Development and Distribution of Test Method Documentation for ToxCast HTT Assays

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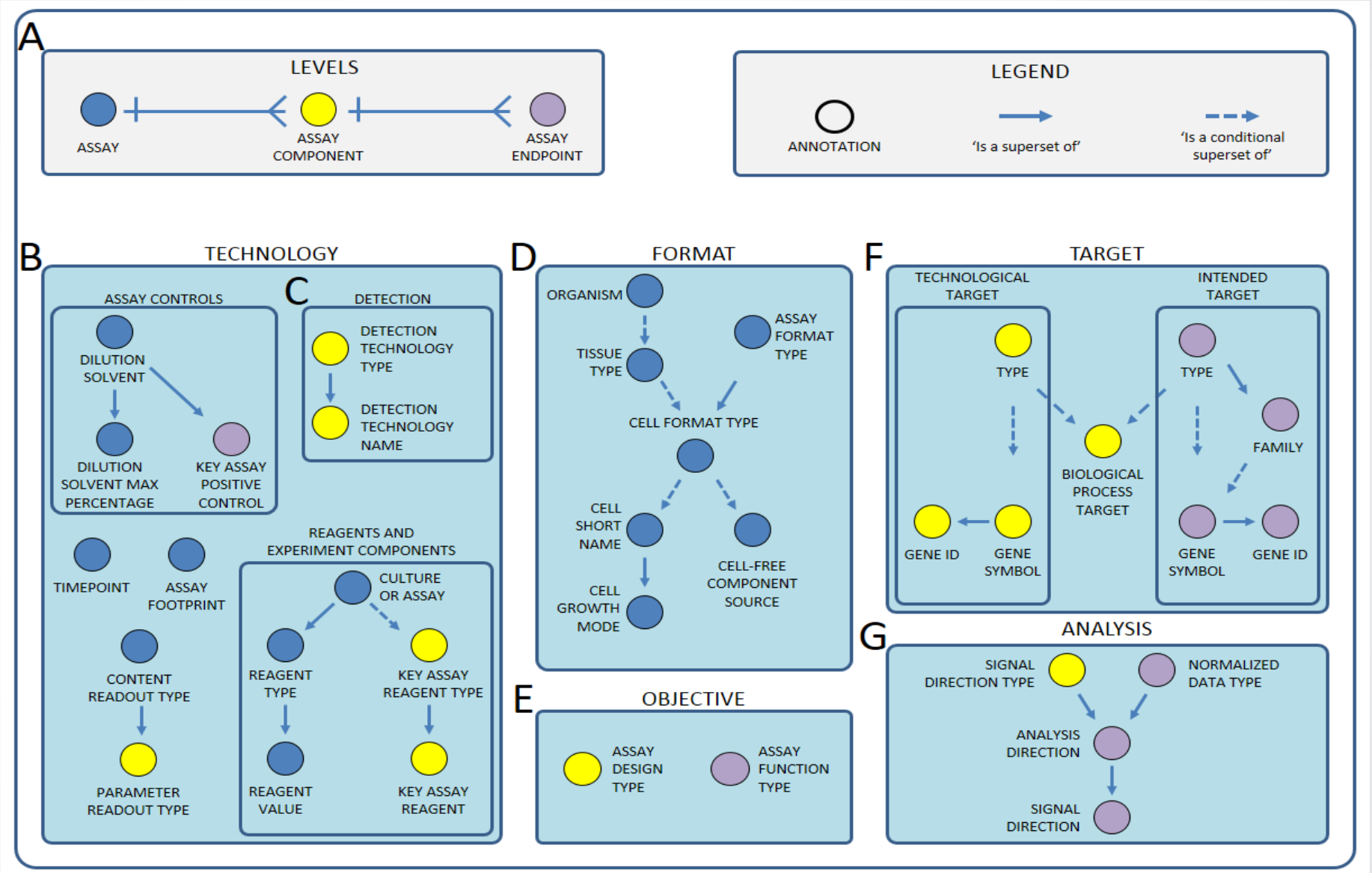
ToxCast/Tox21 Data Distribution
Assay Description - Objectives

- Accessibility and transparency – assay interpretation tools
- Encourage public confidence in assay activity
  - ***Comprehensive description of methodologies***
    to facilitate accurate interpretation of results
  - ***Clearly define the response measured***
    and its relevance for impacts on biological systems
- Provide detailed assay documentation to aid external evaluation
- Describe assay ***reliability*** (reproducibility), ***relevance*** (mechanistic modeling, downstream health effects, AOP applicability, etc.), and ***fitness-for-purpose*** (ability to predict outcomes similar to guideline studies)

