Development and Distribution of ToxCast and Tox21 HTS Assay Method Descriptions

Stacie Flood, Oak Ridge Associated Universities (ORAU) and
Keith Houck, National Center for Computational Toxicology (NCCT)

EPA's Computational Toxicology Communities of Practice

January 25th, 2018

U.S. Environmental Protection Agency

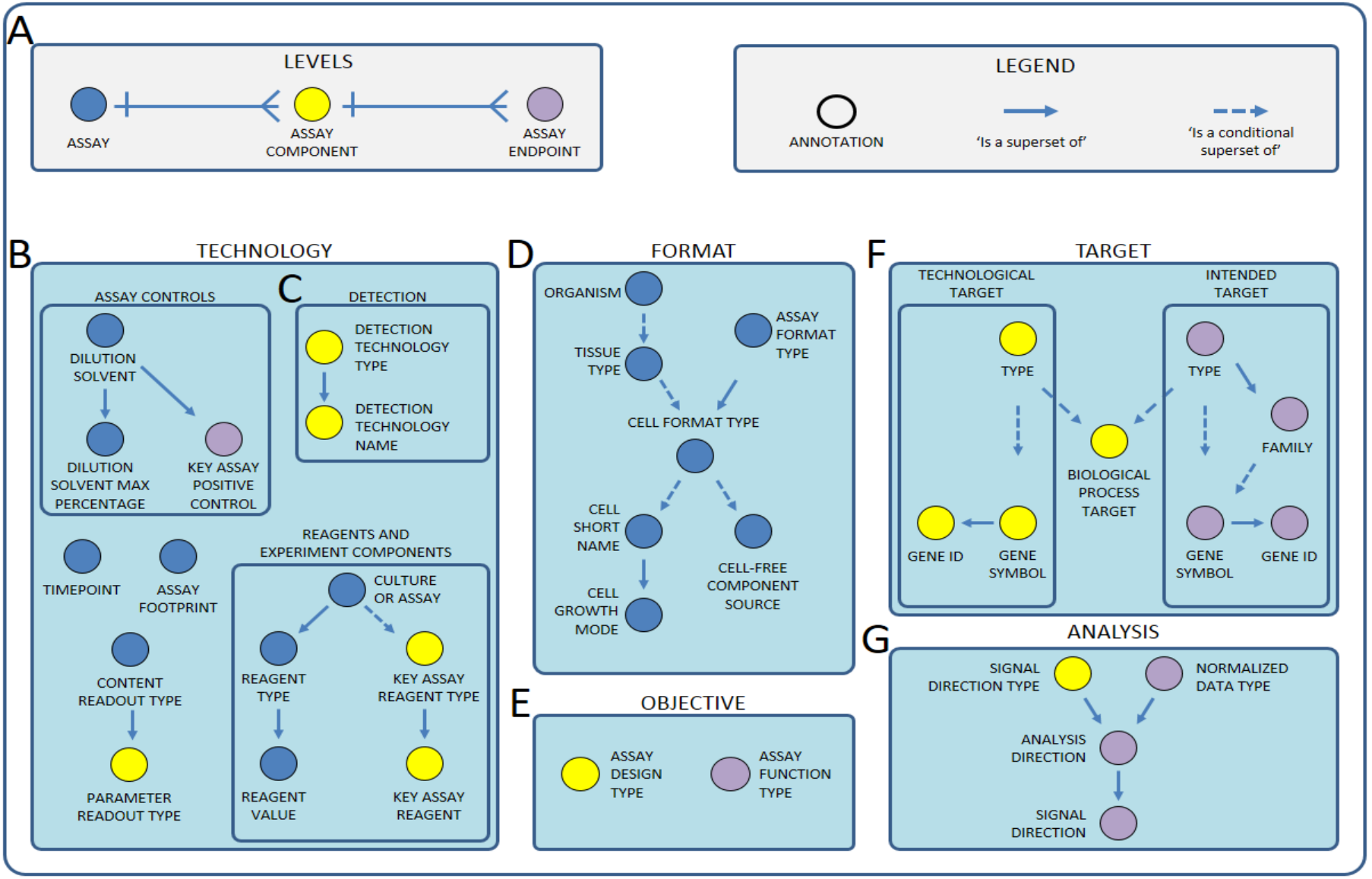
Overview

- ToxCast annotation framework and needs
- Overview of OECD reporting guidelines for *in vitro* assays
- EPA NCCT implementation of OECD recommendations
- Assay Method Descriptions - Reporting Template
- Status of Public Distribution
- Future goals

ToxCast Assay Documentation Objectives

- Provide the highest level of accessibility and transparency
- Encourage confidence in assay activity
  - Need for a ***comprehensive description*** of in vitro assay methods to facilitate knowledgeable / accurate interpretation of results
  - Clearly define the nature of 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)

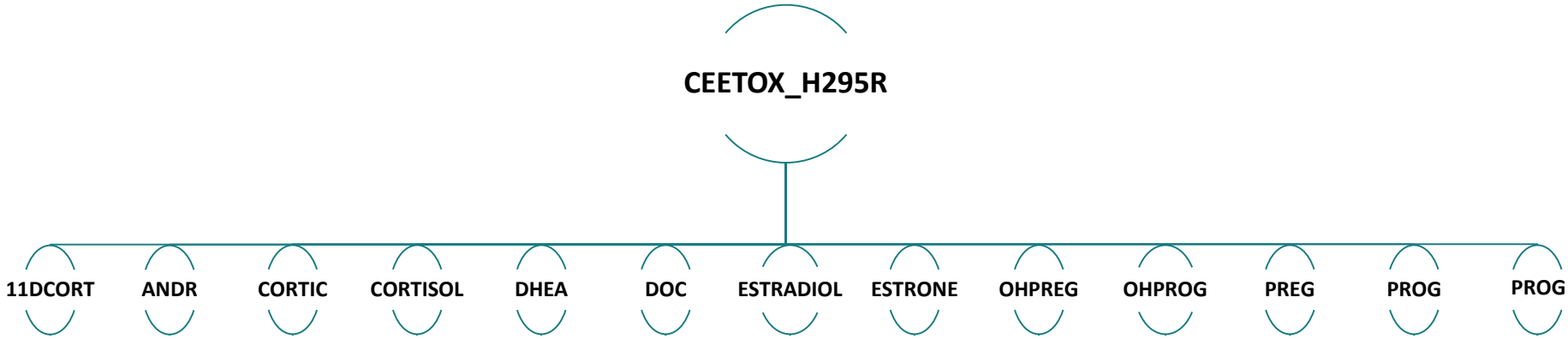
ToxCast Bioassay Ontology (BAO)-based Annotations

