reporter enzyme. When the estrogenic pathway is stimulated, separate ERα proteins form homodimers and the resulting YFP signal can be measured using fluorescence microscopy to screen a diverse chemical library for potential xenobiotic ligand-binding and ERα activation. Changes in protein complex interactions can be impacted by a variety of biochemical events within a pathway, and this assay is designed to track xenobiotic changes at the level of cell functioning which may occur at a number of points along the estrogen signaling pathway following an 8-hour incubation of cells with test compound in 384-well plate, using DMSO as a negative control and baseline signal and 17β-estradiol as a positive control and measure of 100% ligand-binding activity in ERα.

Scientific Principles:

Endocrine disrupting compounds bind to nuclear hormone receptors, leading to a diverse array of transcriptional and signaling pathway alterations. These cell- and tissue-type specific changes affect many aspects of human physiology, including those involved in inflammation, neonatal development and oncogenesis. Among the human NRs, the estrogen receptor family is particularly

susceptible to perturbation by EDCs because of the ER LBD proclivity to bind a disparate set of small hydrophobic molecules commonly found in nature and in man-made materials [6].

Cell-based and in vivo experiments suggest that the ERα isoform is the primary mediator of estrogenic effects by EDCs on reproductive development and on cell proliferation [7]. Thus, a highly sensitive assay that can detect ERα LBD binding in the context of a whole cell would serve as a powerful predictor of human-relevant estrogenic effects. The Odyssey Thera Ligand Binding assays used protein-fragment complementation (PCA) to measure dose-dependent homodimerization of estrogen receptor (ER) α expressed in human embryonic kidney cell line HEK293T. This dimerization and concurrent conformational changes brings into close proximity the fused fragments of split-yellow fluorescent protein (YFP), leading to a dramatic increase in YFP intensity. This intensity change is dose-dependent and shows saturation-binding that plateaus differently for agonists versus antagonists.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with estrogen receptor alpha mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that estrogen receptor agonism is the Molecular Initiating Event (MIE) in an Adverse Outcome Pathway (AOP) leading to reproductive dysfunction in oviparous vertebrates, and there is some evidence that estrogen receptor activation is the MIE for putative AOPs leading to reduced survival due to renal failure and leading to skewed sex ratios due to altered sexual differentiation in males (all AOPs currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive in vivo studies to further investigate the involvement of ER agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Michnick, S. W., Remy, I., Campbell-Valois, F. X., Vallee-Belisle, A., & Pelletier, J. N. (2000). "Detection of protein-protein interactions by protein fragment complementation strategies". Methods Enzymol 328, 208-230. (PMID: 11075347)

Assay Quality Statistics:

Neutral control well median response value, by plate:	0.859
Neutral control median absolute deviation, by plate:	0.256
Positive control well median response value, by plate:	15.56
Positive control well median absolute deviation, by plate:	1.10
Z' (median across all plates, using positive control wells):	0.7
SSMD (median across all plates, using positive control wells):	12
Signal-to-noise (median across all plates, using positive control wells):	53.55
Signal-to-background (median across all plates, using positive control wells):	17.85
CV (median across all plates):	0.3

39. Assay Endpoint Descriptions

39.1. Data Interpretation

Biological Response:

Estrogen receptor homodimerization in response to ligand-binding as measured with protein complementation assay technology by monitoring fluorescence intensity.

Analytical Elements:

OT_ER_ERaERa_0480 was analyzed into in the positive fitting direction (receptor gain-of-signal activity) as a percent of 17b-Estradiol (positive control, 100% activation) and relative to DMSO, negative control and signal baseline for activity. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant

function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Estrogen receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ACEA_T47D_80hr_Positive
ATG_ERE_CIS_up
ATG_ERa_TRANS_up
ATG_ERb_TRANS2_up
NVS_NR_bER
NVS_NR_hER
NVS_NR_mERa
OT_ER_ERaERa_1440
OT_ER_ERaERb_0480
OT_ER_ERaERb_1440
OT_ER_ERbERb_0480
OT_ER_ERbERb_1440
OT_ERa_ERELUC_AG_1440
OT_ERa_ERELUC_ANT_1440
OT_ERa_EREGFP_0120
OT_ERa_EREGFP_0480
OT_ERb_ERELUC_ANT_1440
Tox21_ERa_BLA_Agonist_ratio
Tox21_ERa_BLA_Antagonist_ratio
Tox21_ERa_LUC_BG1_Agonist
Tox21_ERa_LUC_BG1_Antagonist

39.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations: 6
Target (nominal) number of replicates: 3
Standard minimum concentration tested: 0.3 µM
Standard maximum concentration tested: 100 µM
Baseline median absolute deviation for the assay - based on the response values at the 2 lowest tested concentrations (bmad): 1.66
The response cutoff used to derive the hit calls (e.g., 5*bmad, 10*bmad): 20

Reference Chemicals / Predictive Capacity:

CASRN	Chemical Name	*In Vitro* Activity	*In Vivo* Activity	Activity in Assay

57-91-0	17alpha-Estradiol	Moderate	Active	Yes
57-63-6	17alpha-Ethinylestradiol	Strong	Active	Yes
50-28-2	17beta-Estradiol	Strong	Active	Yes
58-18-4	17-Methyltestosterone	Very Weak	Active	Yes
131-55-5	2,2',4,4'-Tetrahydroxybenzophenone	NA	Active	Yes
131-56-6	2,4-Dihydroxybenzophenone	NA	Active	No
5153-25-3	2-Ethylhexylparaben	NA	Active	Yes
140-66-9	4-(1,1,3,3-Tetramethylbutyl)phenol	Moderate	Active	No
80-46-6	4-(2-Methylbutan-2-yl)phenol	NA	Active	Yes
80-09-1	4,4'-Sulfonyldiphenol	NA	Active	Yes
599-64-4	4-Cumylphenol	Weak	Active	Yes
104-43-8	4-Dodecylphenol	NA	Active	No
99-96-7	4-Hydroxybenzoic acid	NA	Inactive	Yes
104-40-5	4-Nonylphenol	Very Weak	Active	Yes
98-54-4	4-tert-Butylphenol	NA	Active	Yes
521-18-6	5alpha-Dihydrotestosterone	Weak	Active	Yes
61-82-5	Amitrole	NA	Inactive	Yes
520-36-5	Apigenin	Very Weak	NA	Yes
85-68-7	Benzyl butyl phthalate	Very Weak	NA	No
103-23-1	Bis(2-ethylhexyl)hexanedioate	NA	Inactive	No
80-05-7	Bisphenol A	Weak	Active	Yes
1478-61-1	Bisphenol AF	NA	Active	Yes
77-40-7	Bisphenol B	Weak	Active	Yes
94-26-8	Butylparaben	NA	Active	No
480-40-0	Chrysin	Very Weak	NA	No
50-22-6	Corticosterone	Inactive	NA	Yes
486-66-8	Daidzein	Weak	NA	No
117-81-7	Di(2-ethylhexyl) phthalate	Very Weak	Inactive	Yes
84-74-2	Dibutyl phthalate	Very Weak	Inactive	Yes
115-32-2	Dicofol	Very Weak	NA	Yes
84-61-7	Dicyclohexyl phthalate	NA	Inactive	Yes
84-66-2	Diethyl phthalate	NA	Inactive	Yes
56-53-1	Diethylstilbestrol	Strong	Active	Yes
84-75-3	Dihexyl phthalate	NA	Inactive	Yes
474-86-2	Equilin	NA	Active	No
50-27-1	Estriol	NA	Active	No
53-16-7	Estrone	Moderate	Active	Yes
120-47-8	Ethylparaben	Very Weak	NA	No
60168-88-9	Fenarimol	Very Weak	NA	Yes
51630-58-1	Fenvalerate	NA	Inactive	No

446-72-0	Genistein	Weak	Active	No
52-86-8	Haloperidol	Inactive	NA	No
520-18-3	Kaempferol	Very Weak	Inactive	Yes
143-50-0	Kepone	Weak	NA	Yes
65277-42-1	Ketoconazole	Inactive	NA	Yes
330-55-2	Linuron	Inactive	NA	No
84-16-2	meso-Hexestrol	Strong	NA	Yes
72-33-3	Mestranol	NA	Active	Yes
72-43-5	Methoxychlor	Very Weak	Active	Yes
68-22-4	Norethindrone	NA	Active	No
789-02-6	o,p'-DDT	Weak	Active	Yes
556-67-2	Octamethylcyclotetrasiloxane	NA	Active	Yes
72-55-9	p,p'-DDE	Very Weak	Weak	No
87-86-5	Pentachlorophenol	NA	Inactive	No
57-30-7	Phenobarbital sodium	Inactive	NA	No
32809-16-8	Procymidone	Inactive	NA	Yes
50-55-5	Reserpine	Inactive	NA	Yes
52-01-7	Spironolactone	Inactive	NA	No
17924-92-4	Zearalenone	NA	Active	Yes

In Vitro Activity	ToxCast Active	ToxCast Inactive
Active	23	6
Inactive	1	7

In Vivo Activity	ToxCast Active	ToxCast Inactive
Active	28	2
Inactive	3	8

In Vitro Sensitivity = 79.3%
In Vitro Specificity = 87.5%
Balanced Accuracy = 83.4%

In Vivo Sensitivity = 93.3%
In Vivo Specificity = 72.7%
Balanced Accuracy = 83.0%

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated

drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [8].

40. Assay Documentation

40.1. References

[1] Graham, F., et al. (1977). J Gen Virol 36(1): 59-72. (PMID: 886304)
[2] Bylund, L., et al. (2004). Cytogenet Genome Res 106(1): 28-32. (PMID: 15218237)
[3] Dai, D., et al. (2015). Die Pharmazie- Inter J Pharma Sci 70(1): 33-37. (PMID: 25975096)
[4] Lin, Y.-C., et al. (2014). Nature Comm 5. (PMID: 25182477)
[5] Katzenellenbogen, B. S., et al. (1993). J Ster Biochem Mol Biol 47(1): 39-48. (PMID: 8274440)
[6] Shanle, E. K. and W. Xu (2010). Chem Res Toxicol 24(1): 6-19. (PMID: 21053929)
[7] Helguero, L. A., et al. (2005). Oncogene 24(44): 6605-6616. (PMID: 16007178)
[8] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

40.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
DBD, DNA Binding Domain
DMSO, Dimethyl Sulfoxide
EDC, Endocrine Disrupting Compounds
ER, Estrogen Receptor
E2, Estradiol
HEK, Human Embryonic Kidney
LBD, Ligand Binding Domain
MIE, Molecular Initiating Event
NHR, Nuclear Hormone Receptors
OT, Odyssey Thera
PCA, Protein-Fragment Complementation
YFP, Yellow Fluorescent Protein

40.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

26 May 2016

Date of Revisions:

25 November 2016

Author of Revisions:

EPA NCCT

41. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:

Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;

Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation
Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;
Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

42. Supporting Information (existing annotations):

OT_ER_ERaERa_1440

Assay Name: Odyssey Thera Estrogen Receptor α/α Homodimer 24-hour Protein-Complementation Assay

43. Assay Descriptions

43.1. Overview

Assay Summary:

The Odyssey Thera estrogen receptor alpha homodimer (ERα/ERα) assay used Protein-Fragment Complementation Assays (PCAs) to investigate the biochemical pathways which bring separate protein fragments into close proximity and result in functional reporter enzyme (yellow fluorescent protein, YFP) signal production when the pathway is stimulated. Changes in protein complex interactions can be impacted by a variety of biochemical events within a pathway, and this assay is designed to track xenobiotic changes at the level of cell functioning which may occur at a number of points along the estrogen signaling pathway following 24-hour incubation of test chemical with transformed HEK293T cells in 384-well plates.

43.2. Assay Definition

Assay Throughput:

HEK-293T cells are seeded into 384-well microtiter plates and allowed to adhere for 24 hours prior to 24 hour chemical exposures.

Experimental System:

This assay monitors the ligand binding domain (LBD) of the wild type human ERα (stably expressed in HEK293T) for xenoestrogenic activation. The HEK-293 cell line are human embryonic epithelial kidney cells (of unknown parentage) transformed with sheared adenovirus 5 DNA by Frank Graham in 1973 [1]. The transformation incorporated approximately 4.5 kilobases from the viral genome into human chromosome 19 of the HEK cells, and subsequent cytogenetic characterization established that the 293 line is pseudotriploid [2]. HEK-293T cells are derived from the HEK293 cell line by the addition of the SV40 large T antigen that has been shown to increase vector production of some viral vectors. HEK293T are reported to have relatively high transfection efficiencies when compared to other cell lines (COS-7 and HepG2) [3] and it is among the most frequently utilized cell lines for in small-scale protein production and in viral vector propagation using the transient transfection method [4].

Xenobiotic Biotransformation Potential:

Constitutive expression of Phase I/II enzymes has not been well characterized in this cell line and metabolic activation/detoxification of test compounds is potentially limited.

Basic Procedure:

The OT ERα / ERα assay is a homodimer PCA of the ligand binding domain (amino acids 310-547) of human ERα stably expressed in HEK293T cells. Cells are seeded into optical quality 384-well poly-D-lysine coated plates in phenol red-free medium supplemented with 10% dextran-treated FBS and allowed to adhere for 24 hours prior to treatment with compounds of interest or controls for 24 hours. Cells are fixed in 4% formaldehyde and stained with Draq5 (BioStatus) to identify cells prior to signal detection. Fluorescent signal in the basal state of the assay is very low and is primarily punctuate and cytoplasmic. Modulation of this assay is quantified as an increase in mean fluorescence intensity in the nucleus (for agonists) or in the nucleus and cytoplasm (antagonists) relative to vehicle controls, and can be quantified on a high content imaging device or a laser scanning cytometer. The latter instrument is favored for this assay because of the rapid mode of data acquisition, the ability to acquire data from the entire cell population in a well (as opposed to a fraction of the well on the high content devices) and the larger dynamic range typically achieved with this instrument. To assess sensitivity of the ERα/ERα LBD assay to ligand, cells were treated with the ER agonist 17-β-estradiol (E2) in 10-point concentration- response format for 24 hours and monitored response on a laser scanning plate cytometer (Acumen eX3; TTP Lab Tech).

Proprietary Elements:

Odyssey Thera assay described here used patented PCA technology.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote estrogen receptor homodimerization, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

43.3. Assay References

Assay Source Contact Information:

Odyssey Thera Inc.
4550 Norris Canyon Road, Suite 140
San Ramon, CA 94583
Tel: (925) 242-5000

Assay Publication Year:

2003

Assay Publication:

MacDonald, M. L., Lamerdin, J., Owens, S., Keon, B. H., Bilter, G. K., Shang, Z., Huang, Z., Yu, H., Dias, J., Minami, T., Michnick, S. W., & Westwick, J. K. (2006). "Identifying off-target effects and hidden phenotypes of drugs in human cells". Nat Chem Biol 2(6), 329-337. (PMID: 16680159)

Yu, H., West, M., Keon, B. H., Bilter, G. K., Owens, S., Lamerdin, J., & Westwick, J. K. (2003). "Measuring drug action in the cellular context using protein-fragment complementation assays". Assay Drug Dev Technol 1(6), 811-822. (PMID: 15090227)

Method Updates / Confirmatory Studies:

None Reported.

44. Assay Component Descriptions

Assay Objectives:

The Odyssey Thera ERα/ERα LBD assay utilized the ability of the ERα to homodimerize upon ligand-binding with estrogenic compounds [5]. This activity is monitored via Protein-Fragment Complementation Assays (PCAs) which investigate the biochemical pathways capable of bringing separate protein fragments into close proximity. Each ERα protein contains a rationally dissected fragment of a yellow-fluorescent protein (YFP) reporter enzyme. When the estrogenic pathway is stimulated, separate ERα proteins form homodimers and the resulting YFP signal can be measured using fluorescence microscopy to screen a diverse chemical library for potential xenobiotic ligand-binding and ERα activation. Changes in protein complex interactions can be impacted by a variety of biochemical events within a pathway, and this assay is designed to track xenobiotic changes at the level of cell functioning which may occur at a number of points along the estrogen signaling pathway following an 8-hour incubation of cells with test compound in 384-well plate, using DMSO as a negative control and baseline signal and 17β-estradiol as a positive control and measure of 100% ligand-binding activity in ERα.

Scientific Principles:

Endocrine disrupting compounds bind to nuclear hormone receptors, leading to a diverse array of transcriptional and signaling pathway alterations. These cell- and tissue-type specific changes affect many aspects of human physiology, including those involved in inflammation, neonatal development and oncogenesis. Among the human NRs, the estrogen receptor family is particularly

susceptible to perturbation by EDCs because of the ER LBD proclivity to bind a disparate set of small hydrophobic molecules commonly found in nature and in man-made materials [6].

Cell-based and in vivo experiments suggest that the ERα isoform is the primary mediator of estrogenic effects by EDCs on reproductive development and on cell proliferation [7]. Thus, a highly sensitive assay that can detect ERα LBD binding in the context of a whole cell would serve as a powerful predictor of human-relevant estrogenic effects. The Odyssey Thera Ligand Binding assays used protein-fragment complementation (PCA) to express a dose-dependent homodimer which binds to estrogen receptor (ER) α expressed in human embryonic kidney cell line HEK293T. This dimizeration and concurrent conformational changes brings into close proximity the fused fragments of split-yellow fluorescent protein (YFP), leading to a dramatic increase in YFP intensity. This intensity change is dose-dependent and shows saturation-binding that plateaus differently for agonists versus antagonists.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with estrogen receptor alpha mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that estrogen receptor agonism is the Molecular Initiating Event (MIE) in an Adverse Outcome Pathway (AOP) leading to reproductive dysfunction in oviparous vertebrates, and there is some evidence that estrogen receptor activation is the MIE for putative AOPs leading to reduced survival due to renal failure and leading to skewed sex ratios due to altered sexual differentiation in males (all AOPs currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive in vivo studies to further investigate the involvement of ER agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Michnick, S. W., Remy, I., Campbell-Valois, F. X., Vallee-Belisle, A., & Pelletier, J. N. (2000). "Detection of protein-protein interactions by protein fragment complementation strategies". Methods Enzymol 328, 208-230. (PMID: 11075347)

Assay Quality Statistics:

Neutral control well median response value, by plate:	0.943
Neutral control median absolute deviation, by plate:	0.250
Positive control well median response value, by plate:	30.36
Positive control well median absolute deviation, by plate:	1.81
Z' (median across all plates, using positive control wells):	0.8
SSMD (median across all plates, using positive control wells):	17
Signal-to-noise (median across all plates, using positive control wells):	137.55
Signal-to-background (median across all plates, using positive control wells):	36.88
CV (median across all plates):	0.25

45. Assay Endpoint Descriptions

45.1. Data Interpretation

Biological Response:

Estrogen signaling pathway protein fragment dimerization and enzyme formation in response to estrogen receptor α ligand-binding as detected by monitoring fluorescence intensity.