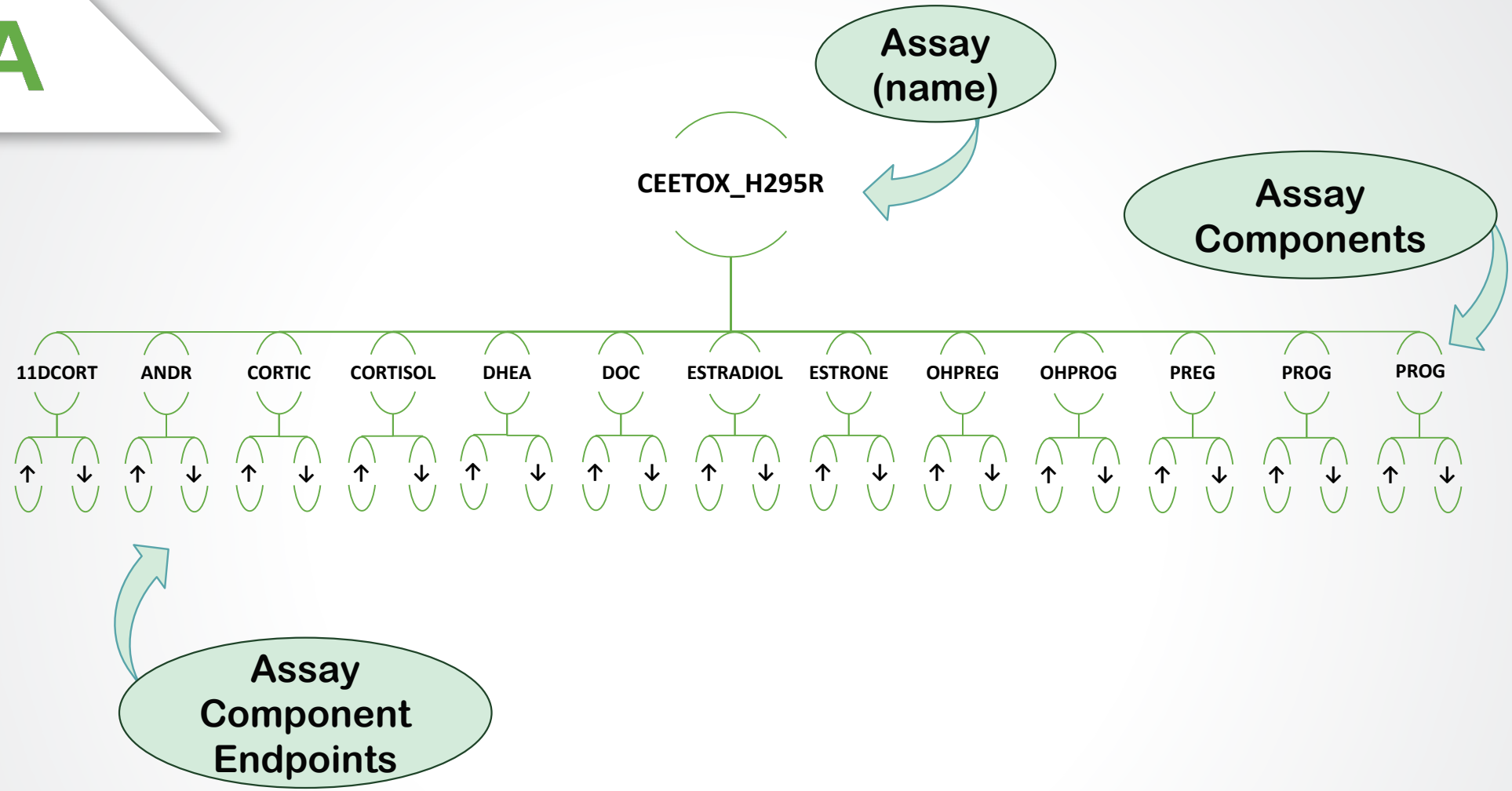
ToxCast/Tox21 Data Distribution
Assay annotation framework and needs

- NCCT's current distribution of ToxCast assay metadata
- **OECD reporting guidelines** for *in vitro* assays
  - NCCT's implementation of OECD recommendations
- **Assay Method Descriptions** – New NCCT Reporting Template
- Public Distribution
- Future goals

ToxCast Bioassay Ontology (BAO)-based annotation structure



(Image from Phuong, J., *et al*. (2014). ToxCast Assay Annotation Version 1.0 Data User Guide. U.S. Environmental Protection Agency., pp. 36. https://www.epa.gov/sites/production/files/2015-08/documents/ toxcast_assay_annotation_data_users_guide_20141021.pdf).