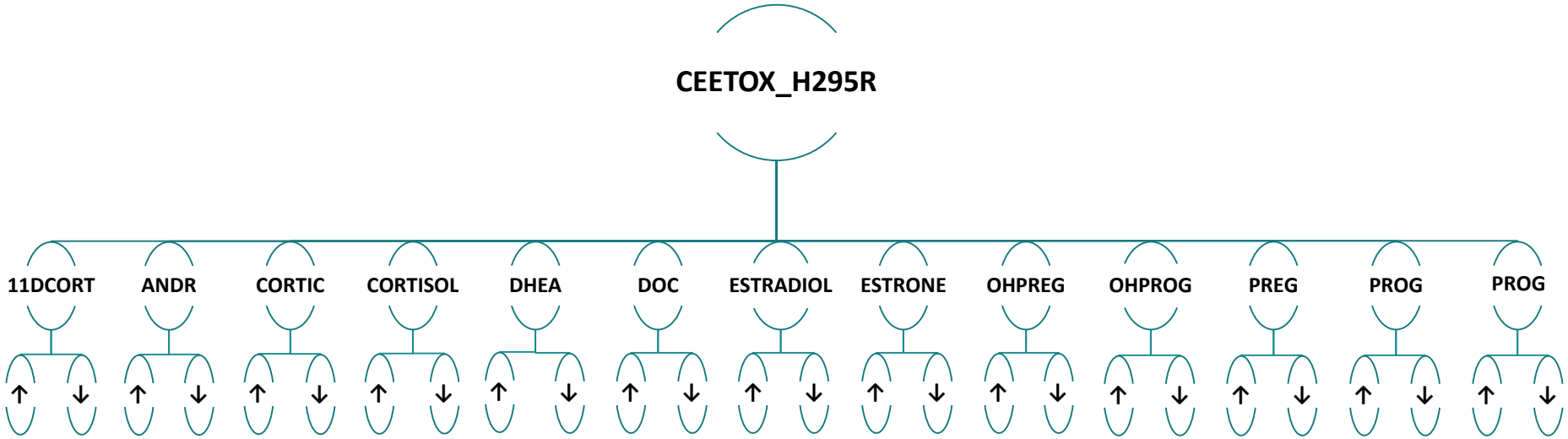


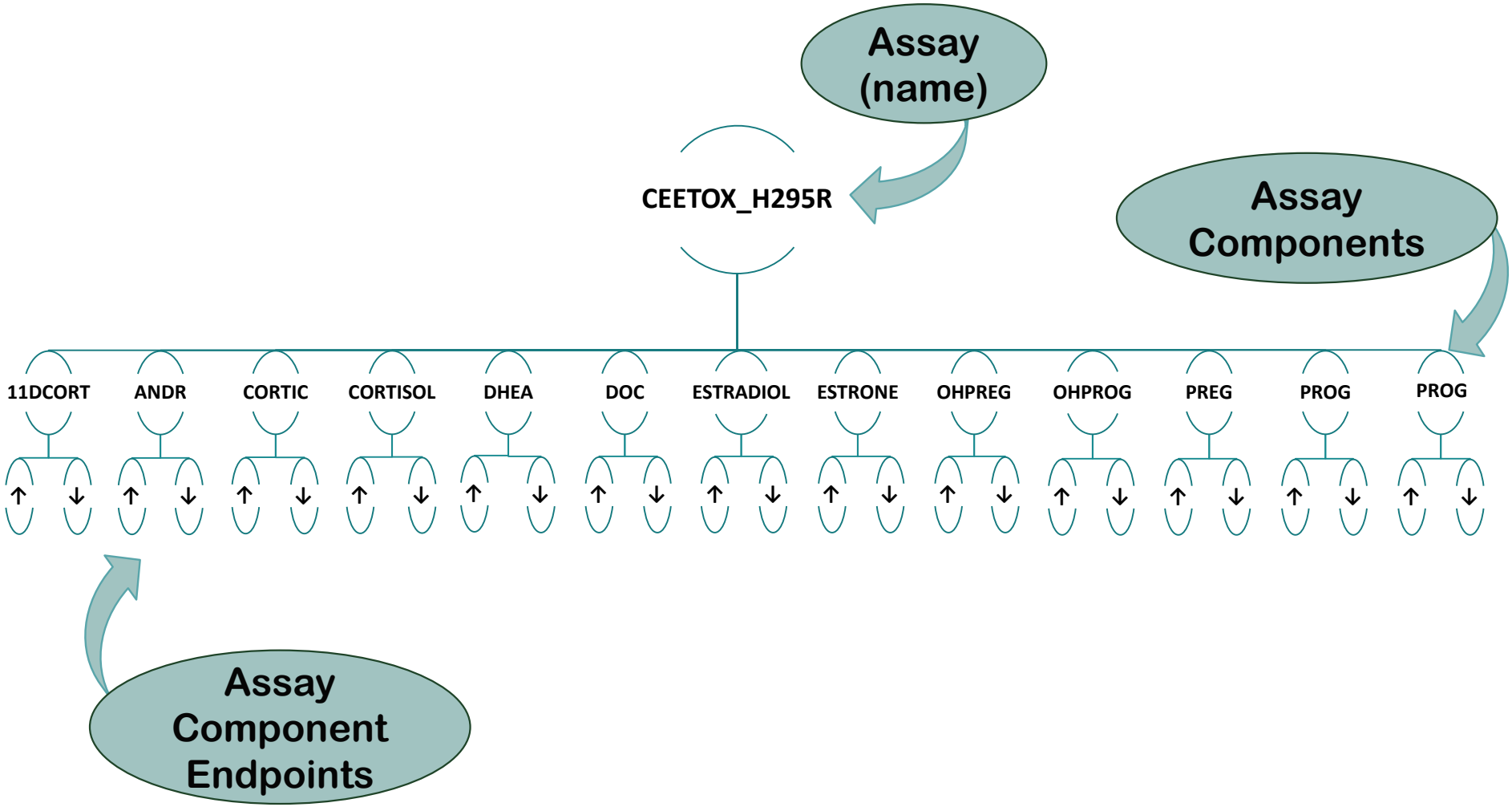
(Image from Phuong, J., *et al*. (2014). ToxCast Assay Annotation Version 1.0 Data User Guide. U.S. Environmental Protection Agency., pp. 36. https://www.epa.gov/sites/production/files/2015-08/documents/ toxcast_assay_annotation_data_users_guide_20141021.pdf).