Analytical Elements:

OT_ER_ERaERa_1440 was analyzed into in the positive fitting direction (receptor gain-of-signal activity) as a percent of 17b-Estradiol (positive control, 100% activation) and relative to DMSO, negative control and baseline activity. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant

function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Estrogen receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ACEA_T47D_80hr_Positive
ATG_ERE_CIS_up
ATG_ERa_TRANS_up
ATG_ERb_TRANS2_up
NVS_NR_bER
NVS_NR_hER
NVS_NR_mERa
OT_ER_ERaERa_0480
OT_ER_ERaERb_0480
OT_ER_ERaERb_1440
OT_ER_ERbERb_0480
OT_ER_ERbERb_1440
OT_ERa_ERELUC_AG_1440
OT_ERa_ERELUC_ANT_1440
OT_ERa_EREGFP_0120
OT_ERa_EREGFP_0480
OT_ERb_ERELUC_ANT_1440
Tox21_ERa_BLA_Agonist_ratio
Tox21_ERa_BLA_Antagonist_ratio
Tox21_ERa_LUC_BG1_Agonist
Tox21_ERa_LUC_BG1_Antagonist

45.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations: 6
Target (nominal) number of replicates: 3
Standard minimum concentration tested: 0.3 µM
Standard maximum concentration tested: 100 µM
Baseline median absolute deviation for the assay - based on the response values at the 2 lowest tested concentrations (bmad): 0.640
The response cutoff used to derive the hit calls (e.g., 5*bmad, 10*bmad): 20

Reference Chemicals / Predictive Capacity:

CASRN	Chemical Name	*In Vitro* Activity	*In Vivo* Activity	Activity in Assay

57-91-0	17alpha-Estradiol	Moderate	Active	Yes
57-63-6	17alpha-Ethinylestradiol	Strong	Active	Yes
50-28-2	17beta-Estradiol	Strong	Active	Yes
58-18-4	17-Methyltestosterone	Very Weak	Active	Yes
131-55-5	2,2',4,4'-Tetrahydroxybenzophenone	NA	Active	Yes
131-56-6	2,4-Dihydroxybenzophenone	NA	Active	No
5153-25-3	2-Ethylhexylparaben	NA	Active	Yes
140-66-9	4-(1,1,3,3-Tetramethylbutyl)phenol	Moderate	Active	No
80-46-6	4-(2-Methylbutan-2-yl)phenol	NA	Active	Yes
80-09-1	4,4'-Sulfonyldiphenol	NA	Active	Yes
599-64-4	4-Cumylphenol	Weak	Active	Yes
104-43-8	4-Dodecylphenol	NA	Active	No
99-96-7	4-Hydroxybenzoic acid	NA	Inactive	Yes
104-40-5	4-Nonylphenol	Very Weak	Active	Yes
98-54-4	4-tert-Butylphenol	NA	Active	Yes
521-18-6	5alpha-Dihydrotestosterone	Weak	Active	Yes
61-82-5	Amitrole	NA	Inactive	Yes
520-36-5	Apigenin	Very Weak	NA	Yes
85-68-7	Benzyl butyl phthalate	Very Weak	NA	No
103-23-1	Bis(2-ethylhexyl)hexanedioate	NA	Inactive	No
80-05-7	Bisphenol A	Weak	Active	Yes
1478-61-1	Bisphenol AF	NA	Active	No
77-40-7	Bisphenol B	Weak	Active	Yes
94-26-8	Butylparaben	NA	Active	No
480-40-0	Chrysin	Very Weak	NA	Yes
50-22-6	Corticosterone	Inactive	NA	Yes
486-66-8	Daidzein	Weak	NA	No
117-81-7	Di(2-ethylhexyl) phthalate	Very Weak	Inactive	Yes
84-74-2	Dibutyl phthalate	Very Weak	Inactive	Yes
115-32-2	Dicofol	Very Weak	NA	Yes
84-61-7	Dicyclohexyl phthalate	NA	Inactive	Yes
84-66-2	Diethyl phthalate	NA	Inactive	Yes
56-53-1	Diethylstilbestrol	Strong	Active	Yes
84-75-3	Dihexyl phthalate	NA	Inactive	Yes
474-86-2	Equilin	NA	Active	No
50-27-1	Estriol	NA	Active	No
53-16-7	Estrone	Moderate	Active	Yes
120-47-8	Ethylparaben	Very Weak	NA	No
60168-88-9	Fenarimol	Very Weak	NA	Yes
51630-58-1	Fenvalerate	NA	Inactive	No

446-72-0	Genistein	Weak	Active	No
52-86-8	Haloperidol	Inactive	NA	No
520-18-3	Kaempferol	Very Weak	Inactive	Yes
143-50-0	Kepone	Weak	NA	Yes
65277-42-1	Ketoconazole	Inactive	NA	Yes
330-55-2	Linuron	Inactive	NA	No
84-16-2	meso-Hexestrol	Strong	NA	Yes
72-33-3	Mestranol	NA	Active	Yes
72-43-5	Methoxychlor	Very Weak	Active	Yes
68-22-4	Norethindrone	NA	Active	No
789-02-6	o,p'-DDT	Weak	Active	Yes
556-67-2	Octamethylcyclotetrasiloxane	NA	Active	Yes
72-55-9	p,p'-DDE	Very Weak	Weak	No
87-86-5	Pentachlorophenol	NA	Inactive	No
57-30-7	Phenobarbital sodium	Inactive	NA	No
32809-16-8	Procymidone	Inactive	NA	Yes
50-55-5	Reserpine	Inactive	NA	Yes
52-01-7	Spironolactone	Inactive	NA	No
17924-92-4	Zearalenone	NA	Active	Yes

In Vitro Activity	ToxCast Active	ToxCast Inactive
Active	23	6
Inactive	1	7

In Vivo Activity	ToxCast Active	ToxCast Inactive
Active	28	2
Inactive	4	7

In Vitro Sensitivity = 79.3%
In Vitro Specificity = 87.5%
Balanced Accuracy = 83.4%

In Vivo Sensitivity = 93.3%
In Vivo Specificity = 63.6%
Balanced Accuracy = 78.5%

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated

drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [8].

46. Assay Documentation

46.1. References

[1] Graham, F., et al. (1977). J Gen Virol 36(1): 59-72. (PMID: 886304)
[2] Bylund, L., et al. (2004). Cytogenet Genome Res 106(1): 28-32. (PMID: 15218237)
[3] Dai, D., et al. (2015). Die Pharmazie - Int J of Pharm Sci 70(1): 33-37. (PMID: 25975096)
[4] Lin, Y.-C., et al. (2014). Nature Comm 5. (PMID: 25182477)
[5] Katzenellenbogen, B. S., et al. (1993). J Steroid Biochem Molecul Biol 47(1): 39-48. (PMID: 8274440)
[6] Shanle, E. K. and W. Xu (2010). Chem Res Toxicol 24(1): 6-19. (PMID: 21053929)
[7] Helguero, L. A., et al. (2005). Oncogene 24(44): 6605-6616. (PMID: 16007178)
[8] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

46.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
DBD, DNA Binding Domain
DMSO, Dimethyl Sulfoxide
EDC, Endocrine Disrupting Compounds
ER, Estrogen Receptor
E2, Estradiol
HEK, Human Embryonic Kidney
LBD, Ligand Binding Domain
MIE, Molecular Initiating Event
NHR, Nuclear Hormone Receptors
OT, Odyssey Thera
PCA , Protein-Fragment Complementation
YFP, Yellow Fluorescent Protein

46.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

2016

Date of Revisions:

2016

Author of Revisions:

EPA NCCT

47. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:

Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;
Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation
Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;
Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

48. Supporting Information (existing annotations):

OT_ER_ERaERb_0480

Assay Name: Odyssey Thera Estrogen Receptor α/β Homodimer 8-hour Protein-Complementation Assay

49. Assay Descriptions

49.1. Overview

Assay Summary:

The Odyssey Thera estrogen receptor alpha heterodimer (ERα/ERβ) assay used Protein-Fragment Complementation Assays (PCAs) to investigate the biochemical pathways which bring separate protein fragments into close proximity and result in functional reporter enzyme (yellow fluorescent protein, YFP) signal production when the estrogenic pathway is stimulated. Changes in protein complex interactions can be impacted by a variety of biochemical events within a pathway, and this assay is designed to track xenobiotic changes at the level of cell functioning which may occur at a number of points along the estrogen signaling pathway following 8-hour incubation of test chemical with transformed HEK293T cells in 384-well plates.

49.2. Assay Definition

Assay Throughput:

HEK-293T cells are seeded into 384-well microtiter plates and allowed to adhere for 24 hours prior to 8 hour chemical exposures.

Experimental System:

This assay monitors the ligand binding domain (LBD) of the wild type human ER (isoforms α and β, stably expressed in HEK293T cells) for xenoestrogenic activation. The HEK-293 cell line are human embryonic epithelial kidney cells (of unknown parentage) transformed with sheared adenovirus 5 DNA by Frank Graham in 1973 [1]. The transformation incorporated approximately 4.5 kilobases from the viral genome into human chromosome 19 of the HEK cells, and subsequent cytogenetic characterization established that the 293 line is pseudotriploid [2]. HEK-293T cells are derived from the HEK293 cell line by the addition of the SV40 large T antigen that has been shown to increase vector production of some viral vectors. HEK293T are reported to have relatively high transfection efficiencies when compared to other cell lines COS-7 and HepG2 cell lines [3] and it is among the most frequently utilized cell lines for in small-scale protein production and in viral vector propagation using the transient transfection method [4].

Xenobiotic Biotransformation Potential:

Constitutive expression of Phase I/II enzymes has not been well characterized in this cell line and metabolic activation/detoxification of test compounds is potentially limited.

Basic Procedure:

The OT ERα / ERβ assay is a heterodimer PCA of the ligand binding domains (LBD) of ERα (amino acids 310-547) and human ERβ (amino acids 263-489) stably expressed in HEK293T cells. ERα/ERβ LBD cells are seeded into optical quality 384-well poly-D-lysine coated plates in phenol red-free medium supplemented with 10% dextran-treated FBS and allowed to adhere for 24 hours prior to treatment with compounds of interest or controls for 8 hours. Cells are fixed in 4% formaldehyde and stained with Draq5 (BioStatus) to identify cells prior to signal detection. Fluorescent signal in the basal state of the assay is very low and is primarily punctuate and cytoplasmic. Modulation of this assay is quantified as an increase in mean fluorescence intensity in the nucleus (for agonists) or in the nucleus and cytoplasm (antagonists) relative to vehicle controls, and can be quantified on a high content imaging device or a laser scanning cytometer. The latter instrument is favored for this assay because of the rapid mode of data acquisition, the ability to acquire data from the entire cell population in a well (as opposed to a fraction of the well on the high content devices) and the larger dynamic range typically achieved with this instrument. To assess sensitivity of the ERα/ERβ LBD assay to ligand, cells were treated with the ER agonist 17-β-estradiol (E2) in 10-point concentration- response format for 8 hours and monitored response on a laser scanning plate cytometer (Acumen eX3; TTP Lab Tech).

Proprietary Elements:

Odyssey Thera assay described here used patented PCA technology.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote estrogen receptor heterodimerization, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

49.3. Assay References

Assay Source Contact Information:

Odyssey Thera Inc.
4550 Norris Canyon Road, Suite 140
San Ramon, CA 94583
Tel: (925) 242-5000

Assay Publication Year:

2003

Assay Publication:

MacDonald, M. L., Lamerdin, J., Owens, S., Keon, B. H., Bilter, G. K., Shang, Z., Huang, Z., Yu, H., Dias, J., Minami, T., Michnick, S. W., & Westwick, J. K. (2006). "Identifying off-target effects and hidden phenotypes of drugs in human cells". Nat Chem Biol 2(6), 329-337. (PMID: 16680159)

Yu, H., West, M., Keon, B. H., Bilter, G. K., Owens, S., Lamerdin, J., & Westwick, J. K. (2003). "Measuring drug action in the cellular context using protein-fragment complementation assays". Assay Drug Dev Technol 1(6), 811-822. (PMID: 15090227)

Method Updates / Confirmatory Studies:

None Reported.

50. Assay Component Descriptions

Assay Objectives:

The Odyssey Thera ERα/ERβ LBD assay utilized the ability of ERα and ERβ to form heterodimers following ligand-binding with estrogenic compounds. This activity is monitored via Protein-Fragment Complementation Assays (PCAs) which investigate the biochemical pathways capable of bringing separate protein fragments into close proximity. Each ERα and ERβ protein contains a fragment of a reporter enzyme (YFP) and when both proteins come in contact to form homo- or heterodimers, the resulting YFP signal can be measured using fluorescence microscopy and used to screen a diverse chemical library for potential xenobiotic ligand-binding and ER activation. Changes in protein complex interactions can be impacted by a variety of biochemical events within a pathway, and this assay is designed to track xenobiotic changes at the level of cell functioning which may occur at a number of points along the estrogen signaling pathway following an 8 hour incubation of cells with test compound in 384-well plate, using DMSO as a negative control and baseline signal and 17β-estradiol as a positive control and measure of 100% ligand-binding activity in ER α/β.

Scientific Principles:

Endocrine disrupting compounds bind to nuclear hormone receptors, leading to a diverse array of transcriptional and signaling pathway alterations. These cell- and tissue-type specific changes affect many aspects of human physiology, including those involved in inflammation, neonatal development and oncogenesis. Among the human NRs, the estrogen receptor family is particularly

susceptible to perturbation by EDCs because of the ER LBD proclivity to bind a disparate set of small hydrophobic molecules commonly found in nature and in man-made materials [5].

The estrogen receptor is expressed in two forms, ERα and ERβ which play different roles in mediating the actions of estrogenic compounds. Multiple studies have determined that the two isoforms can form functional homo- and hetero-dimers in vitro and in vivo which are capable of binding DNA [6] and initiating transcription of target genes [7]. Furthermore, ER homo- and heterodimers display ligand-selective activity [8] leading in turn to a unique but overlapping set of dimer-mediated transcriptional changes [9, 10]. Thus, a complete understanding of the potential estrogenic effects of EDCs requires the comprehensive profiling of the three physiological dimers. To assess the activity of the ERα/β heterodimer, this assay was designed to utilize the ability of the coexpressed ERα and ERβ LBDs to heterodimerize upon ligand-binding with estrogenic compounds. This dimerization and concurrent conformational changes brings into close proximity the fused fragments of split-YFP, leading to a dramatic increase in YFP intensity. This intensity change is dose-dependent and shows saturation-binding that plateaus differently for agonists versus antagonists.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with estrogen receptor mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that estrogen receptor agonism is the Molecular Initiating Event (MIE) in an Adverse Outcome Pathway (AOP) leading to reproductive dysfunction in oviparous vertebrates, and there is some evidence that estrogen receptor activation is the MIE for putative AOPs leading to reduced survival due to renal failure and leading to skewed sex ratios due to altered sexual differentiation in males (all AOPs currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive in vivo studies to further investigate the involvement of ER agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Michnick, S. W., Remy, I., Campbell-Valois, F. X., Vallee-Belisle, A., & Pelletier, J. N. (2000). "Detection of protein-protein interactions by protein fragment complementation strategies". Methods Enzymol 328, 208-230. (PMID: 11075347)

Assay Quality Statistics:

Neutral control well median response value, by plate:	0.875
Neutral control median absolute deviation, by plate:	0.126
Positive control well median response value, by plate:	3.22
Positive control well median absolute deviation, by plate:	0.21
Z' (median across all plates, using positive control wells):	0.55
SSMD (median across all plates, using positive control wells):	9
Signal-to-noise (median across all plates, using positive control wells):	19.43
Signal-to-background (median across all plates, using positive control wells):	3.64
CV (median across all plates):	0.15

51. Assay Endpoint Descriptions

51.1. Data Interpretation

Biological Response:

Estrogen signaling pathway protein fragment dimerization and enzyme formation in response to estrogen receptor (α / β) ligand-binding as detected by monitoring fluorescence intensity.

Analytical Elements:

OT_ER_ERaERb_0480 was analyzed into in the positive fitting direction (receptor gain-of-signal activity) as a percent of 17b-Estradiol (positive control, 100% activation) and relative to DMSO,

negative control and baseline signal. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Estrogen receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ACEA_T47D_80hr_Positive
ATG_ERE_CIS_up
ATG_ERa_TRANS_up
ATG_ERb_TRANS2_up
NVS_NR_bER
NVS_NR_hER
NVS_NR_mERa
OT_ER_ERaERa_0480
OT_ER_ERaERa_1440
OT_ER_ERaERb_1440
OT_ER_ERbERb_0480
OT_ER_ERbERb_1440
OT_ERa_ERELUC_AG_1440
OT_ERa_ERELUC_ANT_1440
OT_ERa_EREGFP_0120
OT_ERa_EREGFP_0480
OT_ERb_ERELUC_ANT_1440
Tox21_ERa_BLA_Agonist_ratio
Tox21_ERa_BLA_Antagonist_ratio
Tox21_ERa_LUC_BG1_Agonist
Tox21_ERa_LUC_BG1_Antagonist

51.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations: 6
Target (nominal) number of replicates: 3
Standard minimum concentration tested: 0.3 µM
Standard maximum concentration tested: 100 µM
Baseline median absolute deviation for the assay - based on the response values at the 2 lowest tested concentrations (bmad): 4.66
The response cutoff used to derive the hit calls (e.g., 5*bmad, 10*bmad): 23.30

Reference Chemicals / Predictive Capacity:

CASRN	Chemical Name	*In Vitro* Activity	*In Vivo* Activity	Activity in Assay
57-91-0	17alpha-Estradiol	Moderate	Active	Yes
57-63-6	17alpha-Ethinylestradiol	Strong	Active	Yes
50-28-2	17beta-Estradiol	Strong	Active	Yes
58-18-4	17-Methyltestosterone	Very Weak	Active	Yes
131-55-5	2,2',4,4'-Tetrahydroxybenzophenone	NA	Active	Yes
131-56-6	2,4-Dihydroxybenzophenone	NA	Active	No
5153-25-3	2-Ethylhexylparaben	NA	Active	Yes
140-66-9	4-(1,1,3,3-Tetramethylbutyl)phenol	Moderate	Active	No
80-46-6	4-(2-Methylbutan-2-yl)phenol	NA	Active	Yes
80-09-1	4,4'-Sulfonyldiphenol	NA	Active	Yes
599-64-4	4-Cumylphenol	Weak	Active	Yes
104-43-8	4-Dodecylphenol	NA	Active	No
99-96-7	4-Hydroxybenzoic acid	NA	Inactive	Yes
104-40-5	4-Nonylphenol	Very Weak	Active	Yes
98-54-4	4-tert-Butylphenol	NA	Active	Yes
521-18-6	5alpha-Dihydrotestosterone	Weak	Active	Yes
61-82-5	Amitrole	NA	Inactive	Yes
520-36-5	Apigenin	Very Weak	NA	Yes
85-68-7	Benzyl butyl phthalate	Very Weak	NA	Yes
103-23-1	Bis(2-ethylhexyl)hexanedioate	NA	Inactive	No
80-05-7	Bisphenol A	Weak	Active	Yes
1478-61-1	Bisphenol AF	NA	Active	Yes
77-40-7	Bisphenol B	Weak	Active	Yes
94-26-8	Butylparaben	NA	Active	No
480-40-0	Chrysin	Very Weak	NA	Yes
50-22-6	Corticosterone	Inactive	NA	Yes
486-66-8	Daidzein	Weak	NA	Yes
117-81-7	Di(2-ethylhexyl) phthalate	Very Weak	Inactive	Yes
84-74-2	Dibutyl phthalate	Very Weak	Inactive	Yes
115-32-2	Dicofol	Very Weak	NA	Yes
84-61-7	Dicyclohexyl phthalate	NA	Inactive	Yes
84-66-2	Diethyl phthalate	NA	Inactive	Yes
56-53-1	Diethylstilbestrol	Strong	Active	Yes
84-75-3	Dihexyl phthalate	NA	Inactive	Yes
474-86-2	Equilin	NA	Active	Yes
50-27-1	Estriol	NA	Active	No
53-16-7	Estrone	Moderate	Active	Yes
120-47-8	Ethylparaben	Very Weak	NA	No

60168-88-9	Fenarimol	Very Weak	NA	Yes
51630-58-1	Fenvalerate	NA	Inactive	No
446-72-0	Genistein	Weak	Active	No
52-86-8	Haloperidol	Inactive	NA	No
520-18-3	Kaempferol	Very Weak	Inactive	Yes
143-50-0	Kepone	Weak	NA	Yes
65277-42-1	Ketoconazole	Inactive	NA	Yes
330-55-2	Linuron	Inactive	NA	Yes
84-16-2	meso-Hexestrol	Strong	NA	Yes
72-33-3	Mestranol	NA	Active	Yes
72-43-5	Methoxychlor	Very Weak	Active	Yes
68-22-4	Norethindrone	NA	Active	No
789-02-6	o,p'-DDT	Weak	Active	Yes
556-67-2	Octamethylcyclotetrasiloxane	NA	Active	Yes
72-55-9	p,p'-DDE	Very Weak	Weak	No
87-86-5	Pentachlorophenol	NA	Inactive	No
57-30-7	Phenobarbital sodium	Inactive	NA	No
32809-16-8	Procymidone	Inactive	NA	Yes
50-55-5	Reserpine	Inactive	NA	Yes
52-01-7	Spironolactone	Inactive	NA	No
17924-92-4	Zearalenone	NA	Active	Yes

In Vitro Activity	ToxCast Active	ToxCast Inactive
Active	27	2
Inactive	1	7

In Vivo Activity	ToxCast Active	ToxCast Inactive
Active	29	1
Inactive	5	6

In Vitro Sensitivity = 93.1%
In Vitro Specificity = 87.5%
Balanced Accuracy = 90.3%

In Vivo Sensitivity = 96.7%
In Vivo Specificity = 54.5%
Balanced Accuracy = 75.6%

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical

constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [11].