Assay (name)
CEETOX_H295R
Assay Components
11DCORT
ANDR
CORTIC
CORTISOL
DHEA
DOC
ESTRADIOL
ESTRONE
OHPREG
OHPROG
PREG
PROG
PROG
Assay Component Endpoints

Assay → annotations define the experimental platform

assay_name	CEETOX_H295R
timepoint_hr	48
organism_id	9606
organism	human
tissue	adrenal gland
cell_format	NULL
cell line	cell-based format
cell_free_component_source	multiplexed
cell_short_name	H295R
cell_growth_mode	adherent
assay_footprint	microplate: 96-well plate
assay_format_type	cell-based
assay_format_type_sub	cell-based format
content_readout_type	multiplexed
dilution_solvent	DMSO
dilution_solvent_ percent_max	0.1

Assay Components → technological targets

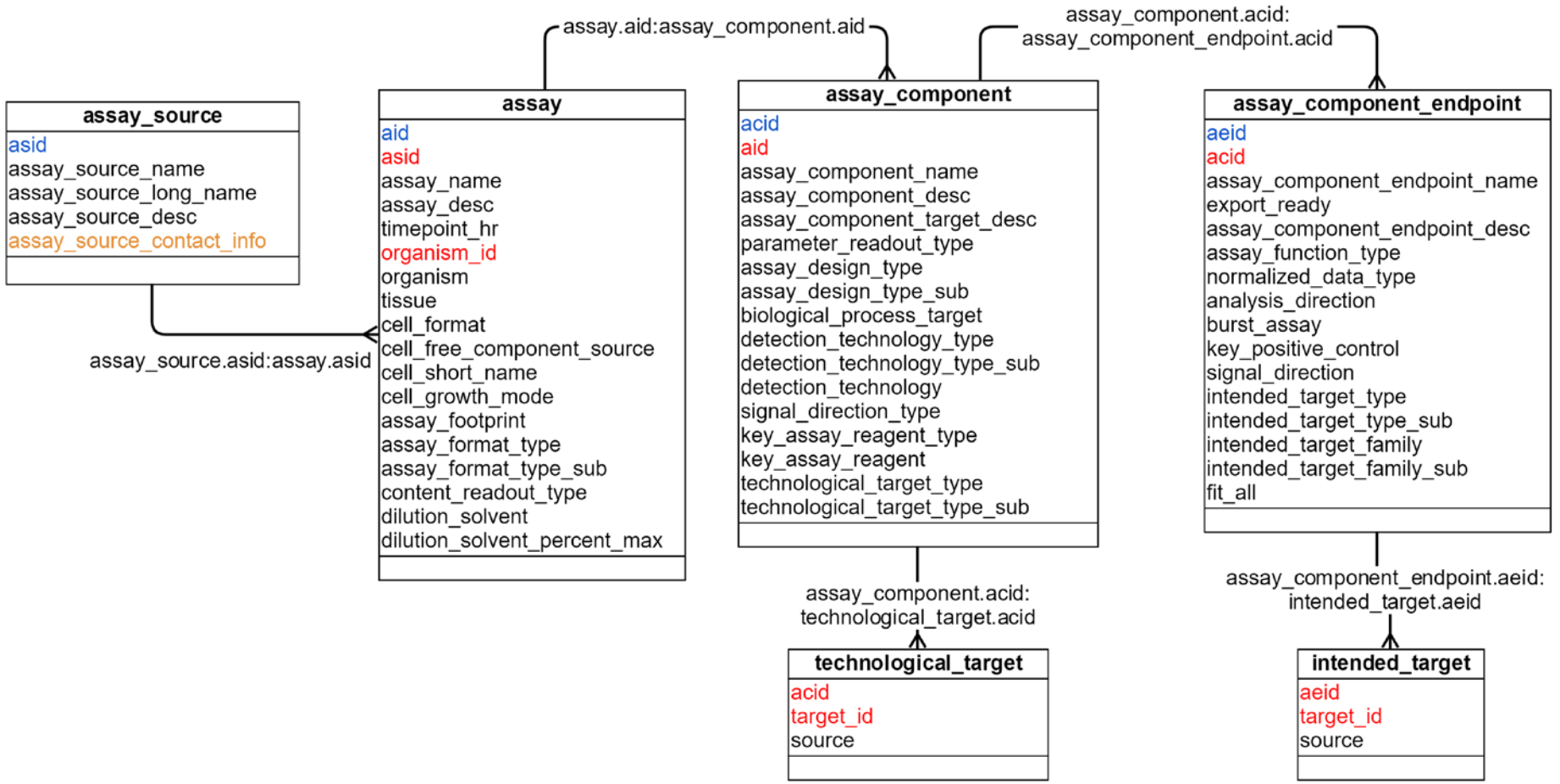
assay_component_name	CEETOX_H295R_ESTRADIOL
parameter_readout_type	single
assay_design_type	NULL
assay_design_type_sub	NULL
biological_process_target	regulation of steroid biosynthetic process
detection_technology_type	Spectrophotometry
detection_technology_type_sub	Absorbance
detection_technology	HPLC-MS-MS
signal_direction_type	both
key_assay_reagent_type	inducer
key_assay_reagent	NULL
technological_target_type	hormone
technological_target_type_sub	Estradiol
assay_component_name	CEETOX_H295R_ESTRADIOL
parameter_readout_type	single
assay_design_type	NULL