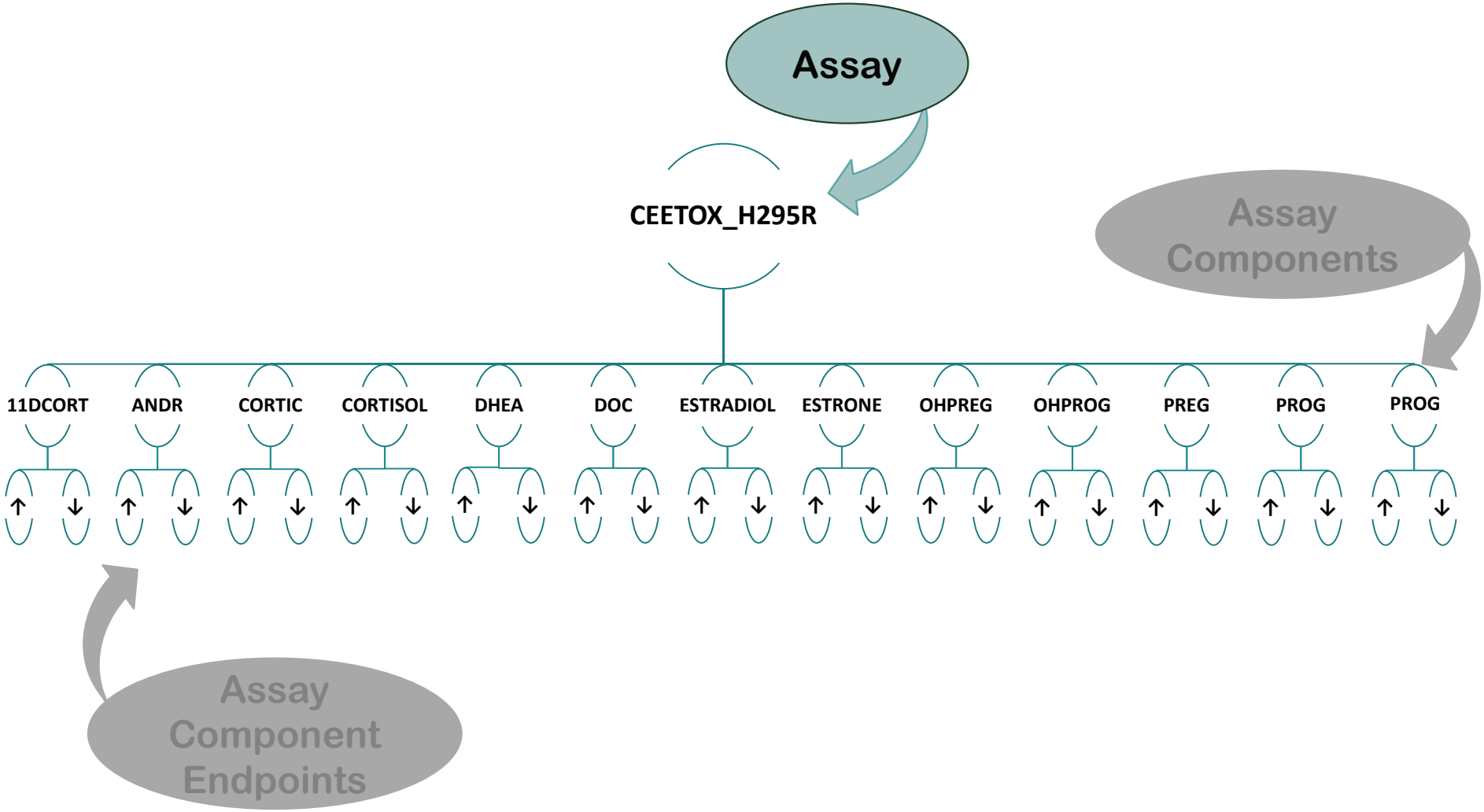
ToxCast Annotations

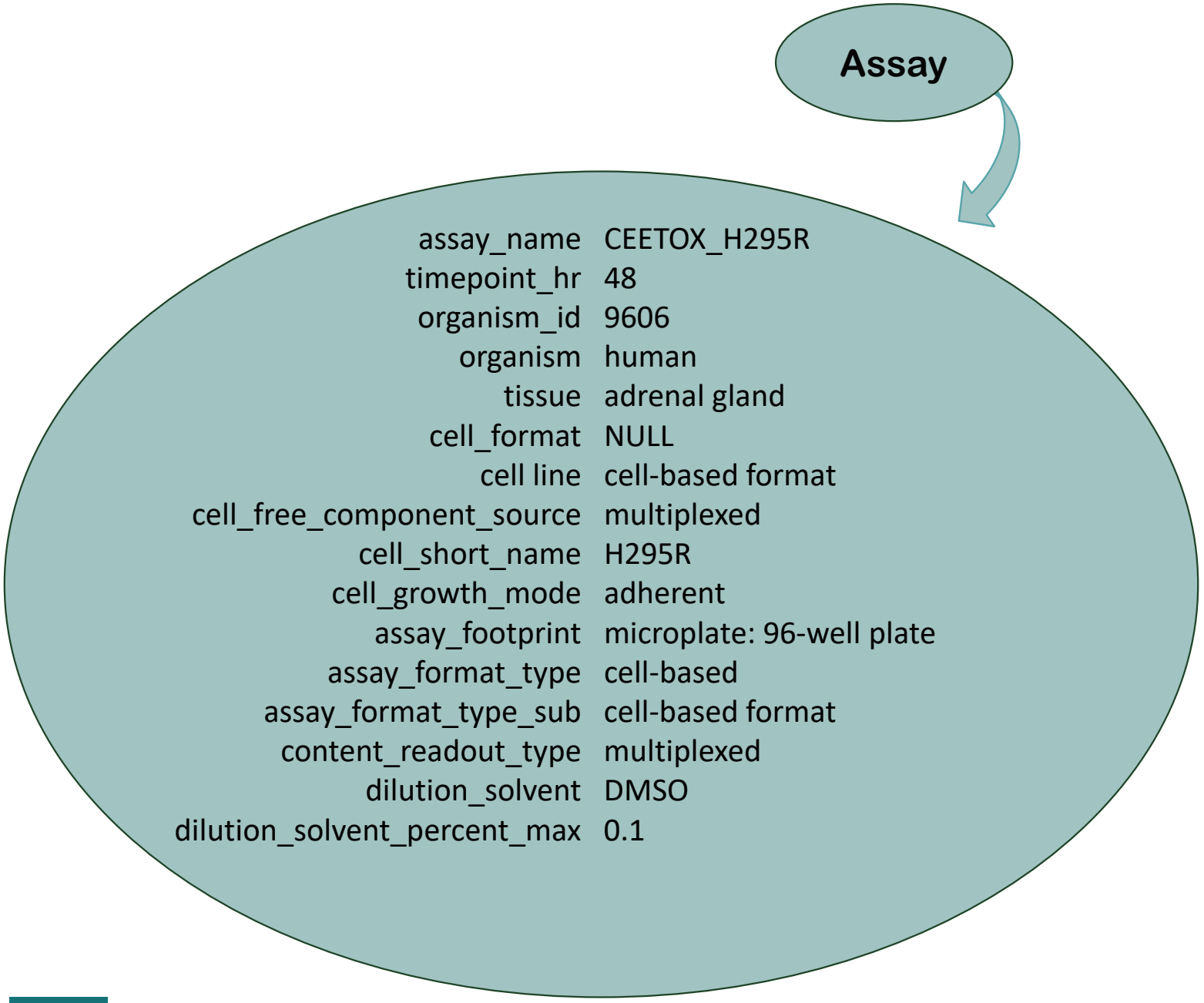
In vitro database (invitrodb) schema and example documentation