52. Assay Documentation

52.1. References

[1] Graham, F., et al. (1977). J Gen Virol 36(1): 59-72. (PMID: 886304)
[2] Bylund, L., et al. (2004). Cytogen Genome Res 106(1): 28-32. (PMID: 15218237)
[3] Dai, D., et al. (2015). Die Pharmazie-Int J Pharm Sci 70(1): 33-37. (PMID: 25975096)
[4] Lin, Y.-C., et al. (2014). Nature Comm 5. (PMID: 25182477)
[5] Shanle, E. K. and W. Xu (2010). Chem Res Toxicol 24(1): 6-19. (PMID: 21053929)
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[8] Powell, E. and W. Xu (2008). PNAS 105(48): 19012-19017. (PMID: 19022902)
[9] Monroe, D. G., et al. (2005). Mol Endocrinol 19(6): 1555-1568. (PMID: 15802376)
[10] Li, X., et al. (2004). Mol Cell Biol 24(17): 7681-7694. (PMID: 15314175)
[11] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

52.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
DBD, DNA Binding Domain
DMSO, Dimethyl Sulfoxide
EDC, Endocrine Disrupting Compounds
ER, Estrogen Receptor
E2, Estradiol
HEK, Human Embryonic Kidney
LBD, Ligand Binding Domain
MIE, Molecular Initiating Event
NHR, Nuclear Hormone Receptors
OT, Odyssey Thera
PCA, Protein-Fragment Complementation
YFP, Yellow Fluorescent Protein

52.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

2016

Date of Revisions:

2016

Author of Revisions:

EPA NCCT

53. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:

Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;

Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation

Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;

Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

54. Supporting Information (existing annotations):

OT_ER_ERaERb_1440

Assay Name: Odyssey Thera Estrogen Receptor α/β Homodimer 24-hour Protein-Complementation Assay

55. Assay Descriptions

55.1. Overview

Assay Summary:

The Odyssey Thera estrogen receptor alpha heterodimer (ERα/ERβ) assay used Protein-Fragment Complementation Assays (PCAs) to investigate the biochemical pathways which bring separate protein fragments into close proximity and result in functional reporter enzyme (yellow fluorescent protein, YFP) signal production when the estrogenic pathway is stimulated. Changes in protein complex interactions can be impacted by a variety of biochemical events within a pathway, and this assay is designed to track xenobiotic changes at the level of cell functioning which may occur at a number of points along the estrogen signaling pathway following 24-hour incubation of test chemical with transformed HEK293T cells in 384-well plates.

55.2. Assay Definition

Assay Throughput:

HEK-293T cells are seeded into 384-well microtiter plates and allowed to adhere for 24 hours prior to 24 hour chemical exposures.

Experimental System:

This assay monitors the ligand binding domain (LBD) of the wild type human ER (isoforms α and β, stably expressed in HEK293T cells) for xenoestrogenic activation. The HEK-293 cell line are human embryonic epithelial kidney cells (of unknown parentage) transformed with sheared adenovirus 5 DNA by Frank Graham in 1973 [1]. The transformation incorporated approximately 4.5 kilobases from the viral genome into human chromosome 19 of the HEK cells, and subsequent cytogenetic characterization established that the 293 line is pseudotriploid [2]. HEK-293T cells are derived from the HEK293 cell line by the addition of the SV40 large T antigen that has been shown to increase vector production of some viral vectors. HEK293T are reported to have relatively high transfection efficiencies when compared to other cell lines (COS-7 and HepG2) [3] and it is among the most frequently utilized cell lines for in small-scale protein production and in viral vector propagation using the transient transfection method [4].

Xenobiotic Biotransformation Potential:

Constitutive expression of Phase I/II enzymes has not been well characterized in this cell line and metabolic activation/detoxification of test compounds is potentially limited.

Basic Procedure:

The OT ERα / ERβ assay is a heterodimer PCA of the ligand binding domains (LBD) of ERα (amino acids 310-547) and human ERβ (amino acids 263-489) stably expressed in HEK293T cells. ERα/ERβ LBD cells are seeded into optical quality 384-well poly-D-lysine coated plates in phenol red-free medium supplemented with 10% dextran-treated FBS and allowed to adhere for 24 hours prior to treatment with compounds of interest or controls for 24 hours. Cells are fixed in 4% formaldehyde and stained with Draq5 (BioStatus) to identify cells prior to signal detection. Fluorescent signal in the basal state of the assay is very low and is primarily punctuate and cytoplasmic. Modulation of this assay is quantified as an increase in mean fluorescence intensity in the nucleus (for agonists) or in the nucleus and cytoplasm (antagonists) relative to vehicle controls, and can be quantified on a high content imaging device or a laser scanning cytometer. The latter instrument is favored for this assay because of the rapid mode of data acquisition, the ability to acquire data from the entire cell population in a well (as opposed to a fraction of the well on the high content devices) and the larger dynamic range typically achieved with this instrument. To assess sensitivity of the ERα/ERβ LBD assay to ligand, cells were treated with the ER agonist 17-β-estradiol (E2) in 10-point concentration- response format for 24 hours and monitored response on a laser scanning plate cytometer (Acumen eX3; TTP Lab Tech).

Proprietary Elements:

Odyssey Thera assay described here used patented PCA technology.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote estrogen receptor heterodimerization, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

55.3. Assay References

Assay Source Contact Information:

Odyssey Thera Inc.
4550 Norris Canyon Road, Suite 140
San Ramon, CA 94583
Tel: (925) 242-5000

Assay Publication Year:

2003

Assay Publication:

MacDonald, M. L., Lamerdin, J., Owens, S., Keon, B. H., Bilter, G. K., Shang, Z., Huang, Z., Yu, H., Dias, J., Minami, T., Michnick, S. W., & Westwick, J. K. (2006). "Identifying off-target effects and hidden phenotypes of drugs in human cells". Nat Chem Biol 2(6), 329-337. (PMID: 16680159)

Yu, H., West, M., Keon, B. H., Bilter, G. K., Owens, S., Lamerdin, J., & Westwick, J. K. (2003). "Measuring drug action in the cellular context using protein-fragment complementation assays". Assay Drug Dev Technol 1(6), 811-822. (PMID: 15090227)

Method Updates / Confirmatory Studies:

None Reported.

56. Assay Component Descriptions

Assay Objectives:

The Odyssey Thera ERα/ERβ LBD assay utilized the ability of ERα and ERβ to form heterodimers following ligand-binding with estrogenic compounds. This activity is monitored via Protein-Fragment Complementation Assays (PCAs) which investigate the biochemical pathways capable of bringing separate protein fragments into close proximity. Each ERα and ERβ protein contains a fragment of a reporter enzyme (YFP) and when both proteins come in contact to form homo- or heterodimers, the resulting YFP signal can be measured using fluorescence microscopy and used to screen a diverse chemical library for potential xenobiotic ligand-binding and ER activation. Changes in protein complex interactions can be impacted by a variety of biochemical events within a pathway, and this assay is designed to track xenobiotic changes at the level of cell functioning which may occur at a number of points along the estrogen signaling pathway following a 24 hour incubation of cells with test compound in 384-well plate, using DMSO as a negative control and baseline signal and 17β-estradiol as a positive control and measure of 100% ligand-binding activity in ER α/β.

Scientific Principles:

Endocrine disrupting compounds bind to nuclear hormone receptors, leading to a diverse array of transcriptional and signaling pathway alterations. These cell- and tissue-type specific changes affect many aspects of human physiology, including those involved in inflammation, neonatal development and oncogenesis. Among the human NRs, the estrogen receptor family is particularly

susceptible to perturbation by EDCs because of the ER LBD proclivity to bind a disparate set of small hydrophobic molecules commonly found in nature and in man-made materials [5].

The estrogen receptor is expressed in two forms, ERα and ERβ which play different roles in mediating the actions of estrogenic compounds. Multiple studies have determined that the two isoforms can form functional homo- and hetero-dimers in vitro and in vivo which are capable of binding DNA [6] and initiating transcription of target genes [7]. Furthermore, ER homo- and heterodimers display ligand-selective activity [8] leading in turn to a unique but overlapping set of dimer-mediated transcriptional changes [9, 10]. Thus, a complete understanding of the potential estrogenic effects of EDCs requires the comprehensive profiling of the three physiological dimers. To assess the activity of the ERα/β heterodimer, this assay was designed to utilize the ability of the coexpressed ERα and ERβ LBDs to heterodimerize upon ligand-binding with estrogenic compounds. This dimerization and concurrent conformational changes brings into close proximity the fused fragments of split-YFP, leading to a dramatic increase in YFP intensity. This intensity change is dose-dependent and shows saturation-binding that plateaus differently for agonists versus antagonists.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with estrogen receptor alpha mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that estrogen receptor agonism is the Molecular Initiating Event (MIE) in an Adverse Outcome Pathway (AOP) leading to reproductive dysfunction in oviparous vertebrates, and there is some evidence that estrogen receptor activation is the MIE for putative AOPs leading to reduced survival due to renal failure and leading to skewed sex ratios due to altered sexual differentiation in males (all AOPs currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive in vivo studies to further investigate the involvement of ER agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Michnick, S. W., Remy, I., Campbell-Valois, F. X., Vallee-Belisle, A., & Pelletier, J. N. (2000). "Detection of protein-protein interactions by protein fragment complementation strategies". Methods Enzymol 328, 208-230. (PMID: 11075347)

Assay Quality Statistics:

Neutral control well median response value, by plate:	1.00
Neutral control median absolute deviation, by plate:	0.140
Positive control well median response value, by plate:	6.79
Positive control well median absolute deviation, by plate:	0.43
Z' (median across all plates, using positive control wells):	0.72
SSMD (median across all plates, using positive control wells):	13
Signal-to-noise (median across all plates, using positive control wells):	43.18
Signal-to-background (median across all plates, using positive control wells):	6.82
CV (median across all plates):	0.14

57. Assay Endpoint Descriptions

57.1. Data Interpretation

Biological Response:

Estrogen signaling pathway protein fragment dimerization and enzyme formation in response to estrogen receptor (α / β) ligand-binding as detected by monitoring fluorescence intensity.

Analytical Elements:

OT_ER_ERaERb_1440 was analyzed into in the positive fitting direction (receptor gain-of-signal activity) as a percent of 17b-Estradiol (positive control, 100% activation) and relative to DMSO,

negative control and baseline signal. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Estrogen receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ACEA_T47D_80hr_Positive
ATG_ERE_CIS_up
ATG_ERa_TRANS_up
ATG_ERb_TRANS2_up
NVS_NR_bER
NVS_NR_hER
NVS_NR_mERa
OT_ER_ERaERa_0480
OT_ER_ERaERa_1440
OT_ER_ERaERb_0480
OT_ER_ERbERb_0480
OT_ER_ERbERb_1440
OT_ERa_ERELUC_AG_1440
OT_ERa_ERELUC_ANT_1440
OT_ERa_EREGFP_0120
OT_ERa_EREGFP_0480
OT_ERb_ERELUC_ANT_1440
Tox21_ERa_BLA_Agonist_ratio
Tox21_ERa_BLA_Antagonist_ratio
Tox21_ERa_LUC_BG1_Agonist
Tox21_ERa_LUC_BG1_Antagonist

57.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations: 6
Target (nominal) number of replicates: 3
Standard minimum concentration tested: 0.3 µM
Standard maximum concentration tested: 100 µM
Baseline median absolute deviation for the assay - based on the response values at the 2 lowest tested concentrations (bmad): 1.91
The response cutoff used to derive the hit calls (e.g., 5*bmad, 10*bmad): 20

Reference Chemicals / Predictive Capacity:

CASRN	Chemical Name	*In Vitro* Activity	*In Vivo* Activity	Activity in Assay
57-91-0	17alpha-Estradiol	Moderate	Active	Yes
57-63-6	17alpha-Ethinylestradiol	Strong	Active	Yes
50-28-2	17beta-Estradiol	Strong	Active	Yes
58-18-4	17-Methyltestosterone	Very Weak	Active	Yes
131-55-5	2,2',4,4'-Tetrahydroxybenzophenone	NA	Active	Yes
131-56-6	2,4-Dihydroxybenzophenone	NA	Active	No
5153-25-3	2-Ethylhexylparaben	NA	Active	Yes
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80-09-1	4,4'-Sulfonyldiphenol	NA	Active	Yes
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99-96-7	4-Hydroxybenzoic acid	NA	Inactive	Yes
104-40-5	4-Nonylphenol	Very Weak	Active	Yes
98-54-4	4-tert-Butylphenol	NA	Active	Yes
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61-82-5	Amitrole	NA	Inactive	Yes
520-36-5	Apigenin	Very Weak	NA	Yes
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1478-61-1	Bisphenol AF	NA	Active	Yes
77-40-7	Bisphenol B	Weak	Active	Yes
94-26-8	Butylparaben	NA	Active	No
480-40-0	Chrysin	Very Weak	NA	Yes
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115-32-2	Dicofol	Very Weak	NA	Yes
84-61-7	Dicyclohexyl phthalate	NA	Inactive	Yes
84-66-2	Diethyl phthalate	NA	Inactive	Yes
56-53-1	Diethylstilbestrol	Strong	Active	Yes
84-75-3	Dihexyl phthalate	NA	Inactive	Yes
474-86-2	Equilin	NA	Active	Yes
50-27-1	Estriol	NA	Active	No
53-16-7	Estrone	Moderate	Active	Yes
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60168-88-9	Fenarimol	Very Weak	NA	Yes
51630-58-1	Fenvalerate	NA	Inactive	Yes
446-72-0	Genistein	Weak	Active	No
52-86-8	Haloperidol	Inactive	NA	No
520-18-3	Kaempferol	Very Weak	Inactive	Yes
143-50-0	Kepone	Weak	NA	Yes
65277-42-1	Ketoconazole	Inactive	NA	Yes
330-55-2	Linuron	Inactive	NA	Yes
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72-43-5	Methoxychlor	Very Weak	Active	Yes
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87-86-5	Pentachlorophenol	NA	Inactive	No
57-30-7	Phenobarbital sodium	Inactive	NA	No
32809-16-8	Procymidone	Inactive	NA	Yes
50-55-5	Reserpine	Inactive	NA	Yes
52-01-7	Spironolactone	Inactive	NA	No
17924-92-4	Zearalenone	NA	Active	Yes

In Vitro Activity	ToxCast Active	ToxCast Inactive
Active	28	1
Inactive	2	6

In Vivo Activity	ToxCast Active	ToxCast Inactive
Active	29	1
Inactive	5	6

In Vitro Sensitivity = 96.6%
In Vitro Specificity = 75.0%
Balanced Accuracy = 85.8%

In Vivo Sensitivity = 96.7%
In Vivo Specificity = 54.5%
Balanced Accuracy = 75.6%

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical

constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [11].

58. Assay Documentation

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[1] Graham, F., et al. (1977). J Gen Virol 36(1): 59-72. (PMID: 886304)
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[4] Lin, Y.-C., et al. (2014). Nature Comm 5. (PMID: 25182477)
[5] Shanle, E. K. and W. Xu (2010). Chem Res Toxicol 24(1): 6-19. (PMID: 21053929)
[6] Papoutsi, Z., et al. (2009). J Molecul Endocrin 43(2): 65-72. (PMID: 19376833)
[7] Cowley, S. M., et al. (1997). J Biol Chem 272(32): 19858-19862. (PMID: 9242648)
[8] Powell, E. and W. Xu (2008). PNAS 105(48): 19012-19017. (PMID: 19022902)
[9] Monroe, D. G., et al. (2005). Mol Endocrinol 19(6): 1555-1568. (PMID: 15802376)
[10] Li, X., et al. (2004). Mol Cell Biol 24(17): 7681-7694. (PMID: 15314175)
[11] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

58.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
DBD, DNA Binding Domain
DMSO, Dimethyl Sulfoxide
EDC, Endocrine Disrupting Compounds
ER, Estrogen Receptor
E2, Estradiol
HEK, Human Embryonic Kidney
LBD, Ligand Binding Domain
MIE, Molecular Initiating Event
NHR, Nuclear Hormone Receptors
OT, Odyssey Thera
PCA , Protein-Fragment Complementation
YFP, Yellow Fluorescent Protein

58.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

2016

Date of Revisions:

2016

Author of Revisions:

EPA NCCT

59. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:

Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;

Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation

Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;

Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

60. Supporting Information (existing annotations):

OT_ER_ERbERb_0480

Assay Name: Odyssey Thera Estrogen Receptor β/β Homodimer 8-hour Protein-Complementation Assay

61. Assay Descriptions

61.1. Overview

Assay Summary:

The Odyssey Thera estrogen receptor beta (ERβ/ERβ) ligand-binding assay used Protein-Fragment Complementation Assays (PCAs) to probe estrogen receptor beta (ERβ) for xenoestrogenic nuclear receptor binding and subsequent homodimer formation in stably transfected human embryonic kidney cells (cell line HEK293T). This assay format is designed to investigate the biochemical pathways capable of bringing separate, rationally dissected yellow fluorescent protein (YFP) fragments, which are linked in-frame to ERβ genes, into close proximity. When the reporter enzyme fragments are physically adjacent, the result is YFP reassembly and functional signal production. This fluorescence signal is only produced when the estrogen receptor beta pathway is stimulated. Changes in protein complex interactions can be impacted by a variety of biochemical events within a pathway, and this assay is designed to track xenoestrogenic impacts on whole cell functioning which may affect functioning at a number of points along the estrogen signaling pathway. This activity was monitored following 8-hour incubation of test chemical or solvent with transformed HEK293T cells in 384-well plates.

61.2. Assay Definition

Assay Throughput:

HEK-293T cells are seeded into 384-well microtiter plates and allowed to adhere for 24 hours prior to 8 hour chemical exposures.

Experimental System:

This assay monitors the ligand binding domain (LBD) of the wild type human ERβ (stably expressed in HEK293T cells) for xenoestrogenic activation. The HEK-293 cell line are human embryonic epithelial kidney cells (of unknown parentage) transformed with sheared adenovirus 5 DNA by Frank Graham in 1973 [1]. The transformation incorporated approximately 4.5 kilobases from the viral genome into human chromosome 19 of the HEK cells, and subsequent cytogenetic characterization established that the 293 line is pseudotriploid [2]. HEK-293T cells are derived from the HEK293 cell line by the addition of the SV40 large T antigen that has been shown to increase vector production of some viral vectors. HEK293T are reported to have relatively high transfection efficiencies when compared to other cell lines (COS-7 and HepG2) [3] and it is among the most frequently utilized cell lines for in small-scale protein production and in viral vector propagation using the transient transfection method [4].

Xenobiotic Biotransformation Potential:

Constitutive expression of Phase I/II enzymes has not been well characterized in this cell line and metabolic activation/detoxification of test compounds is potentially limited.

Basic Procedure:

The OT ERβ / ERβ assay is a homodimer PCA of the ligand binding domain (LBD; amino acids 310-547) of human ERβ stably expressed in HEK293T cells. Cells are seeded into optical quality 384-well poly-D-lysine coated plates in phenol red-free medium supplemented with 10% dextran-treated FBS and allowed to adhere for 8 hours prior to treatment with compounds of interest or controls for 8 hours. Cells are fixed in 4% formaldehyde and stained with Draq5 (BioStatus) to identify cells prior to signal detection. Fluorescent signal in the basal state of the assay is very low and is primarily punctuate and cytoplasmic. Modulation of this assay is quantified as an increase in mean fluorescence intensity in the nucleus (for agonists) or in the nucleus and cytoplasm (antagonists) relative to vehicle controls, and can be quantified on a high content imaging device or a laser scanning cytometer. The latter instrument is favored for this assay because of the rapid mode of data acquisition, the ability to acquire data from the entire cell population in a well (as

opposed to a fraction of the well on the high content devices) and the larger dynamic range typically achieved with this instrument. To assess sensitivity of the ERβ/ERβ LBD assay to ligand, cells were treated with the ER agonist 17-β-estradiol (E2) in 10-point concentration- response format for 8 hours and monitored response on a laser scanning plate cytometer (Acumen eX3; TTP Lab Tech).