Assay Component Endpoints→ biological ("intended") targets

assay_component_endpoint_name	CEETOX_H295R_ESTRADIOL_up
assay_function_type	steroidogenesis
normalized_data_type	log2_fold_induction
analysis_direction	positive
key_positive_control	Prochloraz;Forskolin
signal_direction	gain
intended_target_type	hormone
intended_target_type_sub	Estradiol
intended_target_family	steroid hormone
intended_target_family_sub	estrogens

ToxCast Annotations in invitrodb

Assay information is currently provided with focus on bioinformatics and data aggregation



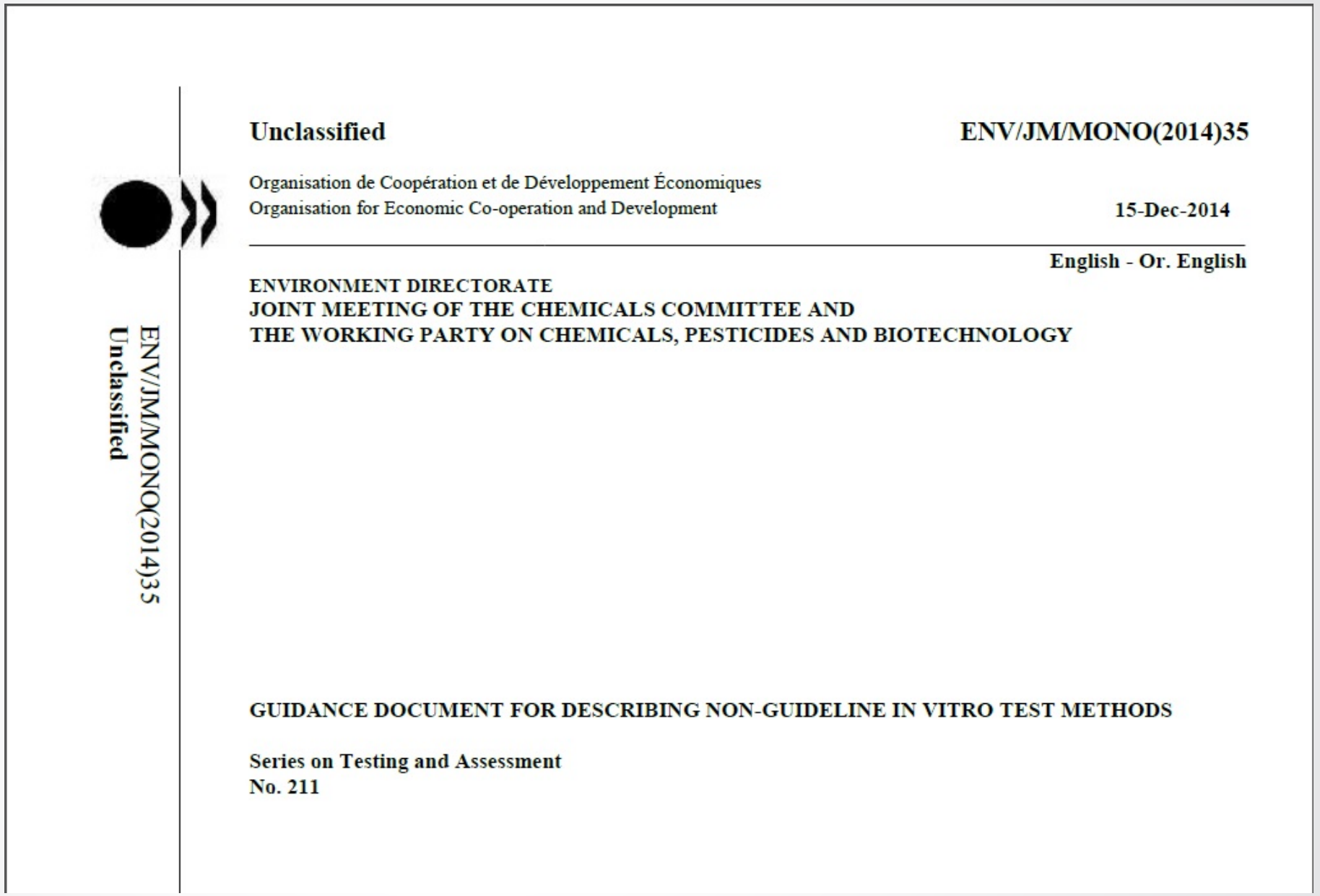
ToxCast Annotations

Regulatory / Chemical Assessment Needs

- HTS studies need to be assessed as part of a ***larger biological context***
- ***Gaps in information*** exist about how in vitro activity should be considered in relation to in vivo studies
  - High-throughput assay technologies allow for screening of large (previously untested) chemical libraries, but may not provide same level of context / information on reproducibility / replication as OECD-validated/PBTG studies

→ Accurate interpretation of data generated by quickly advancing technologies may be more labor-intensive and not as readily available for end-users as information provided with guideline studies.