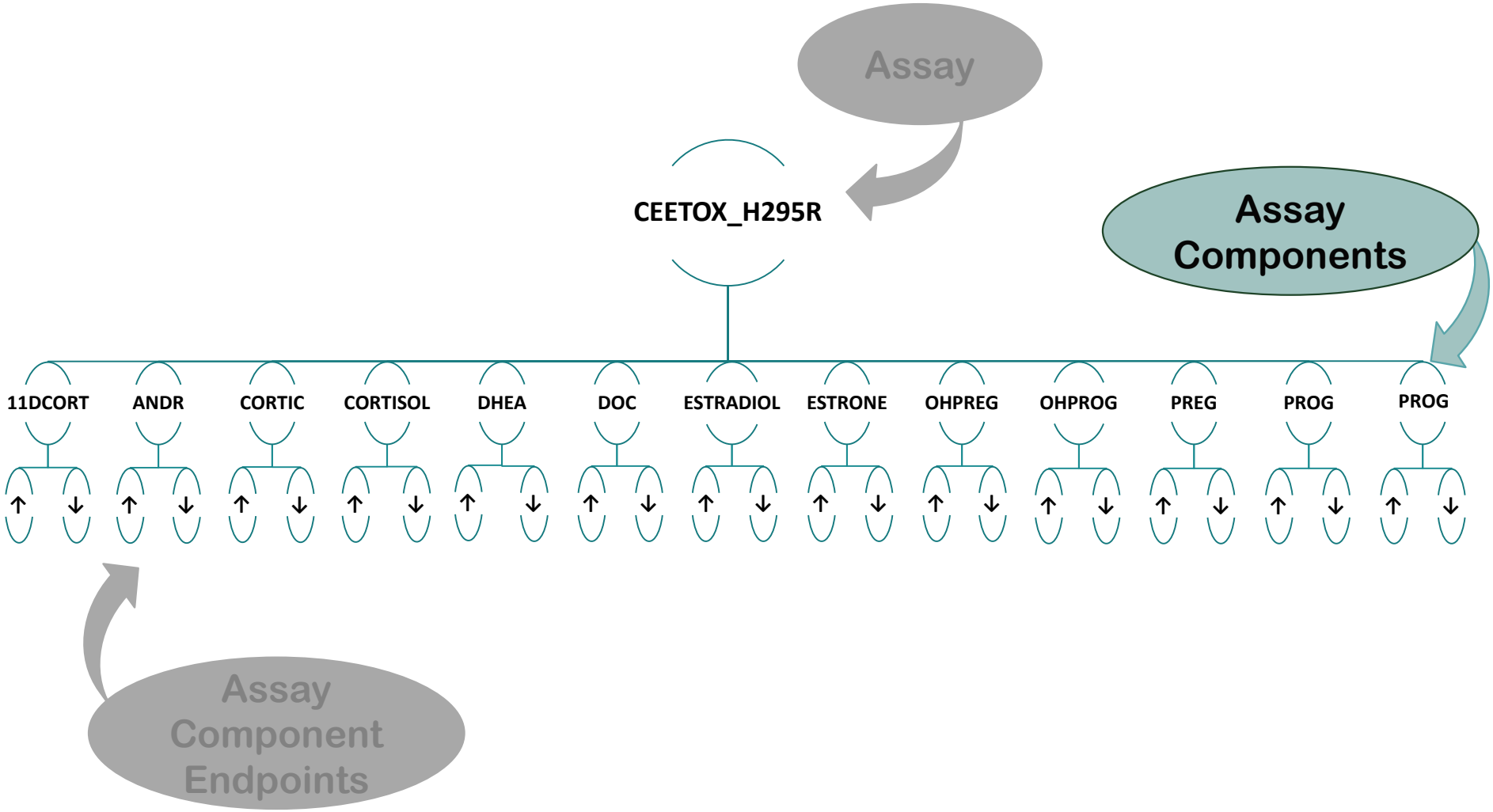

CEETOX_H295R
11DCORT
ANDR
CORTIC
CORTISOL
DHEA
DOC
ESTRADIOL
ESTRONE
OHPREG
OHPROG
PREG
PROG
PROG

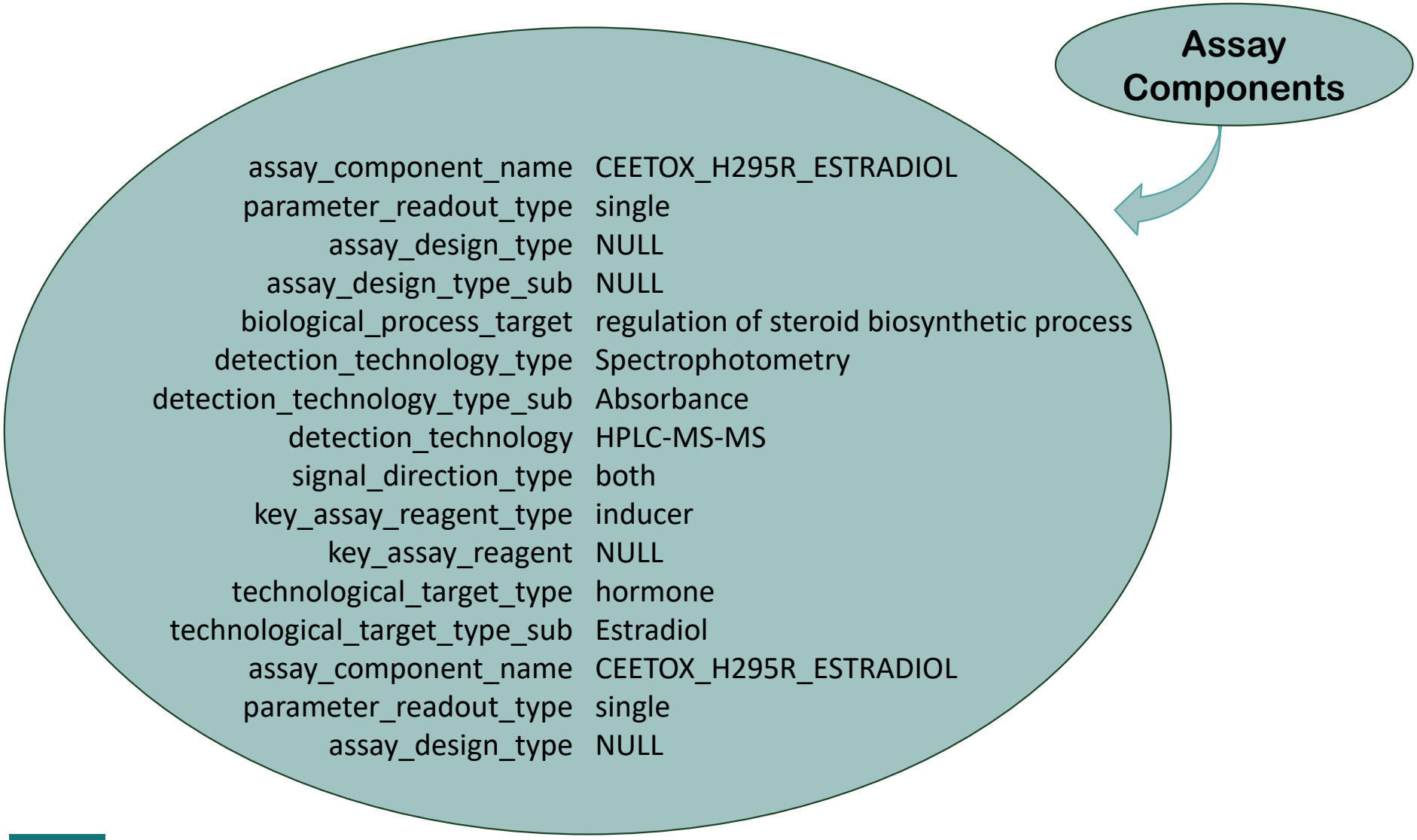

CEETOX_H295R
11DCORT
ANDR
CORTIC
CORTISOL
DHEA
DOC
ESTRADIOL