Proprietary Elements:

Odyssey Thera assay described here used patented PCA technology.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote estrogen receptor homodimerization, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

61.3. Assay References

Assay Source Contact Information:

Odyssey Thera Inc.
4550 Norris Canyon Road, Suite 140
San Ramon, CA 94583
Tel: (925) 242-5000

Assay Publication Year:

2003

Assay Publication:

MacDonald, M. L., Lamerdin, J., Owens, S., Keon, B. H., Bilter, G. K., Shang, Z., Huang, Z., Yu, H., Dias, J., Minami, T., Michnick, S. W., & Westwick, J. K. (2006). "Identifying off-target effects and hidden phenotypes of drugs in human cells". Nat Chem Biol 2(6), 329-337. (PMID: 16680159)

Yu, H., West, M., Keon, B. H., Bilter, G. K., Owens, S., Lamerdin, J., & Westwick, J. K. (2003). "Measuring drug action in the cellular context using protein-fragment complementation assays". Assay Drug Dev Technol 1(6), 811-822. (PMID: 15090227)

Method Updates / Confirmatory Studies:

None Reported.

62. Assay Component Descriptions

Assay Objectives:

Endocrine disrupting compounds bind to nuclear hormone receptors, leading to a diverse array of transcriptional and signaling pathway alterations. These cell- and tissue-type specific changes affect many aspects of human physiology, including those involved in inflammation, neonatal development and oncogenesis. Among the human NRs, the estrogen receptor family is particularly susceptible to perturbation by EDCs because of the ER LBD proclivity to bind a disparate set of small hydrophobic molecules commonly found in nature and in man-made materials [5].

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with estrogen receptor mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that estrogen receptor agonism is the Molecular Initiating Event (MIE) in an Adverse Outcome Pathway (AOP) leading to reproductive dysfunction in oviparous vertebrates, and there is some evidence that estrogen receptor activation is the MIE for putative AOPs leading to reduced survival due to renal failure and leading to skewed sex ratios due to altered sexual

differentiation in males (all AOPs currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive in vivo studies to further investigate the involvement of ER agonism in pathways leading to hazardous outcomes in biological systems.

Scientific Principles:

Endocrine disrupting compounds bind to nuclear hormone receptors, leading to a diverse array of transcriptional and signaling pathway alterations. These cell- and tissue-type specific changes affect many aspects of human physiology, including those involved in inflammation, neonatal development and oncogenesis. Among the human NRs, the estrogen receptor family is particularly susceptible to perturbation by EDCs because of the ER LBD proclivity to bind a disparate set of small hydrophobic molecules commonly found in nature and in man-made materials [5].

The estrogen receptor is expressed in two forms, ERα and ERβ, which play different roles in mediating the actions of estrogenic compounds. Cell-based and in vivo experiments suggest that the ERα isoform is the primary mediator of estrogenic effects by EDCs on reproductive development and on cell proliferation, while ERβ is thought to inhibit estrogen dependent cell growth, especially in breast cancer cells [6-8]. Several studies have associated a loss of ERβ or a decreased ratio of ERβ/ERα with other cancer types, including ovarian and colorectal cancers [9], suggesting a tumor suppressor role for ERβ in several cell types. Several isotype-selective ligands that bind one receptor with higher affinity than the other have been identified [10, 11] that produce distinct physiological effects when tested in animal models [9]. Therefore, a highly sensitive assay that can detect ERβ transcriptional changes in the context of a whole cell would be a useful tool to differentiate EDC's that preferentially activate ERβ-specific pathways. The OT ERβ/ERβ LBD PCA utilizes the ability of the ERβ LBD to homodimerize upon binding to estrogenic compounds. This dimizeration and concurrent conformational changes brings into close proximity the fused fragments of split-YFP, leading to a dramatic increase in YFP intensity. This intensity change is dose-dependent and shows saturation-binding that plateaus differently for agonists versus antagonists.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with estrogen receptor mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that estrogen receptor agonism is the Molecular Initiating Event (MIE) in an Adverse Outcome Pathway (AOP) leading to reproductive dysfunction in oviparous vertebrates, and there is some evidence that estrogen receptor activation is the MIE for putative AOPs leading to reduced survival due to renal failure and leading to skewed sex ratios due to altered sexual differentiation in males (all AOPs currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive in vivo studies to further investigate the involvement of ER agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Michnick, S. W., Remy, I., Campbell-Valois, F. X., Vallee-Belisle, A., & Pelletier, J. N. (2000). "Detection of protein-protein interactions by protein fragment complementation strategies". Methods Enzymol 328, 208-230. (PMID: 11075347)

Assay Quality Statistics:

Neutral control well median response value, by plate:	1.11
Neutral control median absolute deviation, by plate:	0.19
Positive control well median response value, by plate:	6.75
Positive control well median absolute deviation, by plate:	0.37
Z' (median across all plates, using positive control wells):	0.7

SSMD (median across all plates, using positive control wells):	13
Signal-to-noise (median across all plates, using positive control wells):	27.9
Signal-to-background (median across all plates, using positive control wells):	5.81
CV (median across all plates):	0.18

63. Assay Endpoint Descriptions

63.1. Data Interpretation

Biological Response:

Estrogen signaling pathway protein fragment dimerization and enzyme formation in response to estrogen receptor beta ligand-binding as detected by monitoring fluorescence intensity.

Analytical Elements:

OT_ER_ERbERb_0480 assay was analyzed into in the positive fitting direction (receptor gain-of-signal activity) as a percent of 17b-Estradiol (positive control, 100% activation) and relative to DMSO, negative control and baseline signal. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Estrogen receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ACEA_T47D_80hr_Positive
ATG_ERE_CIS_up
ATG_ERa_TRANS_up
ATG_ERb_TRANS2_up
NVS_NR_bER
NVS_NR_hER
NVS_NR_mERa
OT_ER_ERaERa_0480
OT_ER_ERaERa_1440
OT_ER_ERaERb_1440
OT_ER_ERaERb_0480
OT_ER_ERbERb_1440
OT_ERa_ERELUC_AG_1440
OT_ERa_ERELUC_ANT_1440
OT_ERa_EREGFP_0120
OT_ERa_EREGFP_0480
OT_ERb_ERELUC_ANT_1440
Tox21_ERa_BLA_Agonist_ratio
Tox21_ERa_BLA_Antagonist_ratio
Tox21_ERa_LUC_BG1_Agonist

Tox21_ERa_LUC_BG1_Antagonist

63.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations: 6
Target (nominal) number of replicates: 3
Standard minimum concentration tested: 0.3 µM
Standard maximum concentration tested: 100 µM
Baseline median absolute deviation for the assay - based on the response values at the 2 lowest tested concentrations (bmad): 3.04
The response cutoff used to derive the hit calls (e.g., 5*bmad, 10*bmad): 20

Reference Chemicals / Predictive Capacity:

CASRN	Chemical Name	*In Vitro* Activity	*In Vivo* Activity	Activity in Assay
57-91-0	17alpha-Estradiol	Moderate	Active	Yes
57-63-6	17alpha-Ethinylestradiol	Strong	Active	Yes
50-28-2	17beta-Estradiol	Strong	Active	Yes
58-18-4	17-Methyltestosterone	Very Weak	Active	Yes
131-55-5	2,2',4,4'-Tetrahydroxybenzophenone	NA	Active	Yes
131-56-6	2,4-Dihydroxybenzophenone	NA	Active	No
5153-25-3	2-Ethylhexylparaben	NA	Active	Yes
140-66-9	4-(1,1,3,3-Tetramethylbutyl)phenol	Moderate	Active	No
80-46-6	4-(2-Methylbutan-2-yl)phenol	NA	Active	Yes
80-09-1	4,4'-Sulfonyldiphenol	NA	Active	Yes
599-64-4	4-Cumylphenol	Weak	Active	Yes
104-43-8	4-Dodecylphenol	NA	Active	No
99-96-7	4-Hydroxybenzoic acid	NA	Inactive	Yes
104-40-5	4-Nonylphenol	Very Weak	Active	Yes
98-54-4	4-tert-Butylphenol	NA	Active	Yes
521-18-6	5alpha-Dihydrotestosterone	Weak	Active	Yes
61-82-5	Amitrole	NA	Inactive	Yes
520-36-5	Apigenin	Very Weak	NA	Yes
85-68-7	Benzyl butyl phthalate	Very Weak	NA	Yes
103-23-1	Bis(2-ethylhexyl)hexanedioate	NA	Inactive	No
80-05-7	Bisphenol A	Weak	Active	Yes
1478-61-1	Bisphenol AF	NA	Active	Yes
77-40-7	Bisphenol B	Weak	Active	Yes
94-26-8	Butylparaben	NA	Active	No
480-40-0	Chrysin	Very Weak	NA	No
50-22-6	Corticosterone	Inactive	NA	Yes
486-66-8	Daidzein	Weak	NA	Yes
117-81-7	Di(2-ethylhexyl) phthalate	Very Weak	Inactive	Yes
84-74-2	Dibutyl phthalate	Very Weak	Inactive	Yes

115-32-2	Dicofol	Very Weak	NA	Yes
84-61-7	Dicyclohexyl phthalate	NA	Inactive	Yes
84-66-2	Diethyl phthalate	NA	Inactive	Yes
56-53-1	Diethylstilbestrol	Strong	Active	Yes
84-75-3	Dihexyl phthalate	NA	Inactive	Yes
474-86-2	Equilin	NA	Active	Yes
50-27-1	Estriol	NA	Active	No
53-16-7	Estrone	Moderate	Active	Yes
120-47-8	Ethylparaben	Very Weak	NA	No
60168-88-9	Fenarimol	Very Weak	NA	Yes
51630-58-1	Fenvalerate	NA	Inactive	No
446-72-0	Genistein	Weak	Active	No
52-86-8	Haloperidol	Inactive	NA	No
520-18-3	Kaempferol	Very Weak	Inactive	Yes
143-50-0	Kepone	Weak	NA	Yes
65277-42-1	Ketoconazole	Inactive	NA	Yes
330-55-2	Linuron	Inactive	NA	Yes
84-16-2	meso-Hexestrol	Strong	NA	Yes
72-33-3	Mestranol	NA	Active	Yes
72-43-5	Methoxychlor	Very Weak	Active	Yes
68-22-4	Norethindrone	NA	Active	No
789-02-6	o,p'-DDT	Weak	Active	Yes
556-67-2	Octamethylcyclotetrasiloxane	NA	Active	Yes
72-55-9	p,p'-DDE	Very Weak	Weak	No
87-86-5	Pentachlorophenol	NA	Inactive	No
57-30-7	Phenobarbital sodium	Inactive	NA	No
32809-16-8	Procymidone	Inactive	NA	Yes
50-55-5	Reserpine	Inactive	NA	Yes
52-01-7	Spironolactone	Inactive	NA	No
17924-92-4	Zearalenone	NA	Active	Yes

In Vitro Activity	ToxCast Active	ToxCast Inactive
Active	26	3
Inactive	1	7

In Vivo Activity	ToxCast Active	ToxCast Inactive
Active	29	1
Inactive	4	7

In Vitro Sensitivity = 89.7%
In Vitro Specificity = 87.5%
Balanced Accuracy = 88.6%

In Vivo Sensitivity = 96.7%

In Vivo Specificity = 63.6%
Balanced Accuracy = 80.2%

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [12].

64. Assay Documentation

64.1. References

[1] Graham, F., et al. (1977). J Gen Virol 36(1): 59-72. (PMID: 886304)
[2] Bylund, L., et al. (2004). Cytogenet Genome Res 106(1): 28-32. (PMID: 15218237)
[3] Dai, D., et al. (2015). Die Pharmazie- Int J Pharm Sci 70(1): 33-37. (PMID: 25975096)
[4] Lin, Y.-C., et al. (2014). Nature Comm 5. (PMID: 25182477)
[5] Shanle, E. K. and W. Xu (2010). Chem Res Toxicol 24(1): 6-19. (PMID: 21053929)
[6] Papoutsi, Z., et al. (2009). J Mol Endocrin 43(2): 65-72. (PMID: 19376833)
[7] Cowley, S. M., et al. (1997). J Biol Chem 272(32): 19858-19862. (PMID: 9242648)
[8] Powell, E. and W. Xu (2008). PNAS105(48): 19012-19017. (PMID: 19022902)
[9] Monroe, D. G., et al. (2005). Mol Endocrinol 19(6): 1555-1568. (PMID: 15802376)
[10] Li, X., et al. (2004). Mol Cell Biol 24(17): 7681-7694. (PMID: 15314175)
[11] Kraichely, D. M., et al. (2000). Endocrinology 141(10): 3534-3545. (PMID: 11014206)
[12] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

64.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
DBD, DNA Binding Domain
DMSO, Dimethyl Sulfoxide
EDC, Endocrine Disrupting Compounds
ER, Estrogen Receptor
E2, Estradiol
HEK, Human Embryonic Kidney
LBD, Ligand Binding Domain
MIE, Molecular Initiating Event
NHR, Nuclear Hormone Receptors
OT, Odyssey Thera
PCA, Protein-Fragment Complementation
YFP, Yellow Fluorescent Protein

64.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

26 May 2016

Date of Revisions:

25 November 2016

Author of Revisions:

EPA NCCT

65. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:

Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;

Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation

Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;

Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

66. Supporting Information (existing annotations):

OT_ER_ERbERb_1440

Assay Name: Odyssey Thera Estrogen Receptor ß/ß Homodimer 24-hour Protein-Complementation Assay

67. Assay Descriptions

67.1. Overview

Assay Summary:

The Odyssey Thera estrogen receptor beta (ERβ/ERβ) ligand-binding assay used Protein-Fragment Complementation Assays (PCAs) to probe estrogen receptor beta (ERβ) for xenoestrogenic nuclear receptor binding and subsequent homodimer formation in stably transfected human embryonic kidney cells (cell line HEK293T). This assay format is designed to investigate the biochemical pathways capable of bringing separate, rationally dissected yellow fluorescent protein (YFP) fragments which are linked in-frame to ERβ genes into close proximity. When the reporter enzyme fragments are physically adjacent, the result is YFP reassembly and functional signal production. This fluorescence signal is only produced when the estrogen receptor beta pathway is stimulated. Changes in protein complex interactions can be impacted by a variety of biochemical events within a pathway, and this assay is designed to track xenoestrogenic impacts on whole cell functioning which may affect functioning at a number of points along the estrogen signaling pathway. This activity was monitored following 24-hour incubation of test chemical or solvent with transformed HEK293T cells in 384-well plates.

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HEK-293T cells are seeded into 384-well microtiter plates and allowed to adhere for 24 hours prior to 24 hour chemical exposures.

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This assay monitors the ligand binding domain (LBD) of the wild type human ERβ (stably expressed in HEK293T cells) for xenoestrogenic activation. The HEK-293 cell line are human embryonic epithelial kidney cells (of unknown parentage) transformed with sheared adenovirus 5 DNA by Frank Graham in 1973 [1]. The transformation incorporated approximately 4.5 kilobases from the viral genome into human chromosome 19 of the HEK cells, and subsequent cytogenetic characterization established that the 293 line is pseudotriploid [2]. HEK-293T cells are derived from the HEK293 cell line by the addition of the SV40 large T antigen that has been shown to increase vector production of some viral vectors. HEK293T are reported to have relatively high transfection efficiencies when compared to other cell lines (COS-7 and HepG2) [3] and it is among the most frequently utilized cell lines for in small-scale protein production and in viral vector propagation using the transient transfection method [4].

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Constitutive expression of Phase I/II enzymes has not been well characterized in this cell line and metabolic activation/detoxification of test compounds is potentially limited.

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Proprietary Elements:

Odyssey Thera assay described here used patented PCA technology.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote estrogen receptor homodimerization, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

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Tel: (925) 242-5000

Assay Publication Year:

2003

Assay Publication:

MacDonald, M. L., Lamerdin, J., Owens, S., Keon, B. H., Bilter, G. K., Shang, Z., Huang, Z., Yu, H., Dias, J., Minami, T., Michnick, S. W., & Westwick, J. K. (2006). "Identifying off-target effects and hidden phenotypes of drugs in human cells". Nat Chem Biol 2(6), 329-337. (PMID: 16680159)

Yu, H., West, M., Keon, B. H., Bilter, G. K., Owens, S., Lamerdin, J., & Westwick, J. K. (2003). "Measuring drug action in the cellular context using protein-fragment complementation assays". Assay Drug Dev Technol 1(6), 811-822. (PMID: 15090227)

Method Updates / Confirmatory Studies:

None Reported.

68. Assay Component Descriptions

Assay Objectives:

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Endocrine disrupting compounds bind to nuclear hormone receptors, leading to a diverse array of transcriptional and signaling pathway alterations. These cell- and tissue-type specific changes affect many aspects of human physiology, including those involved in inflammation, neonatal development and oncogenesis. Among the human NRs, the estrogen receptor family is particularly susceptible to perturbation by EDCs because of the ER LBD proclivity to bind a disparate set of small hydrophobic molecules commonly found in nature and in man-made materials [5].

The estrogen receptor is expressed in two forms, ERα and ERβ which play different roles in mediating the actions of estrogenic compounds. Cell-based and in vivo experiments suggest that the ERα isoform is the primary mediator of estrogenic effects by EDCs on reproductive development and on cell proliferation, while ERβ is thought to inhibit estrogen dependent cell growth, especially in breast cancer cells [6-8]. Several studies have associated a loss of ERβ or a decreased ratio of ERβ/ERα with other cancer types, including ovarian and colorectal cancers [9], suggesting a tumor suppressor role for ERβ in several cell types. Several isotype-selective ligands that bind one receptor with higher affinity than the other have been identified [10, 11] that produce distinct physiological effects when tested in animal models [9]. Therefore, a highly sensitive assay that can detect ERβ transcriptional changes in the context of a whole cell would be a useful tool to differentiate EDC's that preferentially activate ERβ-specific pathways. The OT ERβ/ERβ LBD PCA utilizes the ability of the ERβ LBD to homodimerize upon binding to estrogenic compounds. This dimizeration and concurrent conformational changes brings into close proximity the fused fragments of split-YFP, leading to a dramatic increase in YFP intensity. This intensity change is dose-dependent and shows saturation-binding that plateaus differently for agonists versus antagonists.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with estrogen receptor mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that estrogen receptor agonism is the Molecular Initiating Event (MIE) in an Adverse Outcome Pathway (AOP) leading to reproductive dysfunction in oviparous vertebrates, and there is some evidence that estrogen receptor activation is the MIE for putative AOPs leading to reduced survival due to renal failure and leading to skewed sex ratios due to altered sexual differentiation in males (all AOPs currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive in vivo studies to further investigate the involvement of ER agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Michnick, S. W., Remy, I., Campbell-Valois, F. X., Vallee-Belisle, A., & Pelletier, J. N. (2000). "Detection of protein-protein interactions by protein fragment complementation strategies". Methods Enzymol 328, 208-230. (PMID: 11075347)

Assay Quality Statistics:

Neutral control well median response value, by plate:	1.25
Neutral control median absolute deviation, by plate:	0.19
Positive control well median response value, by plate:	14.01
Positive control well median absolute deviation, by plate:	0.65

Z' (median across all plates, using positive control wells):	0.79
SSMD (median across all plates, using positive control wells):	18
Signal-to-noise (median across all plates, using positive control wells):	67.22
Signal-to-background (median across all plates, using positive control wells):	10.89
CV (median across all plates):	0.15

69. Assay Endpoint Descriptions

69.1. Data Interpretation

Biological Response:

Estrogen signaling pathway protein fragment dimerization and enzyme formation in response to estrogen receptor beta ligand-binding as detected by monitoring fluorescence intensity.