OECD recommendations for describing non-guideline in vitro tests (2014)



ENV/JM/MONO(2014)35
Unclassified

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

15-Dec-2014

English - Or. English

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

GUIDANCE DOCUMENT FOR DESCRIBING NON-GUIDELINE IN VITRO TEST METHODS

Series on Testing and Assessment
No. 211

Harmonious Descriptions

"The purpose … is to harmonize the way non-guideline *in vitro* methods are described"

- To enable accurate assessments of:
  - the ***relevance of the test methods*** for biological activities and responses of interest
  - the ***quality of data produced*** and the potential ***utility in regulatory applications***

"How can these methods be practically utilized whilst ensuring that the resulting data are ***scientifically robust*** and ***interpreted in the appropriate context***?"

"This guidance is not intended to be prescriptive nor does it endorse a particular structure for reporting the information"

OECD Test Method Descriptions

1. General Information

- 1.1. Assay Name
- 1.2. Summary
- 1.3. Date of Method Description (MD)
- 1.4. MD author(s) and contact details
- 1.5. Date of MD update(s) and contacts
- 1.6. Assay developer(s)/laboratory and contact details
- 1.7. Date of assay development and/or publication
- 1.8. Reference(s) to main scientific paper
- 1.9. Availability of information about proprietary elements
- 1.10. Information about the throughput of the assay
- 1.11. Status of method development and uses
- 1.12. Abbreviations and Definitions

2. Test Method Definition

- 2.1. Purpose of the test method
- 2.2. Scientific principle of the method
- 2.3. Tissues, Cells or Extracts utilized in the assay
- 2.4. Metabolic competence of the test system
- 2.5. Description of the experimental system exposure regime
- 2.6. Response and response measurement
- 2.7. Quality / Acceptance criteria
- 2.8. Known technical limitations and strengths
- 2.9. Other related assays that characterize the same event as in Section 2.1

3. Data Interpretation and Prediction Models

- 3.1. Assay response captured by the prediction model
- 3.2. Data analysis
- 3.3. Explicit prediction model
- 3.4. Software name and version for algorithm/prediction model generation

4. Test Method Performance

- 4.1. Robustness of the method
- 4.2. Reference chemicals / chemical libraries, and rationale for selection
- 4.3. Performance measures / predictive capacity (where known)
- 4.4. Scope and limitations of the assay, if known

5. Potential Regulatory Use

- 5.1. Context of Use

6. Bibliography

7. Supporting Information

OECD recommended description format (brief outline)

- General information
- Test method definition
- Data interpretation and prediction model
- Test method performance
- Potential regulatory applications

ToxCast adapted description format (brief outline)

- Assay Summary Information

  Assay overview, source lab and contact info, references to publications, experimental design, materials and methods, review of platform biotransformation potential, propriety elements, production status and caveats

- Test Component Definition

  Assay objectives, description of technological platform, intended biological targets and scientific basis for how the platform monitors the targets. Provides references for method development.

- Component Endpoint Descriptions

  Data interpretation and analytical approaches to effect (hit-call) determinations

- Description Documentation

  References, abbreviations and definitions

- Potential regulatory applications

ToxCast Description detailed (right), with corresponding OECD recommendations (left)

OECD Guidance Document № 211 Template:	
1.	**General Information**
1.1.	Assay Name
1.2.	Summary
1.3.	Date of Method Description (MD)
1.4.	MD author(s) and contact details
1.5.	Date of MD update(s) and contacts
1.6.	Assay developer(s)/laboratory and contact details
1.7.	Date of assay development and/or publication
1.8.	Reference(s) to main scientific paper
1.9.	Availability of information about proprietary elements
1.10.	Information about the throughput of the assay
1.11.	Status of method development and uses
1.12.	Abbreviations and Definitions
2.	**Test Method Definition**
2.1.	Purpose of the test method
2.2.	Scientific principle of the method
2.3.	Tissues, Cells or Extracts utilized in the assay
2.4.	Metabolic competence of the test system
2.5.	Description of the experimental system exposure regime
2.6.	Response and response measurement
2.7.	Quality / Acceptance criteria
2.8.	Known technical limitations and strengths
2.9.	Other related assays that characterize the same event as in Section 2.1
3.	**Data Interpretation and Prediction Models**
3.1.	Assay response captured by the prediction model
3.2.	Data analysis
3.3.	Explicit prediction model
3.4.	Software name and version for algorithm/prediction model generation
4.	**Test Method Performance**
4.1.	Robustness of the method
4.2.	Reference chemicals / chemical libraries, and rationale for selection
4.3.	Performance measures / predictive capacity (where known)
4.4.	Scope and limitations of the assay, if known
5.	**Potential Regulatory Use**
6.	**Bibliography**
7.	**Supporting Information**

