Assay Endpoint ID: 714

NVS_NR_hER

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

1. Assay Descriptions

1.1. 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.

1.2. 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-HCl (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.

1.3. 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.

2. 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

3. Assay Endpoint Descriptions

3.1. 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

3.2. 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	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	No
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	acid	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	Yes
61-82-5	Amitrole	NA	Inactive	No
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	No
94-26-8	Butylparaben	NA	Active	Yes
480-40-0	Chrysin	Very Weak	NA	No
50-22-6	Corticosterone	Inactive	NA	No
486-66-8	Daidzein	Weak	NA	No
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	No
84-61-7	Dicyclohexyl phthalate	NA	Inactive	No
84-66-2	Diethyl phthalate	NA	Inactive	No
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	Yes
60168-88-9	Fenarimol	Very Weak	NA	No
51630-58-1	Fenvalerate	NA	Inactive	No
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	No
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	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	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	18	17
Inactive	8	10

In Vivo Activity	**ToxCast Active**	**ToxCast Inactive**
Active	22	15
Inactive	4	12

In Vitro Sensitivity = 51.4%
In Vitro Specificity = 55.6%
Balanced Accuracy = 53.5%

In Vivo Sensitivity = 59.5%
In Vivo Specificity = 75%
Balanced Accuracy = 67.3%

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].

Assay Documentation

4.1. References

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

4.2. 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

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:

25 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):