Analytical Elements:

OT_ER_ERbERb_1440 was analyzed into in the positive fitting direction (receptor gain-of-signal activity) as a percent of 17b-Estradiol (positive control, 100% activation) and relative to DMSO, negative control and baseline signal. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Estrogen receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ACEA_T47D_80hr_Positive
ATG_ERE_CIS_up
ATG_ERa_TRANS_up
ATG_ERb_TRANS2_up
NVS_NR_bER
NVS_NR_hER
NVS_NR_mERa
OT_ER_ERaERa_0480
OT_ER_ERaERa_1440
OT_ER_ERaERb_1440
OT_ER_ERaERb_0480
OT_ER_ERbERb_0480
OT_ERa_ERELUC_AG_1440
OT_ERa_ERELUC_ANT_1440
OT_ERa_EREGFP_0120
OT_ERa_EREGFP_0480
OT_ERb_ERELUC_ANT_1440
Tox21_ERa_BLA_Agonist_ratio
Tox21_ERa_BLA_Antagonist_ratio

Tox21_ERa_LUC_BG1_Agonist
Tox21_ERa_LUC_BG1_Antagonist

69.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations: 6
Target (nominal) number of replicates: 3
Standard minimum concentration tested: 0.3 µM
Standard maximum concentration tested: 100 µM
Baseline median absolute deviation for the assay - based on the response values at the 2 lowest tested concentrations (bmad): 1.48
The response cutoff used to derive the hit calls (e.g., 5*bmad, 10*bmad): 20

Reference Chemicals / Predictive Capacity:

CASRN	Chemical Name	*In Vitro* Activity	*In Vivo* Activity	Activity in Assay
57-91-0	17alpha-Estradiol	Moderate	Active	Yes
57-63-6	17alpha-Ethinylestradiol	Strong	Active	Yes
50-28-2	17beta-Estradiol	Strong	Active	Yes
58-18-4	17-Methyltestosterone	Very Weak	Active	Yes
131-55-5	2,2',4,4'-Tetrahydroxybenzophenone	NA	Active	Yes
131-56-6	2,4-Dihydroxybenzophenone	NA	Active	No
5153-25-3	2-Ethylhexylparaben	NA	Active	Yes
140-66-9	4-(1,1,3,3-Tetramethylbutyl)phenol	Moderate	Active	No
80-46-6	4-(2-Methylbutan-2-yl)phenol	NA	Active	Yes
80-09-1	4,4'-Sulfonyldiphenol	NA	Active	Yes
599-64-4	4-Cumylphenol	Weak	Active	Yes
104-43-8	4-Dodecylphenol	NA	Active	No
99-96-7	4-Hydroxybenzoic acid	NA	Inactive	Yes
104-40-5	4-Nonylphenol	Very Weak	Active	Yes
98-54-4	4-tert-Butylphenol	NA	Active	Yes
521-18-6	5alpha-Dihydrotestosterone	Weak	Active	Yes
61-82-5	Amitrole	NA	Inactive	Yes
520-36-5	Apigenin	Very Weak	NA	Yes
85-68-7	Benzyl butyl phthalate	Very Weak	NA	Yes
103-23-1	Bis(2-ethylhexyl)hexanedioate	NA	Inactive	No
80-05-7	Bisphenol A	Weak	Active	Yes
1478-61-1	Bisphenol AF	NA	Active	Yes
77-40-7	Bisphenol B	Weak	Active	No
94-26-8	Butylparaben	NA	Active	No
480-40-0	Chrysin	Very Weak	NA	No
50-22-6	Corticosterone	Inactive	NA	Yes
486-66-8	Daidzein	Weak	NA	No
117-81-7	Di(2-ethylhexyl) phthalate	Very Weak	Inactive	Yes

84-74-2	Dibutyl phthalate	Very Weak	Inactive	Yes
115-32-2	Dicofol	Very Weak	NA	Yes
84-61-7	Dicyclohexyl phthalate	NA	Inactive	Yes
84-66-2	Diethyl phthalate	NA	Inactive	Yes
56-53-1	Diethylstilbestrol	Strong	Active	Yes
84-75-3	Dihexyl phthalate	NA	Inactive	Yes
474-86-2	Equilin	NA	Active	Yes
50-27-1	Estriol	NA	Active	No
53-16-7	Estrone	Moderate	Active	Yes
120-47-8	Ethylparaben	Very Weak	NA	No
60168-88-9	Fenarimol	Very Weak	NA	Yes
51630-58-1	Fenvalerate	NA	Inactive	No
446-72-0	Genistein	Weak	Active	No
52-86-8	Haloperidol	Inactive	NA	No
520-18-3	Kaempferol	Very Weak	Inactive	Yes
143-50-0	Kepone	Weak	NA	Yes
65277-42-1	Ketoconazole	Inactive	NA	Yes
330-55-2	Linuron	Inactive	NA	Yes
84-16-2	meso-Hexestrol	Strong	NA	Yes
72-33-3	Mestranol	NA	Active	Yes
72-43-5	Methoxychlor	Very Weak	Active	Yes
68-22-4	Norethindrone	NA	Active	No
789-02-6	o,p'-DDT	Weak	Active	Yes
556-67-2	Octamethylcyclotetrasiloxane	NA	Active	Yes
72-55-9	p,p'-DDE	Very Weak	Weak	No
87-86-5	Pentachlorophenol	NA	Inactive	No
57-30-7	Phenobarbital sodium	Inactive	NA	No
32809-16-8	Procymidone	Inactive	NA	Yes
50-55-5	Reserpine	Inactive	NA	Yes
52-01-7	Spironolactone	Inactive	NA	No
17924-92-4	Zearalenone	NA	Active	Yes

In Vitro Activity	ToxCast Active	ToxCast Inactive
Active	26	3
Inactive	1	7

In Vivo Activity	ToxCast Active	ToxCast Inactive
Active	29	1
Inactive	2	9

In Vitro Sensitivity = 89.7%
In Vitro Specificity = 87.5%
Balanced Accuracy = 88.6%

In Vivo Sensitivity = 96.7%
In Vivo Specificity = 81.8%
Balanced Accuracy = 89.2%

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [12].

70. Assay Documentation

70.1. References

[1] Graham, F., et al. (1977). J Gen Virol 36(1): 59-72. (PMID: 886304)
[2] Bylund, L., et al. (2004). Cytogen Genome Res 106(1): 28-32. (PMID: 15218237)
[3] Dai, D., et al. (2015). Die Pharmazie-Int J Pharm Sci 70(1): 33-37. (PMID: 25975096)
[4] Lin, Y.-C., et al. (2014). Nature Comm 5. (PMID: 25182477)
[5] Shanle, E. K. and W. Xu (2010). Chem Res Toxicol 24(1): 6-19. (PMID: 21053929)
[6] Papoutsi, Z., et al. (2009). J Molecul Endocrin 43(2): 65-72. (PMID: 19376833)
[7] Cowley, S. M., et al. (1997). J Biol Chem 272(32): 19858-19862. (PMID: 9242648)
[8] Powell, E. and W. Xu (2008). PNAS 105(48): 19012-19017. (PMID: 19022902)
[9] Monroe, D. G., et al. (2005). Mol Endocrinol 19(6): 1555-1568. (PMID: 15802376)
[10] Li, X., et al. (2004). Mol Cell Biol 24(17): 7681-7694. (PMID: 15314175)
[11] Kraichely, D. M., et al. (2000). Endocrinology 141(10): 3534-3545. (PMID: 11014206)
[12] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

70.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
DBD, DNA Binding Domain
DMSO, Dimethyl Sulfoxide
EDC, Endocrine Disrupting Compounds
ER, Estrogen Receptor
E2, Estradiol
HEK, Human Embryonic Kidney
LBD, Ligand Binding Domain
NHR, Nuclear Hormone Receptors
OT, Odyssey Thera
PCA, Protein-Fragment Complementation
YFP, Yellow Fluorescent Protein

70.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

26 May 2016

Date of Revisions:

25 November 2016

Author of Revisions:

EPA NCCT

71. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:

Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;

Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation

Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;

Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

72. Supporting Information (existing annotations):

OT_ERa_EREGFP_0120

Assay Name: Odyssey Thera HeLa cell-based high content GFP:Prolactin 2-hour assay to monitor active Estrogen Receptor (ER) transcriptional units

73. Assay Descriptions

73.1. Overview

Assay Summary:

The Odyssey Thera estrogen receptor alpha (ERα) GFP estrogen response element (ERE) assay used human cervical adenocarcinoma (HeLa) cells stably expressing both full-length, fluorescently-tagged human ERα transcription factor and multiple tandem prolactin promotor sequences to screen a diverse chemical library for potential xenoestrogenic activity. Sensitive microscopic visualization of prolactin promoter occupancy by a GFP-tagged ERα was capable of measuring agonist- and antagonist- ERα-ligand binding following a 2-hour incubation of test compounds with cells in a 384-well plate.

73.2. Assay Definition

Assay Throughput:

HeLa cells are seeded into 384-well microtiter plates and allowed to adhere for 24 hours prior to treatment with test compounds or controls for 2 hours.

Experimental System:

GFP_ERα:PRL-HeLa cells are cervical adenocarcinoma epithelial cells, isolated from a 31 year old African-American female in February, 1951 [1], which constitutively express fluorescently-tagged full length human ERα and multiple integrated prolactin promotor sequences. The stable ER:PRL-HeLa cell line was developed by the Mancini lab at Baylor College of Medicine [2].

Xenobiotic Biotransformation Potential:

Constitutive expression of CYP1A1 and CYP1B1 mRNA; CYP1A2 expression was examined but not detected in HeLa cells [3, 4]. Expression of tumor-suppressing p53 and pRB proteins has been reported to be low [5].

Basic Procedure:

The stable ERα:PRL-HeLa line is seeded into optical quality 384-well poly-D-lysine coated plates in phenol red-free medium with 10% dextran-treated FBS and allowed to adhere for 24 hours prior to treatment with test compounds or controls for 2 hours. Cells are fixed in 4% formaldehyde and stained with Draq5 (BioStatus) to identify cells prior to signal detection. All assay endpoints are quantified using high content image analysis algorithms.

Proprietary Elements:

This assay is not proprietary.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote estrogen receptor mediated DNA transcription, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource-intensive toxicity studies.

73.3. Assay References

Assay Source Contact Information:

Odyssey Thera Inc.
4550 Norris Canyon Road, Suite 140
San Ramon, CA 94583
Tel: (925) 242-5000

Assay Publication Year:

2014

Assay Publication:

Stossi, F., Bolt, M. J., Ashcroft, F. J., Lamerdin, J. E., Melnick, J. S., Powell, R. T., Dandekar, R. D., Mancini, M. G., Walker, C. L., Westwick, J. K., & Mancini, M. A. (2014). "Defining estrogenic mechanisms of bisphenol A analogs through high throughput microscopy-based contextual assays". Chem Biol 21(6), 743-753. (PMID: 24856822)

Method Updates / Confirmatory Studies:

74. Assay Component Descriptions

Assay Objectives:

This Odyssey Thera estrogen receptor α green fluorescent protein (GFP) estrogen response element (ERE) assay was developed to measure transcriptional changes induced by ligand-binding as detected in a cervical adenocarcinoma cell line stably expressing both full-length human ERα and multiple estrogen responsive prolactin promoter sequences. ERα interacts with estrogenic ligands and following 2-hour incubation of test compound with cells in a 384-well plate, xenoestrogenic activation of a microscopically visible reporter gene is detected as an increase in mean signal relative to baseline activity (DMSO control) using a 10-point concentration-response assay format.

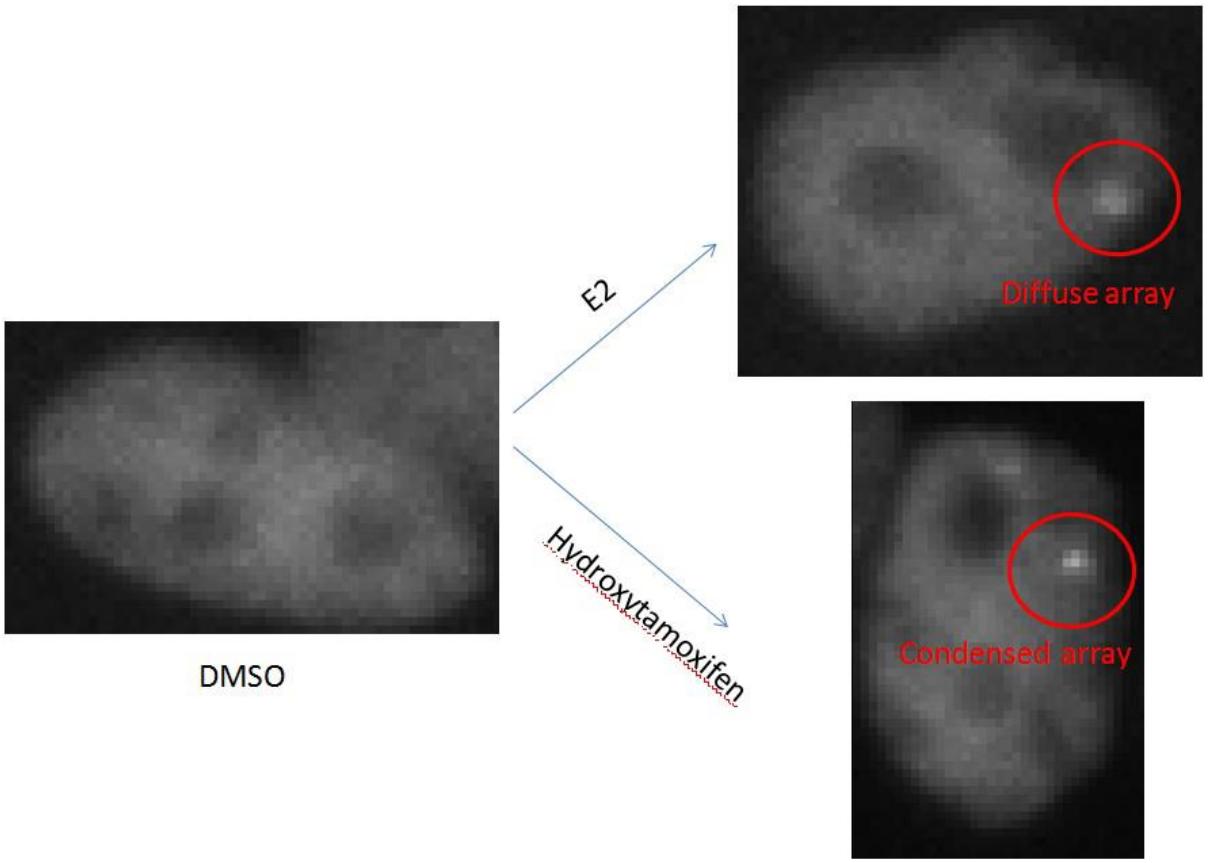

Figure 1. Single-cell views of the ER:PRL-HeLa cell line showing GFP-ERα accumulation on ERE arrays in response to vehicle (DMSO), agonist (E2) or antagonist (4-hydroxytamoxifen). Images were taken on an InCell 2000 with a 20X 0.75NA objective, provided curtesy of Odyssey Thera, Inc.

The ER:PRL-HeLa line constitutively expresses physiologically-relevant levels of fluorescently-tagged, full-length human ERα, and contains multi-copy genomic insertions of the prolactin promoter containg estrogen receptor response elements. When stimulated by agonists, tagged ERα accumulates on the prolactin array in an open (transcriptionally-active) binding mode, leading to a bright, micron-sized spot while antagonist-treated cells lead to tagged-ERα binding in its closed (transcriptionally-repressive) binding-mode, leading to a condensed array that appears as a sub-micron-sized point.

Scientific Principles:

Endocrine disrupting compounds bind to nuclear hormone receptors, leading to a diverse array of transcriptional and signaling pathway alterations. These cell- and tissue-type specific changes affect many aspects of human physiology, including those involved in inflammation, neonatal development and oncogenesis. Among the human NRs, the estrogen receptor family is particularly susceptible to perturbation by EDCs because of the ER LBD's proclivity to bind a disparate set of small hydrophobic molecules commonly found in nature and in man-made materials [6]. Cell-based and in vivo experiments suggest that the ERα isoform is the primary mediator of estrogenic effects by EDCs.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with estrogen receptor mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that estrogen receptor agonism is the MIE leading to reproductive dysfunction in oviparous vertebrates, and there is some evidence that estrogen receptor activation is the MIE for putative adverse outcome pathways leading to reduced survival due to renal failure and leading to skewed sex ratios due to altered sexual differentiation in males. Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive *in vivo* studies to further investigate the involvement of ER agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Ashcroft, F. J., Newberg, J. Y., Jones, E. D., Mikic, I., & Mancini, M. A. (2011). "High content imaging-based assay to classify estrogen receptor-α ligands based on defined mechanistic outcomes". Gene 477(1-2), 42-52. (PMID: 21256200)

Sharp, Z. D., Mancini, M. G., Hinojos, C. A., Dai, F., Berno, V., Szafran, A. T., et al. (2006). Estrogen-receptor-α exchange and chromatin dynamics are ligand-and domain-dependent. Journal of cell science, 119(19), 4101-4116. (PMID: 16968748)

Assay Quality Statistics:

Neutral control well median response value, by plate:	8.75
Neutral control median absolute deviation, by plate:	1.48
Positive control well median response value, by plate:	321.5
Positive control well median absolute deviation, by plate:	16.49
Z' (median across all plates, using positive control wells):	0.81
SSMD (median across all plates, using positive control wells):	19
Signal-to-noise (median across all plates, using positive control wells):	129.09
Signal-to-background (median across all plates, using positive control wells):	35.68
CV (median across all plates):	0.17

75. Assay Endpoint Descriptions

75.1. Data Interpretation

Biological Response:

Ligand binding of estrogen receptor α and xenoestrogenic effects on transcriptional regulation of a fluorescent reporter gene.

Analytical Elements:

OT_ERa_GFPERaERE_0120 readout data was analyzed in the positive (gain of signal) fitting direction using percent activity 4-Hydroxytamoxifen as positive control (100% activity) over DMSO controls. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Estrogen receptor transactivation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ACEA_T47D_80hr_Positive
ATG_ERE_CIS_up
ATG_ERa_TRANS_up
ATG_ERb_TRANS2_up
NVS_NR_bER
NVS_NR_hER
NVS_NR_mERa
OT_ER_ERaERa_0480
OT_ER_ERaERa_1440
OT_ER_ERaERb_0480
OT_ER_ERaERb_1440
OT_ER_ERbERb_0480
OT_ER_ERbERb_1440
OT_ERa_ERELUC_AG_1440
OT_ERa_ERELUC_ANT_1440
OT_ERa_EREGFP_0480
OT_ERb_ERELUC_ANT_1440
Tox21_ERa_BLA_Agonist_ratio
Tox21_ERa_BLA_Antagonist_ratio
Tox21_ERa_LUC_BG1_Agonist
Tox21_ERa_LUC_BG1_Antagonist

75.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations: 6
Target (nominal) number of replicates: 3
Standard minimum concentration tested: 0.3 µM
Standard maximum concentration tested: 100 µM
Baseline median absolute deviation for the assay - based on the response values at the 2 lowest tested concentrations (bmad): 0.488
The response cutoff used to derive the hit calls (e.g., 5*bmad, 10*bmad): 20

Reference Chemicals / Predictive Capacity:

CASRN	Chemical Name	*In Vitro* Activity	*In Vivo* Activity	Activity in Assay
57-91-0	17alpha-Estradiol	Moderate	Active	Yes
57-63-6	17alpha-Ethinylestradiol	Strong	Active	Yes
50-28-2	17beta-Estradiol	Strong	Active	Yes
58-18-4	17-Methyltestosterone	Very Weak	Active	Yes
131-55-5	2,2',4,4'-Tetrahydroxybenzophenone	NA	Active	Yes
131-56-6	2,4-Dihydroxybenzophenone	NA	Active	No
5153-25-3	2-Ethylhexylparaben	NA	Active	Yes
140-66-9	4-(1,1,3,3-Tetramethylbutyl)phenol	Moderate	Active	No
80-46-6	4-(2-Methylbutan-2-yl)phenol	NA	Active	Yes
80-09-1	4,4'-Sulfonyldiphenol	NA	Active	Yes
599-64-4	4-Cumylphenol	Weak	Active	Yes
104-43-8	4-Dodecylphenol	NA	Active	No
99-96-7	4-Hydroxybenzoic acid	NA	Inactive	Yes
104-40-5	4-Nonylphenol	Very Weak	Active	Yes
98-54-4	4-tert-Butylphenol	NA	Active	Yes
521-18-6	5alpha-Dihydrotestosterone	Weak	Active	Yes
61-82-5	Amitrole	NA	Inactive	Yes
520-36-5	Apigenin	Very Weak	NA	No
85-68-7	Benzyl butyl phthalate	Very Weak	NA	No
103-23-1	Bis(2-ethylhexyl)hexanedioate	NA	Inactive	No
80-05-7	Bisphenol A	Weak	Active	Yes
1478-61-1	Bisphenol AF	NA	Active	No
77-40-7	Bisphenol B	Weak	Active	Yes
94-26-8	Butylparaben	NA	Active	No
480-40-0	Chrysin	Very Weak	NA	No
50-22-6	Corticosterone	Inactive	NA	Yes
486-66-8	Daidzein	Weak	NA	No
117-81-7	Di(2-ethylhexyl) phthalate	Very Weak	Inactive	Yes
84-74-2	Dibutyl phthalate	Very Weak	Inactive	Yes
115-32-2	Dicofol	Very Weak	NA	Yes
84-61-7	Dicyclohexyl phthalate	NA	Inactive	Yes
84-66-2	Diethyl phthalate	NA	Inactive	Yes
56-53-1	Diethylstilbestrol	Strong	Active	Yes
84-75-3	Dihexyl phthalate	NA	Inactive	No
474-86-2	Equilin	NA	Active	No
50-27-1	Estriol	NA	Active	No
53-16-7	Estrone	Moderate	Active	Yes
120-47-8	Ethylparaben	Very Weak	NA	No

60168-88-9	Fenarimol	Very Weak	NA	Yes
51630-58-1	Fenvalerate	NA	Inactive	Yes
446-72-0	Genistein	Weak	Active	Yes
52-86-8	Haloperidol	Inactive	NA	No
520-18-3	Kaempferol	Very Weak	Inactive	Yes
143-50-0	Kepone	Weak	NA	Yes
65277-42-1	Ketoconazole	Inactive	NA	Yes
330-55-2	Linuron	Inactive	NA	No
84-16-2	meso-Hexestrol	Strong	NA	No
72-33-3	Mestranol	NA	Active	Yes
72-43-5	Methoxychlor	Very Weak	Active	Yes
68-22-4	Norethindrone	NA	Active	No
789-02-6	o,p'-DDT	Weak	Active	Yes
556-67-2	Octamethylcyclotetrasiloxane	NA	Active	Yes
72-55-9	p,p'-DDE	Very Weak	Weak	No
87-86-5	Pentachlorophenol	NA	Inactive	No
57-30-7	Phenobarbital sodium	Inactive	NA	No
32809-16-8	Procymidone	Inactive	NA	Yes
50-55-5	Reserpine	Inactive	NA	Yes
52-01-7	Spironolactone	Inactive	NA	No
17924-92-4	Zearalenone	NA	Active	Yes

In Vitro Activity	ToxCast Active	ToxCast Inactive
Active	21	8
Inactive	2	6

In Vivo Activity	ToxCast Active	ToxCast Inactive
Active	26	4
Inactive	3	8

In Vitro Sensitivity = 72.4%
In Vitro Specificity = 75.0%
Balanced Accuracy = 73.7%

In Vivo Sensitivity = 86.7%
In Vivo Specificity = 72.7%
Balanced Accuracy = 79.7%

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical

constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [7].