EPA NCCT ToxCast Assay Documentation Outline:

1. Assay Descriptions

- **Assay Overview** (OECD № 211 outline equivalent)
  - Assay Title (1.1)
  - Assay Summary (1.2)
- **Assay Definition**
  - Assay Throughput (1.10)
  - Experimental System (2.3)
  - Xenobiotic Biotransformation Potential (2.4)
  - Basic Procedure (2.5)
  - Proprietary Elements (1.9)
  - Caveats (2.8)
- **Assay References**
  - Assay Source Contact Information (1.6)
  - Assay Publication Year (1.7)
  - Assay Publication Citation (1.8)
  - Method Updates / Confirmatory Studies (1.11)

2. Assay Component Descriptions

- Assay Objectives (2.1; 2.7; 4.2)
- Scientific Principles (2.2)
- Method Development References (2.2)

3. Assay Endpoint Descriptions

- **Data Interpretation**
  - Biological Response (2.6)
  - Analytical Elements (3.2; 3.4; 4.3)
  - Related ToxCast Assays (2.9)
- **Assay Performance**
  - Assay Quality Statistics (Robustness) (4.1)
  - Assay Performance Measures (4.3)
  - Reference Chemicals (4.2)
  - Rationale For Selection Of Chemical Library (4.2; 4.4)

4. Assay Documentation

- **Assay Documentation Definition**
  - References (Section 6)
  - Definitions / Abbreviations (1.12)
- **Assay Documentation Source**
  - Contact Information (1.4)
  - Date of Assay Document Creation (1.3)
  - Date/ Author of Revisions (1.5)

5. Supporting Information

- Existing ToxCast Annotations (Section 7)

Assay level descriptions

CEETOX_H295R_ESTRADIOL_up

Assay Title: CeeTox H295R High-throughput Steroidogenesis Assay – Estradiol Induction

1. Assay Descriptions

1.1. Overview

Assay Summary:

Endocrine disrupting chemicals have largely been studied by examining effects on nuclear receptor (primarily estrogen and androgen receptor) ligand binding or signaling pathways, but environmental chemicals can also disrupt endocrine systems by interfering with steroid hormone biosynthesis and metabolism (steroidogenesis). The CeeTox H295R steroidogenesis assays evaluate a human-derived, steroidogenically-competent adenocarcinoma cell line (H295R) in high-throughput (96-well) format to detect substances with the potential to disrupt steroid hormone production. To detect interference with steroidogenesis, this assay quantified 13 different steroid hormones using high-throughput HPLC-MS/MS. The assay is a modified version of the OECD H295R test guideline (OECD TG 456). Briefly, cells were seeded into 96-well plates and after overnight incubation were pre-treated with 10 µM forskolin for 48 hours to stimulate steroidogenesis. Following the pre-treatment, medium was replaced with test chemical for 48 hours. Following chemical exposure, medium was removed for steroid hormone analysis, and cells were subjected to the MTT assay as an indicator of cell viability. This assay involved a three-tiered screening strategy. The initial stage was designed to identify a maximum tolerated concentration (MTC) of test chemical in H295R cells. Briefly, chemicals were evaluated at up to 100 µM (solubility permitting) using the MTT assay, in order to determine the maximum concentration that maintained ≥70% cell viability. Following MTC determination, each chemical was screened for effects on steroidogenesis by quantifying changes in hormone levels at the MTC (single test concentration). Chemicals selected for follow-up were evaluated in dose-response to establish whether changes in hormone levels were concentration-dependent. In an initial screening exercise, chemicals were selected for concentration-response follow-up if at least 4 different hormones were significantly affected when screened at the MTC. Concentration-response experiments were conducted using six concentrations, serially diluted down from the MTC at half-log increments.

Assay level descriptions

1.2. Assay Definition

Assay Throughput:

Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times (maximum passage of 10). Cells were seeded into 96-well plates at 50-60% confluency and allowed to adhere overnight. Prior to test chemical exposure, cells were pre-stimulated with forskolin for 48 hours, and chemical exposures were subsequently conducted for 48 hours in forskolin-free media. Following concentration-response and MTC assays, MTT cytotoxicity assays were conducted. The media samples were stored at -80 °C prior to HPLC-MS/MS quantification of steroid hormones.