ESTRONE
OHPREG
OHPROG
PREG
PROG
PROG

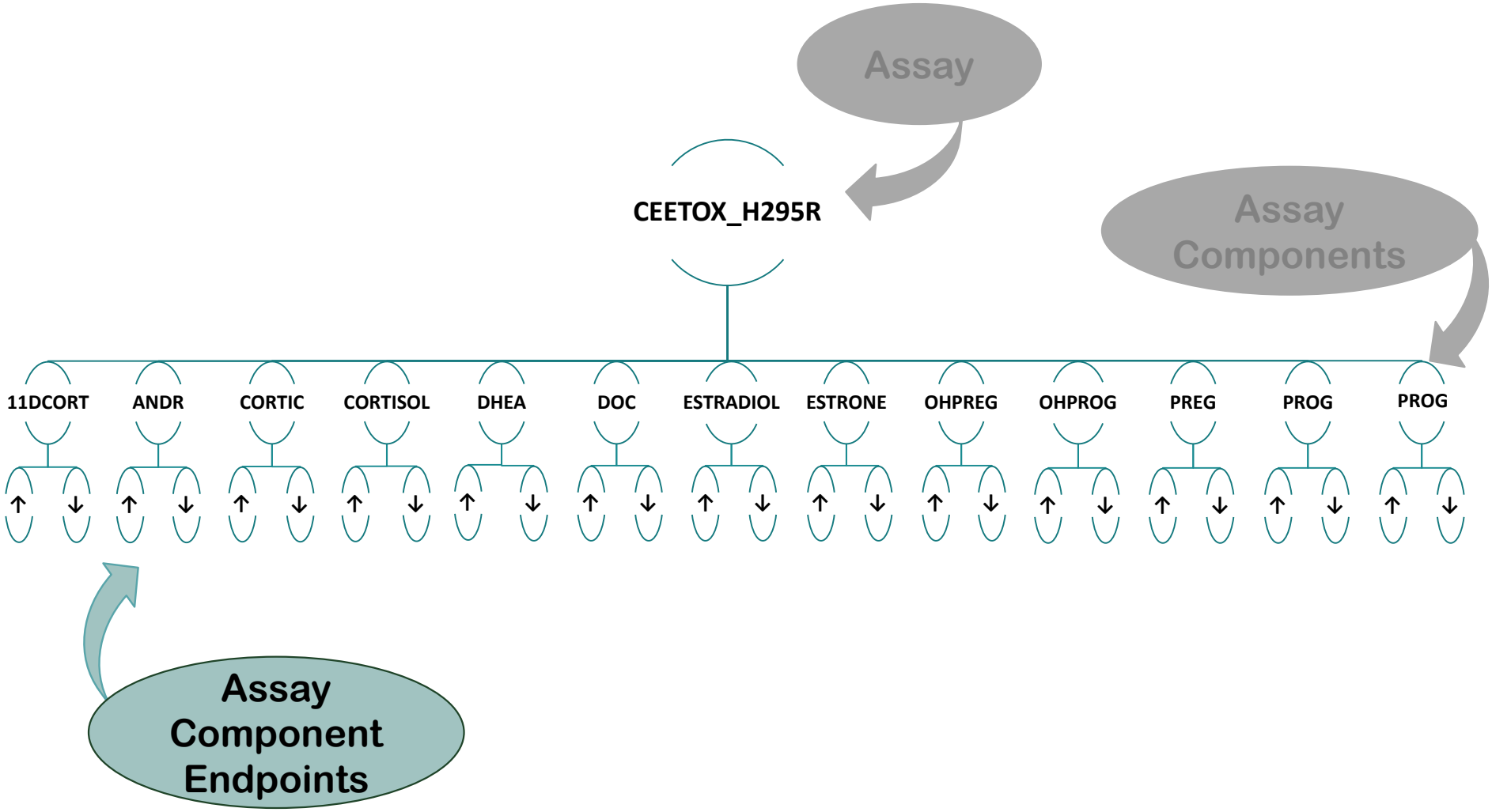

Assay (name)
CEETOX_H295R
Assay Components
11DCORT
ANDR
CORTIC
CORTISOL
DHEA
DOC
ESTRADIOL
ESTRONE
OHPREG
OHPROG
PREG
PROG
PROG
Assay Component Endpoints