76. Assay Documentation

76.1. References

[1] Jones, H. W., et al. (1971). Obstetrics & Gynecology 38(6): 945-949. (PMID: 4942173)
[2] Nakajima, M., et al. (2003). Toxicol Lett 144(2): 247-256. (PMID: 12927368)
[3] Iwanari, M., et al. (2002). Arch Toxicol 76(5-6): 287-298. (PMID: 12107646)
[4] Scheffner, M., et al. (1991). PNAS 88(13): 5523-5527. (PMID: 1648218)
[5] Ashcroft, F. J., et al. (2011). Gene 477(1-2): 42-52. (PMID: 21256200)
[6] Shanle, E. K. and W. Xu (2010). Chem Res Toxicol 24(1): 6-19. (PMID: 21053929)
[7] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

76.2. Abbreviations and Definitions

4HT, 4-hydroxytamoxifen
AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathways
DMSO, Dimethyl Sulfoxide
E2, Estradiol
EDC, Endocrine Disrupting Compounds
ER, Estrogen Receptor
ERE, Estrogen Response Element
GFP, Green Fluorescent Protein
DBD, DNA Binding Domain
LBD, Ligand Binding Domain
MIE, Molecular Initiating Event
MTC, Maximum Tolerated Concentrations
NR, Nuclear Receptors
NHR, Nuclear Hormone Receptors
OT, Odyssey Thera
PBS, Phosphate Buffered Saline
TF, Transcription Factor

76.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

26 May 2016

Date of Revisions:

25 November 2016

Author of Revisions:

EPA NCCT

77. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:

Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;

Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation

Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;

Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

78. Supporting Information (existing annotations):

OT_ERa_EREGFP_0480

Assay Name: Odyssey Thera HeLa cell-based high content GFP:Prolactin 8-hour assay to monitor active Estrogen Receptor (ER) transcriptional units

79. Assay Descriptions

79.1. Overview

Assay Summary:

The Odyssey Thera estrogen receptor alpha (ERα) GFP estrogen response element (ERE) assay used human cervical adenocarcinoma (HeLa) cells stably expressing both full-length, fluorescently-tagged human ERα transcription factor and multiple tandem prolactin promotor sequences to screen a diverse chemical library for potential xenoestrogenic activity. Sensitive microscopic visualization of prolactin promoter occupancy by a GFP-tagged ERα was capable of measuring agonist- and antagonist- ERα-ligand binding following a 8-hour incubation of test compounds with cells in a 384-well plate.

79.2. Assay Definition

Assay Throughput:

HeLa cells are seeded into 384-well microtiter plates and allowed to adhere for 24 hours prior to treatment with test compounds or controls for 8 hours.

Experimental System:

GFP_ERα:PRL-HeLa cells are cervical adenocarcinoma epithelial cells, isolated from a 31 year old African-American female in February, 1951 [1], which constitutively express fluorescently-tagged full length human ERα and multiple integrated prolactin promotor sequences.

Xenobiotic Biotransformation Potential:

Constitutive expression of CYP1A1 and CYP1B1 mRNA; CYP1A2 expression was examined but not detected in HeLa cells [2, 3]. Expression of tumor-suppressing p53 and pRB proteins has been reported to be low [4].

Basic Procedure:

The stable ERα:PRL-HeLa line is seeded into optical quality 384-well poly-D-lysine coated plates in phenol red-free medium with 10% dextran-treated FBS and allowed to adhere for 24 hours prior to treatment with test compounds or controls for 8 hours. Cells are fixed in 4% formaldehyde and stained with Draq5 (BioStatus) to identify cells prior to signal detection. All assay endpoints are quantified using high content image analysis algorithms.

Proprietary Elements:

This assay is not proprietary. ER:PRL-HeLa cell line was developed and provided to OT by the Mancini lab at Baylor [5].

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote estrogen receptor mediated DNA transcription, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies

79.3. Assay References

Assay Source Contact Information:

Odyssey Thera Inc.
4550 Norris Canyon Road, Suite 140
San Ramon, CA 94583
Tel: (925) 242-5000

Assay Publication Year:

2014

Assay Publication:

Stossi, F., Bolt, M. J., Ashcroft, F. J., Lamerdin, J. E., Melnick, J. S., Powell, R. T., Dandekar, R. D., Mancini, M. G., Walker, C. L., Westwick, J. K., & Mancini, M. A. (2014). "Defining estrogenic mechanisms of bisphenol A analogs through high throughput microscopy-based contextual assays". Chem Biol 21(6), 743-753. (PMID: 24856822)

Method Updates / Confirmatory Studies:

80. Assay Component Descriptions

Assay Objectives:

This Odyssey Thera estrogen receptor α green fluorescent protein (GFP) estrogen response element (ERE) assay was developed to measure long-term transcriptional changes induced by ligand-binding as detected in a cervical adenocarcinoma cell line stably expressing both full-length human ERα and multiple estrogen responsive prolactin promoter sequences. ERα interacts with estrogenic ligands and following 2-hour incubation of test compound with cells in a 384-well plate, xenoestrogenic activation of a microscopically visible reporter gene is detected as an increase in mean signal relative to baseline activity (DMSO control) using a 10-point concentration-response assay format.

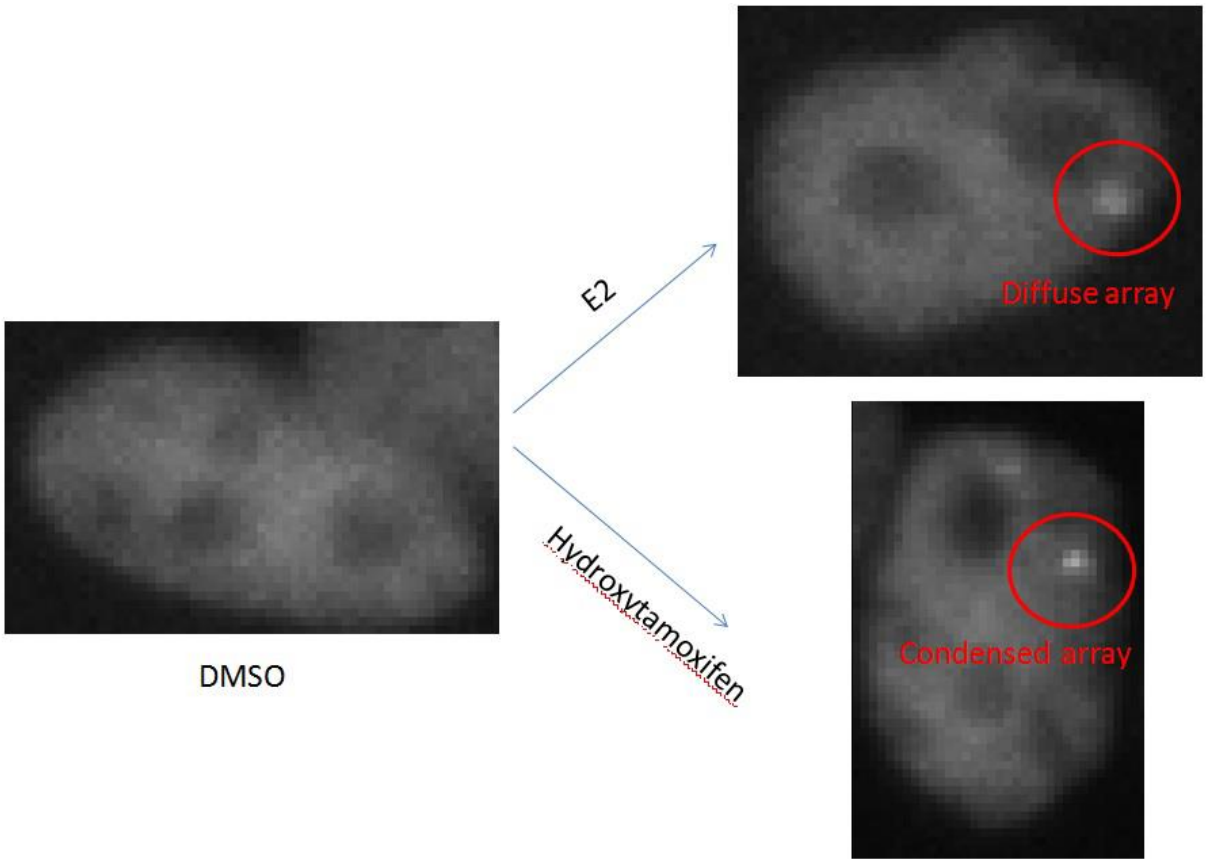

Figure 2. Single-cell views of the ER:PRL-HeLa cell line showing GFP-ERα accumulation on ERE arrays in response to vehicle (DMSO), agonist (E2) or antagonist (4-hydroxytamoxifen). Images were taken on an InCell 2000 with a 20X 0.75NA objective, provided curtesy of Odyssey Thera, Inc.

ER:PRL-HeLa line constitutively expresses physiologically-relevant levels of fluorescently-tagged, full-length human ERα, and contains multi-copy genomic insertions of the prolactin promoter containg estrogen receptor response elements. When stimulated by agonists, tagged ERα accumulates on the prolactin array in an open (transcriptionally-active) binding mode, leading to a bright, micron-sized spot while antagonist-treated cells lead to tagged-ERα binding in its closed (transcriptionally-repressive) binding-mode, leading to a condensed array that appears as a sub-micron-sized point).

Scientific Principles:

Endocrine disrupting compounds bind to nuclear hormone receptors, leading to a diverse array of transcriptional and signaling pathway alterations. These cell- and tissue-type specific changes affect many aspects of human physiology, including those involved in inflammation, neonatal development and oncogenesis. Among the human NHRs, the estrogen receptor family is particularly susceptible to perturbation by EDCs because of the ER LBD's proclivity to bind a disparate set of small hydrophobic molecules commonly found in nature and in man-made materials [6]. Cell-based and in vivo experiments suggest that the ERα isoform is the primary mediator of estrogenic effects by EDCs.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with estrogen receptor mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that estrogen receptor agonism is the MIE leading to reproductive dysfunction in oviparous vertebrates, and there is some evidence that estrogen receptor activation is the MIE for putative adverse outcome pathways leading to reduced survival due to renal failure and leading to skewed sex ratios due to altered sexual differentiation in males. Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive *in vivo* studies to further investigate the involvement of ER agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Ashcroft, F. J., Newberg, J. Y., Jones, E. D., Mikic, I., & Mancini, M. A. (2011). "High content imaging-based assay to classify estrogen receptor-α ligands based on defined mechanistic outcomes". Gene 477(1-2), 42-52. (PMID: 21256200)

Sharp, Z. D., Mancini, M. G., Hinojos, C. A., Dai, F., Berno, V., Szafran, A. T., et al. (2006). Estrogen-receptor-α exchange and chromatin dynamics are ligand-and domain-dependent. Journal of cell science, 119(19), 4101-4116. (PMID: 16968748)

Assay Quality Statistics:

Neutral control well median response value, by plate:	6.5
Neutral control median absolute deviation, by plate:	0.741
Positive control well median response value, by plate:	351.25
Positive control well median absolute deviation, by plate:	16.68
Z' (median across all plates, using positive control wells):	0.84
SSMD (median across all plates, using positive control wells):	20
Signal-to-noise (median across all plates, using positive control wells):	410.09
Signal-to-background (median across all plates, using positive control wells):	52.13
CV (median across all plates):	0.15

81. Assay Endpoint Descriptions

81.1. Data Interpretation

Biological Response:

Ligand binding of estrogen receptor α and xenoestrogenic effects on transcriptional regulation of a fluorescent reporter gene.

Analytical Elements:

OT_ERa_GFPERaERE_0480 readout data was analyzed in the positive (gain of signal) fitting direction using percent activity 4-Hydroxytamoxifen as positive control (100% activity) over DMSO controls. All statistical analyses were conducted using R programming language, employing tcpl package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Estrogen receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ACEA_T47D_80hr_Positive
ATG_ERE_CIS_up
ATG_ERa_TRANS_up
ATG_ERb_TRANS2_up
NVS_NR_bER
NVS_NR_hER
NVS_NR_mERa
OT_ER_ERaERa_0480
OT_ER_ERaERa_1440
OT_ER_ERaERb_0480
OT_ER_ERaERb_1440
OT_ER_ERbERb_0480
OT_ER_ERbERb_1440
OT_ERa_ERELUC_AG_1440
OT_ERa_ERELUC_ANT_1440
OT_ERa_EREGFP_0120
OT_ERb_ERELUC_ANT_1440
Tox21_ERa_BLA_Agonist_ratio
Tox21_ERa_BLA_Antagonist_ratio
Tox21_ERa_LUC_BG1_Agonist
Tox21_ERa_LUC_BG1_Antagonist

81.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations: 6
Target (nominal) number of replicates: 3
Standard minimum concentration tested: 0.3 µM
Standard maximum concentration tested: 100 µM
Baseline median absolute deviation for the assay - based on the response values at the 2 lowest tested concentrations (bmad): 0.227
The response cutoff used to derive the hit calls (e.g., 5*bmad, 10*bmad): 20

Reference Chemicals / Predictive Capacity:

CASRN	Chemical Name	*In vitro* Activity	*In Vivo* Activity	Activity in Assay
57-91-0	17alpha-Estradiol	Moderate	Active	Yes
57-63-6	17alpha-Ethinylestradiol	Strong	Active	Yes
50-28-2	17beta-Estradiol	Strong	Active	Yes
58-18-4	17-Methyltestosterone	Very Weak	Active	Yes
131-55-5	2,2',4,4'-Tetrahydroxybenzophenone	NA	Active	Yes
131-56-6	2,4-Dihydroxybenzophenone	NA	Active	No
5153-25-3	2-Ethylhexylparaben	NA	Active	Yes
140-66-9	4-(1,1,3,3-Tetramethylbutyl)phenol	Moderate	Active	No
80-46-6	4-(2-Methylbutan-2-yl)phenol	NA	Active	Yes
80-09-1	4,4'-Sulfonyldiphenol	NA	Active	Yes
599-64-4	4-Cumylphenol	Weak	Active	Yes
104-43-8	4-Dodecylphenol	NA	Active	No
99-96-7	4-Hydroxybenzoic acid	NA	Inactive	Yes
104-40-5	4-Nonylphenol	Very Weak	Active	Yes
98-54-4	4-tert-Butylphenol	NA	Active	Yes
521-18-6	5alpha-Dihydrotestosterone	Weak	Active	Yes
61-82-5	Amitrole	NA	Inactive	Yes
520-36-5	Apigenin	Very Weak	NA	No
85-68-7	Benzyl butyl phthalate	Very Weak	NA	No
103-23-1	Bis(2-ethylhexyl)hexanedioate	NA	Inactive	No
80-05-7	Bisphenol A	Weak	Active	Yes
1478-61-1	Bisphenol AF	NA	Active	No
77-40-7	Bisphenol B	Weak	Active	No
94-26-8	Butylparaben	NA	Active	No
480-40-0	Chrysin	Very Weak	NA	No
50-22-6	Corticosterone	Inactive	NA	Yes
486-66-8	Daidzein	Weak	NA	No
117-81-7	Di(2-ethylhexyl) phthalate	Very Weak	Inactive	Yes
84-74-2	Dibutyl phthalate	Very Weak	Inactive	Yes
115-32-2	Dicofol	Very Weak	NA	Yes
84-61-7	Dicyclohexyl phthalate	NA	Inactive	Yes
84-66-2	Diethyl phthalate	NA	Inactive	Yes
56-53-1	Diethylstilbestrol	Strong	Active	Yes
84-75-3	Dihexyl phthalate	NA	Inactive	No
474-86-2	Equilin	NA	Active	Yes
50-27-1	Estriol	NA	Active	No
53-16-7	Estrone	Moderate	Active	Yes
120-47-8	Ethylparaben	Very Weak	NA	No

60168-88-9	Fenarimol	Very Weak	NA	No
51630-58-1	Fenvalerate	NA	Inactive	Yes
446-72-0	Genistein	Weak	Active	Yes
52-86-8	Haloperidol	Inactive	NA	No
520-18-3	Kaempferol	Very Weak	Inactive	Yes
143-50-0	Kepone	Weak	NA	Yes
65277-42-1	Ketoconazole	Inactive	NA	Yes
330-55-2	Linuron	Inactive	NA	No
84-16-2	meso-Hexestrol	Strong	NA	No
72-33-3	Mestranol	NA	Active	Yes
72-43-5	Methoxychlor	Very Weak	Active	Yes
68-22-4	Norethindrone	NA	Active	No
789-02-6	o,p'-DDT	Weak	Active	No
556-67-2	Octamethylcyclotetrasiloxane	NA	Active	Yes
72-55-9	p,p'-DDE	Very Weak	Weak	No
87-86-5	Pentachlorophenol	NA	Inactive	No
57-30-7	Phenobarbital sodium	Inactive	NA	No
32809-16-8	Procymidone	Inactive	NA	Yes
50-55-5	Reserpine	Inactive	NA	Yes
52-01-7	Spironolactone	Inactive	NA	No
17924-92-4	Zearalenone	NA	Active	Yes

In Vitro Activity	ToxCast Active	ToxCast Inactive
Active	20	9
Inactive	2	6

In Vivo Activity	ToxCast Active	ToxCast Inactive
Active	26	4
Inactive	1	10

In Vitro Sensitivity = 69.0%
In Vitro Specificity = 75.0%
Balanced Accuracy = 72.0%

In Vivo Sensitivity = 86.7%
In Vivo Specificity = 90.9%
Balanced Accuracy = 88.8%

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical

constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [7].