Experimental System:

H295R is an immortalized cell line derived from an adrenocortical carcinoma isolated in 1980 from a 48-year-old African-American female patient. H295R cells express genes that encode for all the key enzymes involved in steroidogenesis [1, 2]. H295R cells have the physiological characteristics of zonally undifferentiated human fetal adrenal cells. H295R cells represent a unique in vitro system, as the cells retain the ability to produce many of the steroid hormones found in the adult adrenal cortex and the gonads (with the exception of dihydrotestosterone, DHT), which allows for analysis of xenobiotic effects on both corticosteroid synthesis and the production of sex steroid hormones, including androgens and estrogens.

Xenobiotic Biotransformation Potential:

H295R cell line expresses CYPs 1A1, 11A, 17, 19, 21, 1B1 and 11B1, which are differentially induced by endocrine-disrupting chemicals [3, 4].

Assay level descriptions – detailed protocols

Basic Procedure:

Cell culture and media preparation procedures were conducted in accordance with OECD Test No. 456 guidelines [1], with minor modifications. H295R cells (ATCC CRL-2128) were expanded for 5 passages and frozen in batches in liquid nitrogen. Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times. The maximum passage was 10. Cells were maintained in a 1:1 mixture of DMEM/F12 supplemented with 5ml/L ITS+ Premix (BD Bioscience) and 12.5 ml/L Nu-Serum (BD Bioscience). After seeding cells into 96-well plates at 50-60% confluency, cells were allowed to adhere overnight. Prior to chemical testing, culture medium was replaced with 175 µL medium containing 10 µM forskolin to stimulate steroidogenesis for 48 hours. Following pre-stimulation, forskolin medium was replaced with medium containing test chemical solubilized in DMSO (ensuring a final concentration of 0.1% DMSO). Test chemicals were incubated for 48 hours, then the medium was removed and split into 2 vials containing approximately 75µL each, and stored at -80 °C prior to hormone analysis. For steroid quantification, media samples were shipped to OpAns, LLC (Durham, NC) on dry ice, and samples were thawed to room temperature prior to liquid-liquid extraction. Steroid hormones were extracted from media samples using methyl tert-butyl ether (MTBE). An extra derivatization with dansyl chloride was included for estrogen (estrone and estradiol) detection only. Steroid hormones were separated by HPLC, eluted using a reverse phase C18 gradient with electrospray positive ionization, followed by quantification by tandem mass spectrometry (MS/MS). Data were acquired on a MassHunter Workstation Acquisition version B03.01 (Agilent Technologies, Inc.) and processed using MassHunter Quantitative Analysis for QQQ. Accuracy was determined for each hormone analyte from 3 standards to determine upper and lower limits of quantification (ULOQ and LLOQ, respectively) using a 7-point standard curve. Precision and accuracy of the extraction and quantification methods were calculated as the percent relative standard deviation (%RSD) of the spiked standards and percent spiked standard recovered, respectively. The goal was to achieve 100% accuracy (i.e., recover all spiked-in standard at quantification with minimal loss during run time) and good precision (i.e., have %RSD <15% to ensure reproducibility). Test medium was removed following chemical exposure, and cell viability was evaluated in the same wells by MTT [5]. MTT procedures were as follows: after removal of test medium, 500 µl of 0.5 mg/ml of 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide solution was added to the cells. Following a 4-hour incubation at 37 °C and 5% CO_2 to allow formazan-crystal formation, the MTT solution was removed and blue formazan salt crystals were solubilized using 500µl anhydrous isopropanol with shaking for 20 minutes. Absorbance was read at 570 and 650nm using a Packard Fusion microplate reader. Background correction of absorbance units was used to determine percent change relative to controls. All assay plates contained multiple control wells including four 10µM forskolin replicates to control for hormone stimulation, four 3µM procholaz replicates to control for hormone inhibition and 4 digitonin replicates to control for cell death. Cell viability, as indicated by the MTT assay, was used to establish maximum tolerated concentration (MTC) initially using a nominal concentration of 100µM (where feasible within solubility limits of the specific test chemical) and targeting cell viability ≥70%. When a test chemical reduced cell viability to 20% - 70%, the test chemical was diluted 10-fold and re-evaluated, but if cell viability was <20%, then the test chemical was diluted 100-fold and re-evaluated. Test chemical dilutions were made until cell viability was ≥70%. MTT assays were also conducted for duplicates of all concentrations of chemicals tested in concentration-response studies.