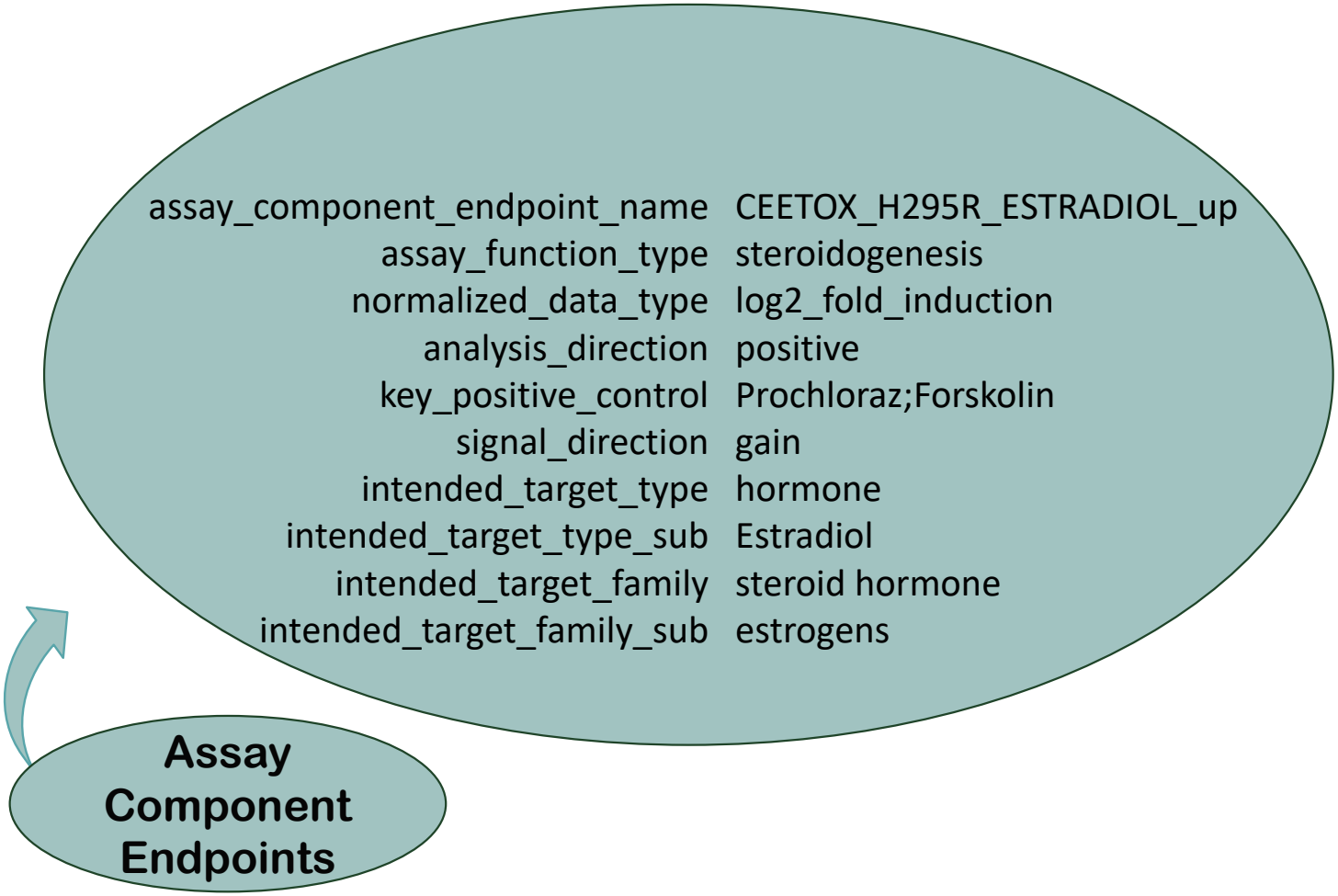

Assay
CEETOX_H295R
Assay Components
11DCORT
ANDR
CORTIC
CORTISOL
DHEA
DOC
ESTRADIOL
ESTRONE
OHPREG
OHPROG
PREG
PROG
PROG
Assay Component Endpoints

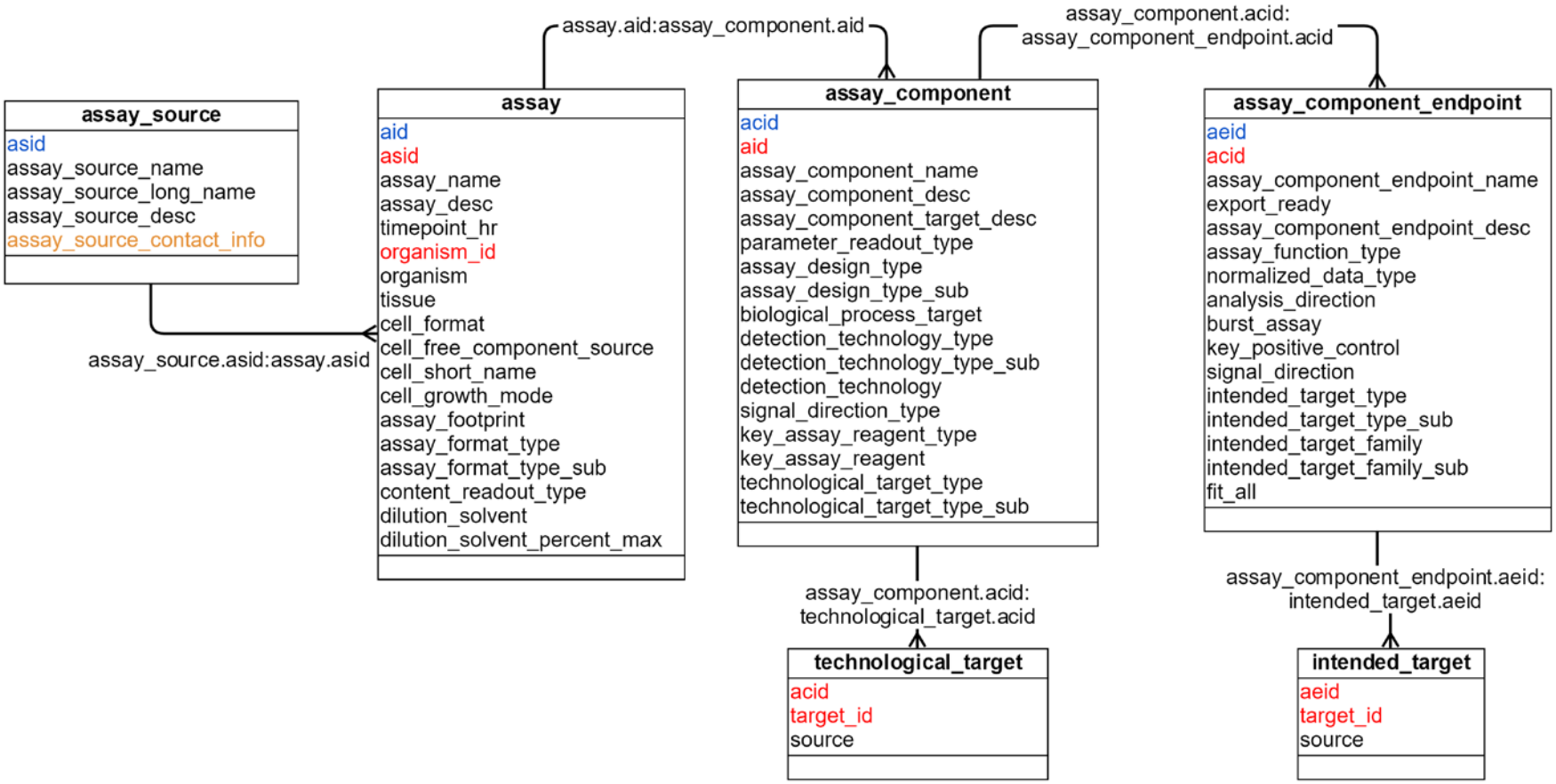

Assay
assay_name CEETOX_H295R
timepoint_hr 48
organism_id 9606
organism human
tissue adrenal gland
cell_format NULL
cell line cell-based format
cell_free_component_source multiplexed
cell_short_name H295R
cell_growth_mode adherent
assay_footprint microplate: 96-well plate
assay_format_type cell-based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