82. Assay Documentation

82.1. References

[1] Jones, H. W., et al. (1971). Obstetrics & Gynecology 38(6): 945-949. (PMID: 4942173)
[2] Nakajima, M., et al. (2003). Toxicol Lett 144(2): 247-256. (PMID: 12927368)
[3] Iwanari, M., et al. (2002). Arch Toxicol 76(5-6): 287-298. (PMID: 12107646)
[4] Scheffner, M., et al. (1991). PNAS 88(13): 5523-5527. (PMID: 1648218)
[5] Ashcroft, F. J., et al. (2011). Gene 477(1-2): 42-52. (PMID: 21256200)
[6] Shanle, E. K. and W. Xu (2010). Chem Res Toxicol 24(1): 6-19. (PMID: 21053929)
[7] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

82.2. Abbreviations and Definitions

4HT, 4-hydroxytamoxifen
AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathways
DMSO, Dimethyl Sulfoxide
E2, Estradiol
EDC, Endocrine Disrupting Compounds
ER, Estrogen Receptor
ERE, Estrogen Response Element
GFP, Green Fluorescent Protein
DBD, DNA Binding Domain
LBD, Ligand Binding Domain
MIE, Molecular Initiating Event
MTC, Maximum Tolerated Concentrations
NR, Nuclear Receptors
NHR, Nuclear Hormone Receptors
OT, Odyssey Thera
PBS, Phosphate Buffered Saline
TF, Transcription Factor

82.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

2016

Date of Revisions:

2016

Author of Revisions:

EPA NCCT

83. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:

Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;

Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation

Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;

Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

84. Supporting Information (existing annotations):

Tox21_ERa_BLA_Agonist_ratio

Assay Name: Tox21 Beta-Lactamase-HEK293T Cell-Based qHTS Assay to Identify Small Molecule Agonists of the Estrogen Receptor Alpha (ER-alpha) Signaling Pathway

85. Assay Descriptions

85.1. Overview

Assay Summary:

Estrogen receptor (ER), a nuclear hormone receptor, plays an important role in development, metabolic homeostasis and reproduction. Endocrine disrupting chemicals (EDCs) and their interactions with steroid hormone receptors like ER causes disruption of normal endocrine function. Therefore, it is important to understand the effect of environmental chemicals on the ER signaling pathway. To identify the compounds that inhibit ER signaling, an ER-alpha-UAS-bla GripTite™ cell line (Invitrogen, Carlsbad, CA, USA) containing a beta-lactamase reporter gene under the control of an Upstream Activator Sequence (UAS) responsive to a GAL4-ER fusion protein stably integrated into HEK293 cells was used to screen the Tox21 chemical library. This experimental system constitutively co-expresses the fusion protein, thel human estrogen receptor alpha (ERα) ligand-binding domain coupled to GAL4 DNA-binding domain which, when activated by xenoestrogenic compounds, stimulates β-lactamase reporter gene expression.

85.2. Assay Definition

Assay Throughput:

Stably transfected HEK293T cells are aliquoted into 1536-well microtiter plates, incubated with test compounds for 16 hours, and reporter gene substrate added prior to monitoring fluorescence emission resulting from substrate cleavage by ERα-stimulated reporter gene expression.

Experimental System:

GeneBLAzer® ER alpha-UAS-bla GripTite™ cells contain the ligand-binding domain of the human estrogen receptor alpha (ERα) fused to the DNA-binding domain of GAL4 stably integrated into the cell line, commonly called a mammalian one-hybrid assay. These cells stably express a β-lactamase reporter gene (*bla*) under the transcriptional control of an upstream activator sequence (UAS). When an agonist binds to the LBD of the GAL4-ERα fusion protein, the protein binds to the UAS, resulting in expression of β-lactamase. The HEK-293 cell line is a human embryonic kidney cell line (of unknown parentage) transformed with sheared adenovirus 5 DNA by Frank Graham in 1973 [1]. The transformation incorporated approximately 4.5 kilobases from the viral genome into human chromosome 19 of the HEK cells, and subsequent cytogenetic characterization established that the 293 line is pseudotriploid [2]. HEK293 cells are popular for their ease of growth and transfection cells, and are frequently used to produce exogenous proteins or viruses for pharmaceutical and biomedical research purposes.

Xenobiotic Biotransformation Potential:

The intrinsic production of Phase I/II enzymes has not been well characterized in this cell line and metabolic activation/detoxification of test compounds is potentially limited.

Basic Procedure:

MATERIALS and INSTRUMENTS:

Supplies/Medium/Reagent	Manufacturer	Vender/Catalog Number
Phenol red-free DMEM	Invitrogen	Invitrogen/21063
DMEM	Invitrogen	Invitrogen/11965
Dialyzed FBS	Invitrogen	Invitrogen/26400
Charcoal stripped FBS	Invitrogen	Invitrogen/12676
NEAA	Invitrogen	Invitrogen/11140
Sodium pyruvate	Invitrogen	Invitrogen/11360
Penicillin and Streptomycin	Invitrogen	Invitrogen/15140
Hygromycin B	Invitrogen	Invitrogen/10687
Zeocin	Invitrogen	Invitrogen/R25001

Black-clear bottom 1536 well plates	Greiner	Greiner/789092F
LiveBLAzer B/G FRET substrate	Invitrogen	Invitrogen/K1028
Recovery Cell Culture Freezing Medium	Invitrogen	Invitrogen/12648
0.05% Trypsin-EDTA	Invitrogen	Invitrogen/25300
Envision Plate Reader	Perkin Elmer	Perkin Elmer
BioRAPTR FRD dispenser	Beckman Coulter	Beckman Coulter
Multidrop COMBI	Thermo Electron Corporation	Thermo Electron Corporation
Beta-Estradiol (Agonist control compound)	Sigma	Sigma/E8875

PROCEDURE:

1. Cell handling:

1.1. Media Required:

Component	Growth Medium	Assay Medium	Thaw Medium	Freezing Medium
Phenol red-free DMEM	-	98%	-	-
DMEM	90%	-	90%	-
Dialyzed FBS	10%	-	10%	-
Charcoal stripped FBS	-	2%	-	-
NEAA	0.1mM	0.1mM	0.1mM	-
Sodium pyruvate	1mM	1mM	1mM	-
Penicillin and Streptomycin	100U/ml-100µg/ml	100U/ml-100µg/ml	100U/ml-100µg/ml	-
Hygromycin B	80µg/ml	-	-	-
Zeocin	100µg/ml	-	-	-
Recovery Cell Culture Freezing Medium	-	-	-	100%

1.2. Thawing method

1.2.1 1ml frozen cells of ERalpha-bla were taken in pre-warmed 10ml of thaw medium for centrifuging.

1.2.2 2-3ml of the thaw medium is taken to resuspend the pellet

1.2.3 The cells were seeded in T-75 flask at 2 million cells

1.3. Propagation method

1.3.1 The cells are detached using 0.05% Trypsin

1.3.2 Cells are further passaged at a density of 4-5 million cells per T-225 flask

2. Assay Protocol

2.1 Rinse the cells twice with DPBS and detach them using 0.05% Trypsin and centrifuge

2.2 Resuspend the pellet with assay buffer

2.3 Plate the cells in black-clear bottom 1536 well plate at 5000/well/5µL through 8 tip plate dispenser (Multi drop)

2.4 Incubate at 37°C for 5hrs

2.5 Transfer 23nL of the compounds from the library collection and positive control through Pintool

2.6 Incubate at 37°C for 18hrs

2.7 Add 1µL of CCF4 dye using a single tip of a plate dispenser (Bioraptr)

2.8 Incubate at room temperature for 2hrs

2.9 Read the fluorescence intensity through Envision plate reader

ER-alpha-bla cells were cultured in assay medium containing 2% charcoal stripped FBS overnight in the culture flasks before the assay was performed. The cells were dispensed at 5,000 cells/6 uL of assay medium per well into black wall/clear-bottom 1536-well plates using a Multidrop Combi (ThermoFisher Scientific, Waltham, MA) dispenser. After the assay plates were incubated at a 37 °C and 5% CO2 for 4 hr, 23 nL of compounds dissolved in DMSO, positive controls or DMSO only was transferred to the assay plate by a Pintool station (Kalypsys, San Diego, CA). The plates were incubated at 37C and 5% CO2 for 18 hr. Then 1 uL of LiveBLAzer™ B/G FRET substrate was added using a Flying Reagent Dispenser (Aurora Discovery, San Diego, CA). After the plates were incubated at room temperature for 2 hours, fluorescence intensity was measured by an Envision plate reader (PerkinElmer, Shelton, CT).

Proprietary Elements:

This assay is not proprietary; The Tox21 qHTS robotic platform has a 1536-well per run capacity and is capable of fully-automated (hands-free) assay execution (liquid dispensing and aspiration, plate centrifugation and incubation, etc.) and signal recording (plate readout) [3]. GeneBLAzer® System is commercially available through Invitrogen.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote estrogen receptor mediated gene expression, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

85.3. Assay References

Assay Source Contact Information:

U.S. Tox21 Program
National Center for Advancing Translational Sciences [NCATS]
NIH Chemical Genomics Center [NCGC]
U.S. Environmental Protection Agency [EPA]
National Institutes of Environmental Health Sciences [NIEHS]
National Toxicology Program [NTP]
U.S. Food and Drug Administration [FDA]

Assay Contact: Ruili Huang
NIH Chemical Genomics Center, National Center for Advancing Translational Sciences, National Institutes of Health, Rockville, MD 20850, USA

Assay Publication Year:

2011

Assay Publication:

Huang, R., Xia, M., Cho, M. H., Sakamuru, S., Shinn, P., Houck, K. A., Dix, D. J., Judson, R. S., Witt, K. L., Kavlock, R. J., Tice, R. R., & Austin, C. P. (2011). "Chemical genomics profiling of environmental chemical modulation of human nuclear receptors". Environ Health Perspect 119(8), 1142-1148. (PMID: 21543282)

Huang, R., Sakamuru, S., Martin, M. T., Reif, D. M., Judson, R. S., Houck, K. A., Casey, W., Hsieh, J. H., Shockley, K. R., Ceger, P., Fostel, J., Witt, K. L., Tong, W., Rotroff, D. M., Zhao, T., Shinn, P., Simeonov, A., Dix, D. J., Austin, C. P., Kavlock, R. J., Tice, R. R., & Xia, M. (2014). "Profiling of the Tox21 10K compound library for agonists and antagonists of the estrogen receptor alpha signaling pathway". Scientific Reports 4, 5664. (PMID: 25012808)

Huang, R., Xia, M., Sakamuru, S., Zhao, J., Shahane, S. A., Attene-Ramos, M., Zhao, T., Austin, C. P., & Simeonov, A. (2016). "Modelling the Tox21 10 K chemical profiles for in vivo toxicity prediction and mechanism characterization". Nature Communications 7, 10425. (PMID: 26811972)

Method Updates / Confirmatory Studies:

86. Assay Component Descriptions

Assay Objectives:

The Tox21 estrogen receptor-α β-lactamase agonist assay screened a library of diverse environmental compounds to probe for xenoestrogenic ligand-binding and potential to induce estrogen-dependent transcription, monitored through *bla* reporter gene signal activation using a mammalian one-hybrid GAL4 system. The assay is run in triplicate on 1536-well microplates. Following 18 hour incubation of cells with test compounds, a cell-permeable, FRET-based substrate, CCF4-AM, is introduced and incubated for 2 hours. Once in the cell, cytoplasmic esterases hydrolyze and trap the negatively charged CCF4 substrate in the cytosol where it can be cleaved by the *bla* reporter gene product. Activity is quantified by measuring the ratio of blue (product) to green (substrate) fluorescence. Fluorescence signals are monitored using an Envision plate reader and CellTiter-Glo assay reagent (Promega) is also incubated with test system for 30 minutes before readout to detect cell viability.

The test compounds were selected based on various criteria, e.g., exposure hazard, physicochemical properties, availability and affordability and each assay incorporated 88 chemical duplications (each derived from the same primary stock solution as a sample chemical) and 39 different reference chemicals with known ER agonistic/antagonistic activities. 10% of the chemicals tested were duplicated chemical structures sourced from separate venders or from different production lots to assess sample variability. Each compound was tested in a concentration-response format, using 15 concentrations ranging from 1.1 nM to 92 μM. Compound auto-fluorescence was monitored using auto-fluorescence assays run at interfering wavelengths to allow for filtering of artifacts.

Scientific Principles:

Estrogen receptor (ER), a nuclear hormone receptor, plays an important role in development, metabolic homeostasis and reproduction. Endocrine disrupting chemicals (EDCs) and their interactions with steroid hormone receptors like ER causes disruption of normal endocrine function. Therefore, it is important to understand the effect of environmental chemicals on the ER signaling pathway. To identify ER agonists, GeneBLAzer® ERα-UAS-bla GripTite™ cell line (Invitrogen) has been used to screen the Tox21 library of diverse environmental compounds. ERα-UAS-bla cell line expresses a partial ERα one-hybrid GAL4 system and is stably transfected with a β-lactamase reporter gene.

The Tox21 ERα bla assays are qHTS format assays which measured the ability of a chemical to interact with estrogen receptor alpha (ERα) by monitoring modulation of fluorescence reporter gene signals. This assay utilized a human embryonic kidney cell line (HEK293T) which expresses a partial ERα and a one-hybrid GAL4 system to quantify xenoestrogenic agonism.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with estrogen receptor alpha mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that estrogen receptor agonism is the Molecular Initiating Event (MIE) in an Adverse Outcome Pathway (AOP) leading to reproductive dysfunction in oviparous vertebrates, and there is some evidence that estrogen receptor activation is the MIE for putative AOPs leading

to reduced survival due to renal failure and leading to skewed sex ratios due to altered sexual differentiation in males (all AOPs currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive in vivo studies to further investigate the involvement of ER agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Inglese, J., Auld, D. S., Jadhav, A., Johnson, R. L., Simeonov, A., Yasgar, A., Zheng, W., & Austin, C. P. (2006). "Quantitative high-throughput screening: a titration-based approach that efficiently identifies biological activities in large chemical libraries". Proc Natl Acad Sci U S A 103(31), 11473-11478. (PMID: 16864780)

Xia, M., Huang, R., Sun, Y., Semenza, G. L., Aldred, S. F., Witt, K. L., Inglese, J., Tice, R. R., & Austin, C. P. (2009). "Identification of chemical compounds that induce HIF-1alpha activity". Toxicol Sci 112(1), 153-163. (PMID: 19502547)

Assay Quality Statistics:

Neutral control well median response value, by plate:	-0.04
Neutral control median absolute deviation, by plate:	1.12
Positive control well median response value, by plate:	99.18
Positive control well median absolute deviation, by plate:	20.81
Z' (median across all plates, using positive control wells):	0.33
SSMD (median across all plates, using positive control wells):	5
Signal-to-noise (median across all plates, using positive control wells):	87.86
Signal-to-background (median across all plates, using positive control wells):	-2485.43
CV (median across all plates):	-27.68

87. Assay Endpoint Descriptions

87.1. Data Interpretation

Biological Response:

Xenoestrogenic ligand-binding and ERα agonism as monitored by FRET emission resulting from GAL4/β-lactamase gene expression.

Analytical Elements:

BLA expression in the ERa_BLA_Agonist assay is quantified by measuring the ratio of product (blue; 460 nm) to substrate (green; 530 nm) fluorescence. Raw plate readouts were normalized relative to positive controls (17 beta-estradiol; 100% activity) and negative controls (DMSO; baseline activity) and then subjected to a NCGC in-house pattern correction algorithm (developed using DMSO-only plates run at the beginning and end of the compound plate stack). Data was analyzed as percentage of 17 beta-estradiol activity, and concentration-response relationships were determined based on a range of 15 chemical concentrations. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Estrogen receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test

chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ACEA_T47D_80hr_Positive
ATG_ERE_CIS_up
ATG_ERa_TRANS_up
ATG_ERb_TRANS2_up
NVS_NR_bER
NVS_NR_hER
NVS_NR_mERa
OT_ER_ERaERa_0480
OT_ER_ERaERa_1440
OT_ER_ERaERb_0480
OT_ER_ERaERb_1440
OT_ER_ERbERb_0480
OT_ER_ERbERb_1440
OT_ERa_ERELUC_AG_1440
OT_ERa_ERELUC_ANT_1440
OT_ERa_EREGFP_0120
OT_ERa_EREGFP_0480
OT_ERb_ERELUC_ANT_1440
Tox21_ERa_BLA_Antagonist_ratio
Tox21_ERa_LUC_BG1_Agonist
Tox21_ERa_LUC_BG1_Antagonist

87.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations: 15
Target (nominal) number of replicates: 3
Standard minimum concentration tested: 0.001 µM
Standard maximum concentration tested: 90 µM
Baseline median absolute deviation for the assay - based on the response values at the 2 lowest tested concentrations (bmad): 0.73
The response cutoff used to derive the hit calls (e.g., 5*bmad, 10*bmad): 20

Reference Chemicals / Predictive Capacity:

CASRN	Chemical Name	*In Vitro* Activity	*In Vivo* Activity	Activity in Assay
57-91-0	17alpha-Estradiol	Moderate	Active	Yes
57-63-6	17alpha-Ethinylestradiol	Strong	Active	Yes
50-28-2	17beta-Estradiol	Strong	Active	Yes
58-18-4	17-Methyltestosterone	Very Weak	Active	No
131-55-5	2,2',4,4'-Tetrahydroxybenzophenone	NA	Active	Yes
131-56-6	2,4-Dihydroxybenzophenone	NA	Active	No
5153-25-3	2-Ethylhexylparaben	NA	Active	Yes
140-66-9	4-(1,1,3,3-Tetramethylbutyl)phenol	Moderate	Active	No
80-46-6	4-(2-Methylbutan-2-yl)phenol	NA	Active	Yes

80-09-1	4,4'-Sulfonyldiphenol	NA	Active	Yes
599-64-4	4-Cumylphenol	Weak	Active	Yes
104-43-8	4-Dodecylphenol	NA	Active	No
99-96-7	4-Hydroxybenzoic acid	NA	Inactive	Yes
104-40-5	4-Nonylphenol	Very Weak	Active	Yes
98-54-4	4-tert-Butylphenol	NA	Active	No
521-18-6	5alpha-Dihydrotestosterone	Weak	Active	Yes
61-82-5	Amitrole	NA	Inactive	Yes
520-36-5	Apigenin	Very Weak	NA	Yes
85-68-7	Benzyl butyl phthalate	Very Weak	NA	Yes
103-23-1	Bis(2-ethylhexyl)hexanedioate	NA	Inactive	No
80-05-7	Bisphenol A	Weak	Active	Yes
1478-61-1	Bisphenol AF	NA	Active	No
77-40-7	Bisphenol B	Weak	Active	No
94-26-8	Butylparaben	NA	Active	No
480-40-0	Chrysin	Very Weak	NA	No
50-22-6	Corticosterone	Inactive	NA	Yes
486-66-8	Daidzein	Weak	NA	No
117-81-7	Di(2-ethylhexyl) phthalate	Very Weak	Inactive	Yes
84-74-2	Dibutyl phthalate	Very Weak	Inactive	Yes
115-32-2	Dicofol	Very Weak	NA	Yes
84-61-7	Dicyclohexyl phthalate	NA	Inactive	Yes
84-66-2	Diethyl phthalate	NA	Inactive	Yes
56-53-1	Diethylstilbestrol	Strong	Active	Yes
84-75-3	Dihexyl phthalate	NA	Inactive	No
474-86-2	Equilin	NA	Active	Yes
50-27-1	Estriol	NA	Active	No
53-16-7	Estrone	Moderate	Active	Yes
120-47-8	Ethylparaben	Very Weak	NA	No
60168-88-9	Fenarimol	Very Weak	NA	Yes
51630-58-1	Fenvalerate	NA	Inactive	Yes
446-72-0	Genistein	Weak	Active	No
52-86-8	Haloperidol	Inactive	NA	No
520-18-3	Kaempferol	Very Weak	Inactive	Yes
143-50-0	Kepone	Weak	NA	Yes
65277-42-1	Ketoconazole	Inactive	NA	No
330-55-2	Linuron	Inactive	NA	Yes
84-16-2	meso-Hexestrol	Strong	NA	No
72-33-3	Mestranol	NA	Active	Yes
72-43-5	Methoxychlor	Very Weak	Active	Yes
68-22-4	Norethindrone	NA	Active	No

789-02-6	o,p'-DDT	Weak	Active	No
556-67-2	Octamethylcyclotetrasiloxane	NA	Active	Yes
72-55-9	p,p'-DDE	Very Weak	Weak	No
87-86-5	Pentachlorophenol	NA	Inactive	No
57-30-7	Phenobarbital sodium	Inactive	NA	No
32809-16-8	Procymidone	Inactive	NA	No
50-55-5	Reserpine	Inactive	NA	No
52-01-7	Spironolactone	Inactive	NA	No
17924-92-4	Zearalenone	NA	Active	Yes

In Vitro Activity	ToxCast Active	ToxCast Inactive
Active	21	8
Inactive	0	8

In Vivo Activity	ToxCast Active	ToxCast Inactive
Active	24	6
Inactive	2	9

In Vitro Sensitivity = 72.4%
In Vitro Specificity = 100.0%
Balanced Accuracy = 86.2%

In Vivo Sensitivity = 80.0%
In Vivo Specificity = 81.8%
Balanced Accuracy = 80.9%

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [4].