Assay level descriptions – schematic protocols

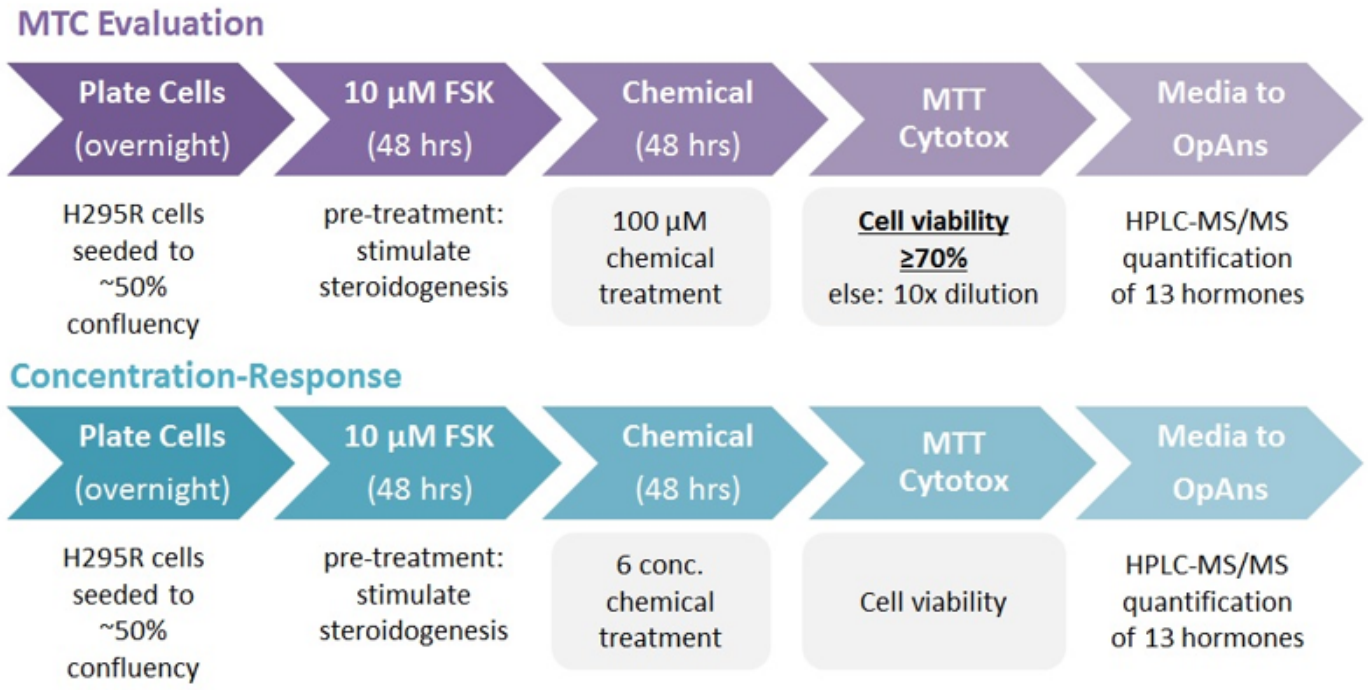

Figure 1. Schematic representation of general testing protocol for CeeTox Steroidogenesis assays. (Adapted from Karmaus et al. 2016.)

Proprietary Elements:

This assay is not proprietary; it is a modification of the existing OECD H295R *in vitro* steroidogenesis assay validated in 2011 (Test Guideline No. 456, [1]). NCI-H295R cells (ATCC CRL-2128) are commercially available from American Type Culture Collection with signed Material Transfer Agreement.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to interfere with endogenous steroidogenesis, and is intended to provide information on a large number of diverse chemicals. 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.

ToxCast descriptions – "first pass" high-throughput documentation; will be build up as descriptions become available

Assay Name: NovaScreen Small Molecule Screening for Nuclear Receptor Activity - Bovine Estrogen Receptor Assay

1. Assay Descriptions

1. Overview

Assay Summary:

NVS_NR_bER is a ***biochemical***, ***single***-readout assay that uses ***extracted gene-proteins*** from ***Bovine*** ***uterine membranes*** in a ***tissue-based cell-free*** assay. Measurements were taken ***18*** hours after chemical dosing in a ***96-well plate***.

1. Assay Definition

Assay Throughput:

High throughput: ***96-well plate microplate***

Experimental System:

tissue-based cell-free; Source: ***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-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 assay described here is intended to provide initial (screening) information on chemical activity, and is intended to provide information on a large number of diverse chemicals. 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:

Assay is mature, and data are publicly available.

Future ToxCast Data Distributions

Ongoing Projects to Improve ToxCast Assay Performance Assessments

- Assay Robustness Metrics
  - Information on control performance, CV, S/B, SSMD and Z'
- Reference Chemicals
  - RefChemDB currently in production; being populated with information on availability and quality of chemical activity data on multiple tox relevant endpoints

→ Will provide a more systematic means of generating assay performance / balanced accuracy information for many more targets than are currently being assessed