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

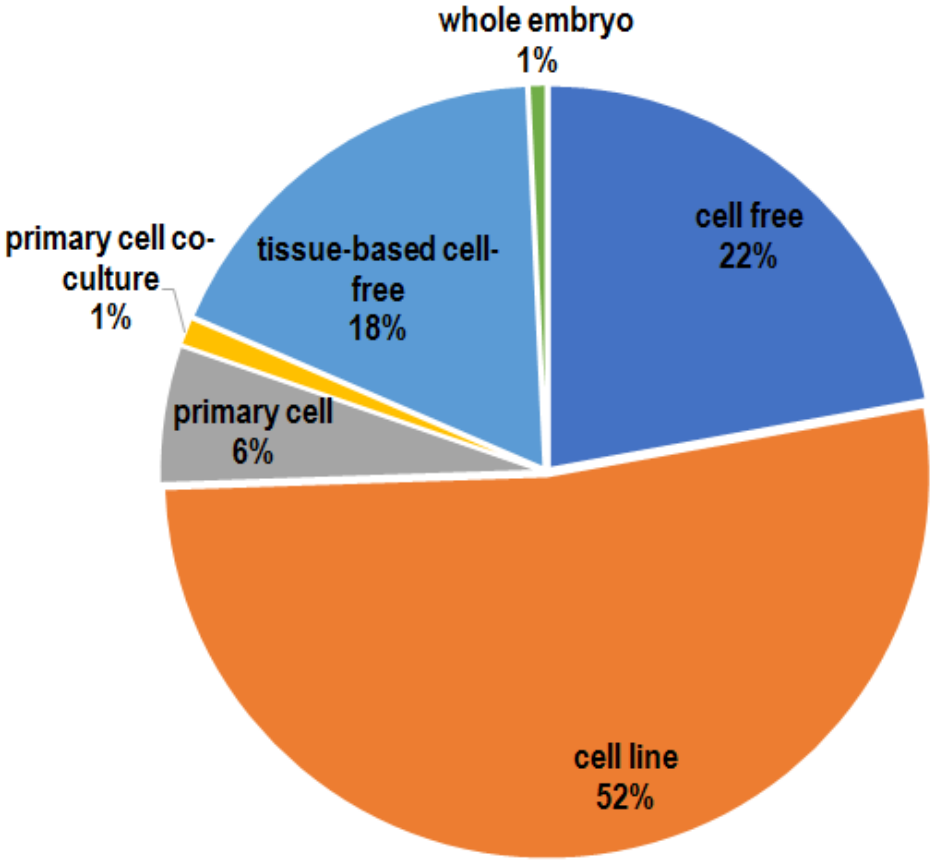

Assay Components
assay_component_name CEETOX_H295R_ESTRADIOL
parameter_readout_type single
assay_design_type NULL
assay_design_type_sub NULL
biological_process_target regulation of steroid biosynthetic process
detection_technology_type Spectrophotometry
detection_technology_type_sub Absorbance
detection_technology 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
CEETOX_H295R
Assay Components
11DCORT
ANDR
CORTIC
CORTISOL
DHEA
DOC
ESTRADIOL
ESTRONE
OHPREG
OHPROG
PREG
PROG
PROG
Assay Component Endpoints

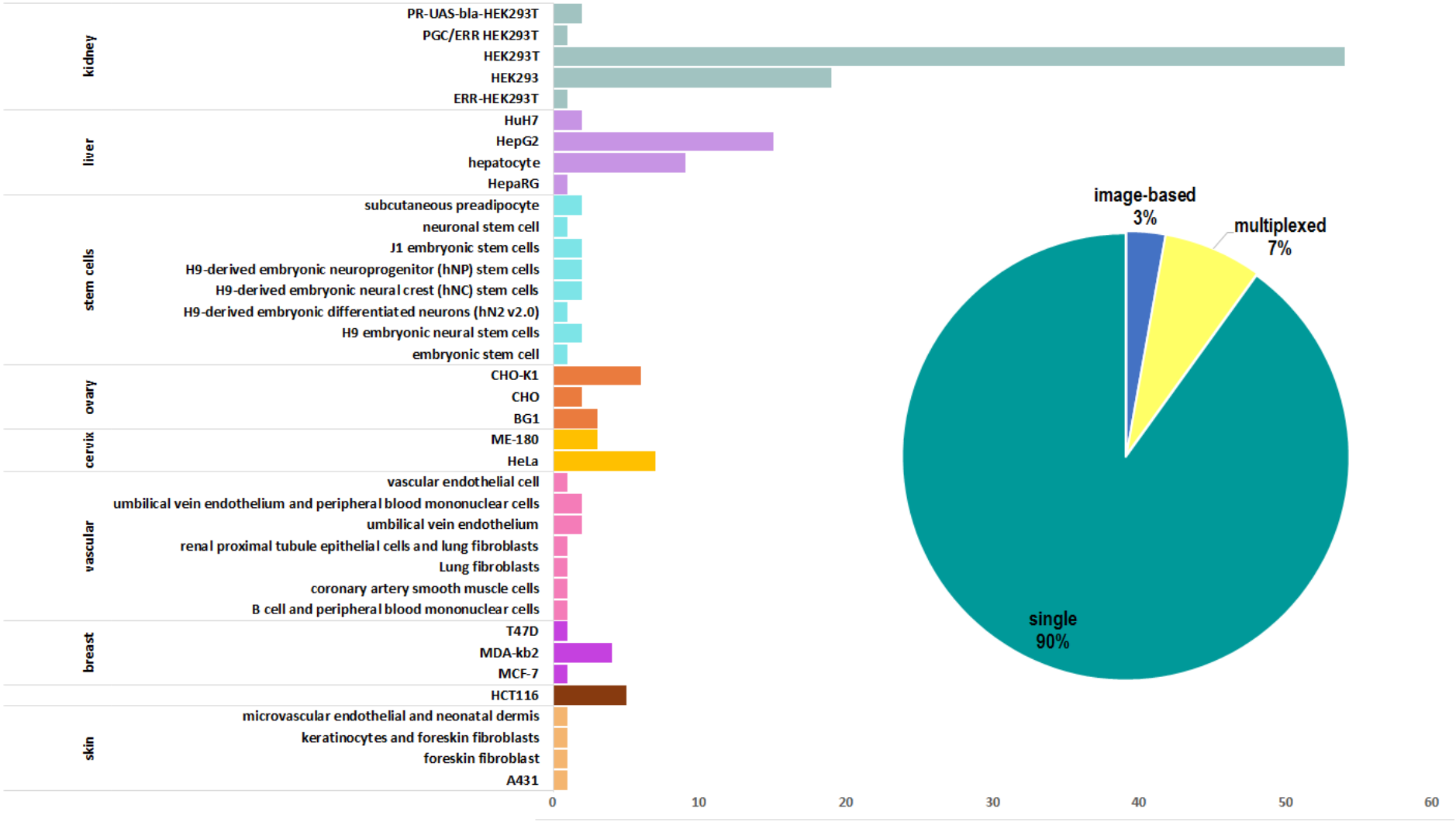

assay_component_endpoint_name CEETOX_H295R_ESTRADIOL_up
assay_function_type steroidogenesis
normalized_data_type log2_fold_induction
analysis_direction positive
key_positive_control Prochloraz;Forskolin
signal_direction gain
intended_target_type hormone
intended_target_type_sub Estradiol
intended_target_family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