88. Assay Documentation

88.1. References

[1] Graham, F., et al. (1977). J Gen Virol 36(1): 59-72. (PMID: 886304)
[2] Bylund, L., et al. (2004). Cytogenet Genome Res 106(1): 28-32. (PMID: 15218237)

[3] Michael, S., et al. (2008). Assay Drug Dev Technol 6(5): 637-657. (PMID: 19035846)
[4] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

88.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
E2, Estradiol
EDC, Endocrine Disrupting Compounds
ER, Estrogen Receptor
ERE, Estrogen Response Element
FBS, Fetal Bovine Serum
FRD, Flying Reagent Dispenser
ICCVAM, Interagency Coordinating Committee on the Validation of Alternative Methods
MIE, Molecular Initiating Event
NICEATM, National Toxicology Program Interagency Center for Evaluation of Alternative Toxicological Methods
NR, Nuclear Receptors
qHTS, quantitative high-throughput screening
TF, Transcription Factor

88.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

7 June 2016

Date of Revisions:

2016

Author of Revisions:

EPA NCCT

89. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:
Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;
Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation
Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;
Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

90. Supporting Information (existing annotations):

Tox21_ERa_LUC_BG1_Agonist

Assay Name: Tox21 BG1 Cell-Based qHTS Luciferase Assay to Identify Small Molecule Agonists of the Estrogen Receptor Alpha (ER-alpha) Signaling Pathway

91. Assay Descriptions

91.1. Overview

Assay Summary:

Estrogen receptor (ER), a nuclear hormone receptor, plays an important role in development, metabolic homeostasis and reproduction. Endocrine disrupting chemicals (EDCs) and their interactions with steroid hormone receptors like ER causes disruption of normal endocrine function. Therefore, it is important to understand the effect of environmental chemicals on the ER signaling pathway. To identify ER agonists, BG1-Luc-4E2 cell line has been used to screen the Tox21 library of environmental compounds. The BG1Luc4E2 cell line endogenously expresses full-length ERα and is stably transfected with an estrogen-responsive luciferase reporter gene plasmid (pGudLuc7ere). This test system was plated into 1536-well microplates and cells were exposed to test chemicals or controls for 24 hours and scanned with a microplate reader to detect bioluminescent signals which result from the enzymatic reaction catalyzed by ONE-Glo™ assay substrate and the induction of an ERα-linked luciferase reporter gene.

91.2. Assay Definition

Assay Throughput:

Stably transfected BG-1 cells are aliquoted into 1536-well microtiter plates and incubated with test compounds for 24 hours prior to monitoring luminescence resulting from ER gene expression.

Experimental System:

Bowman Gray-1 (BG-1) cells are an ovarian adenocarcinoma originating from tumor tissue taken in the early 1980's from a patient with poorly differentiated Stage III ovarian cancer. This is an immortalized cell line which endogenously expresses receptors for estrogen (α and β) and progesterone [1] as well as growth factors EGF and IGF-1 [2], and so provides an alternative to breast cell lines for estrogen-sensitive proliferation assays. BG1-Luc-4E2 cells are BG-1 cells which are stably transfected with plasmid containing four estrogen responsive elements upstream of a luciferase reporter gene [3] to measure transactivation activity occurring along estrogen signaling pathways. The BG1luc estrogen receptor transactivation test method for identifying estrogen receptor activation has been validated by NICEATM and ICCVAM as an appropriate assay for detecting ER agonism [4].

Xenobiotic Biotransformation Potential:

The intrinsic production of Phase I/II enzymes has not been well characterized in this cell line and metabolic activation/detoxification of test compounds is potentially limited.

Basic Procedure:

Materials

Supplies/Medium/Reagent	Manufacturer	Vender/Catalog Number
MEM α medium	Invitrogen	Invitrogen, 12561
10% Premium Fetal Bovine Serum	Atlanta Biologicals	Atlanta Biologicals, S11150
Penicillin/Streptomycin	Invitrogen	Invitrogen, 15140
400mg/l G418 (Geneticin)	Invitrogen	Invitrogen, 10131
DMEM phenol red free - low glucose medium	Invitrogen	Sigma, D5921
10% Charcoal/dextran treated Fetal Bovine Serum	Invitrogen	Invitrogen, 12676
L-Glutamine	Invitrogen	Invitrogen, 25030
0.25% Trypsin-EDTA	Invitrogen	Invitrogen / 25200
1536-well white solid plates	Greiner Bio-One	Greiner Bio-One / 789173-F

MULTIDROP COMBI	Thermo Electron Corporation	Thermo Electron Corporation
BioRAPTR FRD	Beckman Coulter	Beckman Coulter
ViewLux Plate Reader	Perkin Elmer	Perkin Elmer
ONE-Glo Luciferase Assay system	Promega	Promega / E6130
Recovery Cell Culture Freezing Medium	Invitrogen	Invitrogen / 12648

Cell handling:

1.1. Media Required:

Component	Growth Medium	Assay Medium	Thaw Medium	Freezing Medium
MEM α medium	90%	-	90%	-
DMEM phenol red free - low glucose medium	-	90%	-	-
Premium Fetal Bovine Serum	10%	-	10%	-
Charcoal/dextran treated Fetal Bovine Serum	-	10%	-	-
Penicillin/Streptomycin	100U/ml & 100µg/ml	100U/ml & 100µg/ml	100U/ml & 100µg/ml	-
L-Glutamine	-	2mM	-	-
G418 (Geneticin)	400mg/l	-	-	-
Recovery Cell Culture Freezing Medium	-	-	-	100%

1.2. Thawing method

1.2.1 Thaw a frozen vial of cells in 9ml of pre-warmed medium and seed them in T175 flask at 2 million cells

1.3. Propagation method

1.3.1 Trypsinize cells from the flask and centrifuge

1.3.2 Add culture medium to the pellet and passage at 3-4 million per T-225 flask

2.0. Assay Protocol

2.1 Harvest from the 5-day culture in assay medium and re-suspend cells in assay medium

2.2 Dispense 4000 cells/5µL/well into 1536-well tissue treated white/solid bottom plates

2.3 Incubate the plates for 24hrs (22-24hrs) at 37°C and 5% CO2

2.4 Transfer 23nL of compounds from the library collection and positive control to the assay plates through pintool

2.5 Incubate the plates for 22hrs (22-24hrs) at 37°C and 5% CO2

2.6 Add 5µl of ONE-Glo reagent

2.7 Incubate the plates at room temperature for 30min

2.8 Measure luminescence by ViewLux plate reader

Protocol Summary:

BG1-Luc-4E2 cells were cultured in phenol red-free assay medium containing 10% charcoal stripped FBS for 5 days before the assay was performed. The cells were dispensed at 4,000 cells/5 µL of assay medium per well into white wall/solid-bottom 1536-well plates using a Multidrop Combi (ThermoFisher Scientific, Waltham, MA) dispenser. After the assay plates were incubated at a 37C and 5% CO2 for 24 h, 23 nL of compounds dissolved in DMSO, positive controls or DMSO only was transferred to the assay plate by a Pintool station (Kalypsys, San Diego, CA). The plates were incubated at 37C and 5% CO2 for 22 h, followed by addition of 5 µL of ONE-Glo reagent (Promega, Madison, WI) to each well using Flying Reagent Dispenser (Aurora Discovery,

San Diego, CA). The assay plates were incubated at room temperature and luminescence was measured by a ViewLux plate reader (PerkinElmer, Shelton, CT).

Proprietary Elements:

This assay is not proprietary; BG1-Luc-4E2 cell line was provided by Dr. Michael Denison from University of California. The Tox21 qHTS robotic platform has a 1536-well per run capacity and is capable of fully-automated (hands-free) assay execution (liquid dispensing and aspiration, plate centrifugation and incubation, et cetera) and signal recording (plate readout) [5].

Caveats:

It was recently reported that the BG1-Luc-4E2 cell line was derived from MCF7 human breast cancer cells rather than BG-1 cells. Hence, the cell line has been renamed VM7Luc4E2 (https://ntp.niehs.nih.gov/iccvam/methods/endocrine/bg1luc/bg1luc-vm7luc-june2016-508.pdf).
The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote estrogen receptor mediated gene expression, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

91.3. Assay References

Assay Source Contact Information:

U.S. Tox21 Program
National Center for Advancing Translational Sciences [NCATS]
NIH Chemical Genomics Center [NCGC]
U.S. Environmental Protection Agency [EPA]
National Institutes of Environmental Health Sciences [NIEHS]
National Toxicology Program [NTP]
U.S. Food and Drug Administration [FDA]

Ruili Huang
NIH Chemical Genomics Center, National Center for Advancing Translational Sciences, National Institutes of Health, Rockville, MD 20850, USA

Assay Publication Year:

2014

Assay Publication:

Huang, R., Sakamuru, S., Martin, M. T., Reif, D. M., Judson, R. S., Houck, K. A., Casey, W., Hsieh, J. H., Shockley, K. R., Ceger, P., Fostel, J., Witt, K. L., Tong, W., Rotroff, D. M., Zhao, T., Shinn, P., Simeonov, A., Dix, D. J., Austin, C. P., Kavlock, R. J., Tice, R. R., & Xia, M. (2014). "Profiling of the Tox21 10K compound library for agonists and antagonists of the estrogen receptor alpha signaling pathway". Scientific Reports 4, 5664. (PMID: 25012808)

Huang, R., Xia, M., Sakamuru, S., Zhao, J., Shahane, S. A., Attene-Ramos, M., Zhao, T., Austin, C. P., & Simeonov, A. (2016). "Modelling the Tox21 10 K chemical profiles for in vivo toxicity prediction and mechanism characterization". Nature Communications 7, 10425. (PMID: 26811972)

Method Updates / Confirmatory Studies:

92. Assay Component Descriptions

Assay Objectives:

The Tox21 estrogen receptor alpha BG1 luciferase agonist assay screened a library of diverse environmental compounds to probe for xenoestrogenic ligand-binding and potential to induce estrogen-dependent transcription, monitored through luciferase reporter gene signal activation using an endogenous full-length ERα cell line of human ovary origin (BG1). The assay is run in triplicate on 1536-well microplate and bioluminescence was measured following 24-hour incubation of cells with test compounds and 30 min incubation of test system with ONE-Glo™ luciferase assay reagent. The bioluminescent signal was monitored using a Promega ViewLux plate reader. Test compounds were selected based on various criteria, e.g., exposure hazard, physicochemical properties, availability and affordability and each assay incorporated 88 chemical duplications (each derived from the same primary stock solution as a sample chemical) and 39 different reference chemicals with known ER agonistic/antagonistic activities. 10% of the chemicals tested were duplicated chemical structures sourced from separate venders or from different production lots to assess sample variability. Following the incubation period, the cell culture was screened for bioluminescent signals in agonist mode using luciferase detection technology. Each compound was tested in a concentration-response format, using 15 concentrations ranging from 1.1 nM to 92 μM.

Scientific Principles:

Estrogen receptor (ER), a nuclear hormone receptor, plays an important role in development, metabolic homeostasis and reproduction. Endocrine disrupting chemicals (EDCs) and their interactions with steroid hormone receptors like ER causes disruption of normal endocrine function. Therefore, it is important to understand the effect of environmental chemicals on the ER signaling pathway. To identify ER agonists, BG1-Luc-4E2 cell line (provided by Dr. Michael Denison from University of California) has been used to screen the Tox21 library of diverse environmental compounds. BG1Luc4E2 cell line endogenously expresses full-length ER-alpha and is stably transfected with a plasmid containing four estrogen responsive elements (ERE) upstream of a luciferase reporter gene.

The ERα_LUC_BG1 assays are qHTS format assays which measured the ability of a chemical to interact with estrogen receptor alpha (ERα) by monitoring modulation of fluorescence reporter gene signals. This assay utilized a human ovary (BG1) cell line which expresses endogenous full-length ERα and a luciferase reporter gene (ER-luc) to quantify xenoestrogenic activity.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with estrogen receptor alpha mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that estrogen receptor agonism is the Molecular Initiating Event (MIE) in an Adverse Outcome Pathway (AOP) leading to reproductive dysfunction in oviparous vertebrates, and there is some evidence that estrogen receptor activation is the MIE for putative AOPs leading to reduced survival due to renal failure and leading to skewed sex ratios due to altered sexual differentiation in males (all AOPs currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive in vivo studies to further investigate the involvement of ER agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Rogers, J., & Denison, M. (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* Mol Toxicol 13(1), 67-82. (PMID: 10900408)

Assay Quality Statistics:

Neutral control well median response value, by plate:	-0.18
Neutral control median absolute deviation, by plate:	6.87

Positive control well median response value, by plate:	99.58
Positive control well median absolute deviation, by plate:	8.91
Z' (median across all plates, using positive control wells):	0.53
SSMD (median across all plates, using positive control wells):	9
Signal-to-noise (median across all plates, using positive control wells):	14.55
Signal-to-background (median across all plates, using positive control wells):	-548.47
CV (median across all plates):	-39.74

93. Assay Endpoint Descriptions

93.1. Data Interpretation

Biological Response:

Xenoestrogenic ligand-binding and ERα agonism as monitored by measuring changes in luminescence resulting from activation of an estrogen-responsive luciferase reporter gene.

Analytical Elements:

The Tox21 BG1-luciferase ERα agonist assay was monitored for signal increase over the DMSO (negative control) baseline, and activity and was calculated as a percentage of 17β-estradiol (positive control) activity. Concentration-response relationships were determined based on a range of 15 chemical concentrations. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Estrogen receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ACEA_T47D_80hr_Positive
ATG_ERE_CIS_up
ATG_ERa_TRANS_up
ATG_ERb_TRANS2_up
NVS_NR_bER
NVS_NR_hER
NVS_NR_mERa
OT_ER_ERaERa_0480
OT_ER_ERaERa_1440
OT_ER_ERaERb_0480
OT_ER_ERaERb_1440
OT_ER_ERbERb_0480
OT_ER_ERbERb_1440
OT_ERa_ERELUC_AG_1440
OT_ERa_ERELUC_ANT_1440
OT_ERa_EREGFP_0120

OT_ERa_EREGFP_0480
OT_ERb_ERELUC_ANT_1440
Tox21_ERa_BLA_Agonist_ratio
Tox21_ERa_BLA_Antagonist_ratio
Tox21_ERa_LUC_BG1_Antagonist

93.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations: 15
Target (nominal) number of replicates: 3
Standard minimum concentration tested: 0.001 µM
Standard maximum concentration tested: 90 µM
Baseline median absolute deviation for the assay - based on the response values at the 2 lowest tested concentrations (bmad): 2.78
The response cutoff used to derive the hit calls (e.g., 5*bmad, 10*bmad): 20

Reference Chemicals / Predictive Capacity:

CASRN	Chemical Name	*In Vitro* Activity	*In Vivo* Activity	Activity in Assay
57-91-0	17alpha-Estradiol	Moderate	Active	Yes
57-63-6	17alpha-Ethinylestradiol	Strong	Active	Yes
50-28-2	17beta-Estradiol	Strong	Active	Yes
58-18-4	17-Methyltestosterone	Very Weak	Active	Yes
131-55-5	2,2',4,4'-Tetrahydroxybenzophenone	NA	Active	Yes
131-56-6	2,4-Dihydroxybenzophenone	NA	Active	No
5153-25-3	2-Ethylhexylparaben	NA	Active	Yes
140-66-9	4-(1,1,3,3-Tetramethylbutyl)phenol	Moderate	Active	Yes
80-46-6	4-(2-Methylbutan-2-yl)phenol	NA	Active	Yes
80-09-1	4,4'-Sulfonyldiphenol	NA	Active	Yes
599-64-4	4-Cumylphenol	Weak	Active	Yes
104-43-8	4-Dodecylphenol	NA	Active	No
99-96-7	4-Hydroxybenzoic acid	NA	Inactive	Yes
104-40-5	4-Nonylphenol	Very Weak	Active	Yes
98-54-4	4-tert-Butylphenol	NA	Active	Yes
521-18-6	5alpha-Dihydrotestosterone	Weak	Active	Yes
61-82-5	Amitrole	NA	Inactive	Yes
520-36-5	Apigenin	Very Weak	NA	Yes
85-68-7	Benzyl butyl phthalate	Very Weak	NA	Yes
103-23-1	Bis(2-ethylhexyl)hexanedioate	NA	Inactive	No
80-05-7	Bisphenol A	Weak	Active	Yes
1478-61-1	Bisphenol AF	NA	Active	No
77-40-7	Bisphenol B	Weak	Active	No
94-26-8	Butylparaben	NA	Active	No
480-40-0	Chrysin	Very Weak	NA	No

50-22-6	Corticosterone	Inactive	NA	Yes
486-66-8	Daidzein	Weak	NA	No
117-81-7	Di(2-ethylhexyl) phthalate	Very Weak	Inactive	Yes
84-74-2	Dibutyl phthalate	Very Weak	Inactive	Yes
115-32-2	Dicofol	Very Weak	NA	Yes
84-61-7	Dicyclohexyl phthalate	NA	Inactive	Yes
84-66-2	Diethyl phthalate	NA	Inactive	Yes
56-53-1	Diethylstilbestrol	Strong	Active	Yes
84-75-3	Dihexyl phthalate	NA	Inactive	Yes
474-86-2	Equilin	NA	Active	Yes
50-27-1	Estriol	NA	Active	Yes
53-16-7	Estrone	Moderate	Active	Yes
120-47-8	Ethylparaben	Very Weak	NA	No
60168-88-9	Fenarimol	Very Weak	NA	Yes
51630-58-1	Fenvalerate	NA	Inactive	Yes
446-72-0	Genistein	Weak	Active	No
52-86-8	Haloperidol	Inactive	NA	No
520-18-3	Kaempferol	Very Weak	Inactive	Yes
143-50-0	Kepone	Weak	NA	Yes
65277-42-1	Ketoconazole	Inactive	NA	Yes
330-55-2	Linuron	Inactive	NA	Yes
84-16-2	meso-Hexestrol	Strong	NA	Yes
72-33-3	Mestranol	NA	Active	Yes
72-43-5	Methoxychlor	Very Weak	Active	Yes
68-22-4	Norethindrone	NA	Active	No
789-02-6	o,p'-DDT	Weak	Active	Yes
556-67-2	Octamethylcyclotetrasiloxane	NA	Active	Yes
72-55-9	p,p'-DDE	Very Weak	Weak	No
87-86-5	Pentachlorophenol	NA	Inactive	No
57-30-7	Phenobarbital sodium	Inactive	NA	No
32809-16-8	Procymidone	Inactive	NA	No
50-55-5	Reserpine	Inactive	NA	No
52-01-7	Spironolactone	Inactive	NA	No
17924-92-4	Zearalenone	NA	Active	Yes

In Vitro Activity	ToxCast Active	ToxCast Inactive
Active	26	3
Inactive	0	8

In Vivo Activity	ToxCast Active	ToxCast Inactive
Active	28	2
Inactive	4	7

In Vitro Sensitivity = 89.7%
In Vitro Specificity = 100.0%
Balanced Accuracy = 94.8%

In Vivo Sensitivity = 93.3%
In Vivo Specificity = 63.6%
Balanced Accuracy = 78.5%

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [5].

94. Assay Documentation

94.1. References

[1] Geisinger, K. R., et al. (1989). Cancer 63(2): 280-288. (PMID: 2910432)
[2] Baldwin, W. S., et al. (1998). In vitro Cell Develop Biol -Animal 34(8): 649-654. (PMID: 9769151)
[3] Rogers, J. and M. Denison (2000). In vitro Mol Toxicol 13(1): 67-82. (PMID: 10900408)
[4] OECD (2012). Test No. 457: Bg1luc Estrogen Receptor Transactivation Test Method for Identifying Estrogen Receptor Agonists and Antagonists, OECD Publishing.
[5] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

94.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
E2, Estradiol
EDC, Endocrine Disrupting Compounds
ER, Estrogen Receptor
ERE, Estrogen Response Element
FBS, Fetal Bovine Serum
FRD, Flying Reagent Dispenser
ICCVAM, Interagency Coordinating Committee on the Validation of Alternative Methods
MIE, Molecular Initiating Event
NICEATM, National Toxicology Program Interagency Center for Evaluation of Alternative Toxicological Methods
NR, Nuclear Receptors
qHTS, quantitative high-throughput screening

TF, Transcription Factor

94.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

3 June 2016

Date of Revisions:

2016

Author of Revisions:

EPA NCCT

95. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:

Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;

Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation

Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;

Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

96. Supporting Information (existing annotations):