assay.aid:assay_component.aid
assay_component.acid:
assay_component_endpoint.acid
assay_source
asid
assay_source_name
assay_source_long_name
assay_source_desc
assay_source_contact_info
assay_source.asid:assay.asid
assay
aid
asid
assay_name
assay_desc
timepoint_hr
organism_id
organism
tissue
cell_format
cell_free_component_source
cell_short_name
cell_growth_mode
assay_footprint
assay_format_type
assay_format_type_sub
content_readout_type
dilution_solvent
dilution_solvent_percent_max
assay_component
acid
aid
assay_component_name
assay_component_desc
assay_component_target_desc
parameter_readout_type
assay_design_type
assay_design_type_sub
biological_process_target
detection_technology_type
detection_technology_type_sub
detection_technology
signal_direction_type
key_assay_reagent_type
key_assay_reagent
technological_target_type
technological_target_type_sub
assay_component_endpoint
aeid
acid
assay_component_endpoint_name
export_ready
assay_component_endpoint_desc
assay_function_type
normalized_data_type
analysis_direction
burst_assay
key_positive_control
signal_direction
intended_target_type
intended_target_type_sub
intended_target_family
intended_target_family_sub
fit_all
assay_component.acid:
technological_target.acid
assay_component_endpoint.aeid:
intended_target.aeid
technological_target
acid
target_id
source
intended_target
aeid
target_id
source

ToxCast Annotations
Use Case ***Bioinformatics***



ToxCast Annotations - Use Case ***Data Aggregation***



ToxCast Annotations
Regulatory / Chemical Assessment Needs

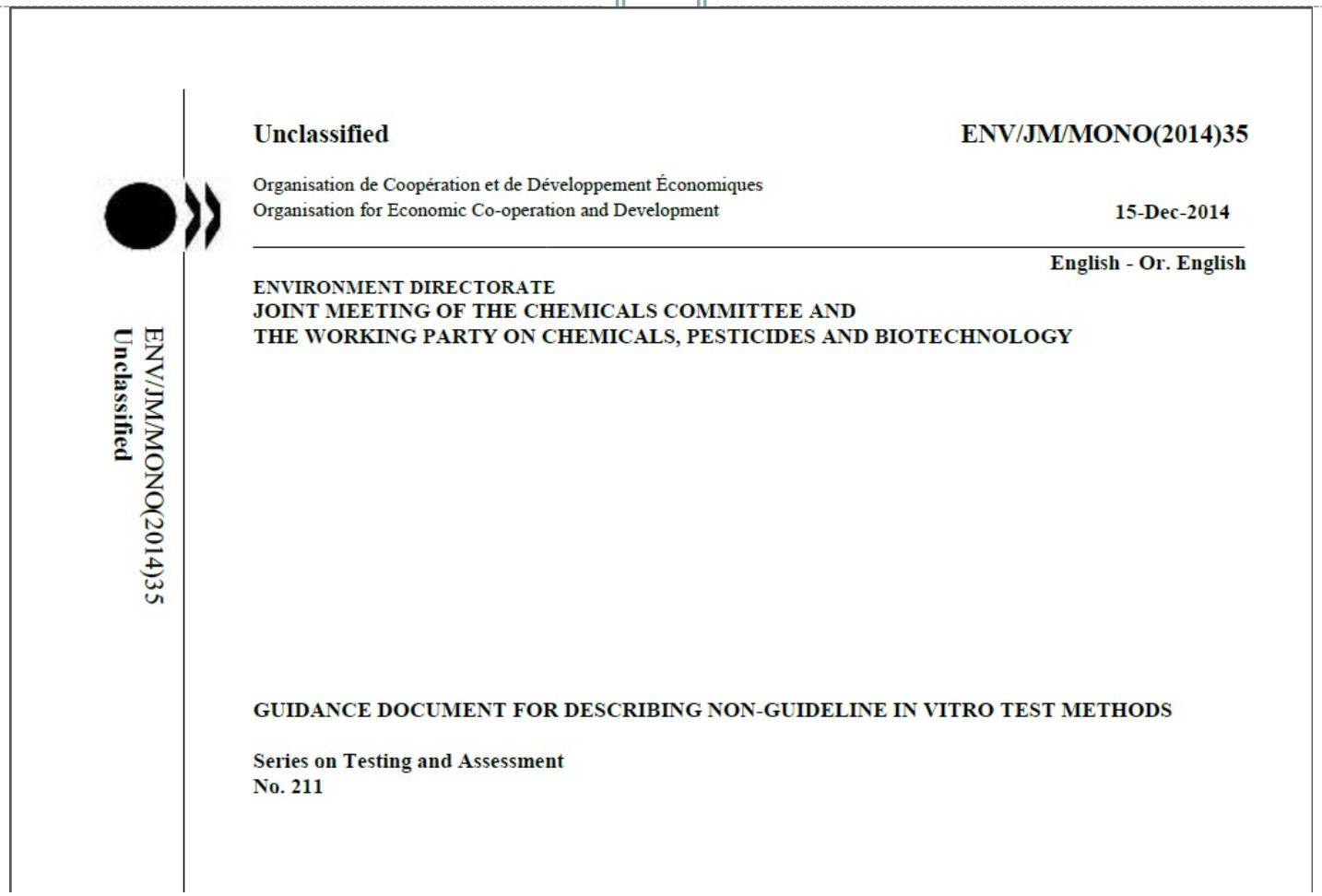
- Information needs to be assessed as part of a ***larger biological context***
- ***Gaps in information*** exist about how in vitro activity should be considered in relation to in vivo studies
- ***High-throughput assay technologies*** allow for screening of large (previously untested) chemical libraries,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

"Solna Principles" for the validation of new or updated test methods for hazard assessment

1) A rationale for the test method should be available…***a clear statement of scientific need*** and ***regulatory purpose***.
2) The ***relationship of the endpoint(s)*** determined by the test method ***to the in vivo biological effect***
3) A ***formal detailed protocol*** must be provided and should be readily available in the public domain. It should be sufficiently detailed … and it should include data analysis and decision criteria. Test methods and results should be available preferably in an independent peer reviewed publication.
4) Intra-test ***variability, repeatability and reproducibility*** of the test method within and amongst laboratories should have been demonstrated
5) The test method's performance must have been demonstrated using a series of ***reference chemicals*** preferably coded to exclude bias.
6) The performance of test methods should have been ***evaluated in relation to existing relevant toxicity data*** as well as information from the ***relevant target species***.
7) ***All data*** supporting the assessment of the validity of the test methods including the full data set collected in the validation study ***must be available for review***.
8) Normally, these data should have been obtained in accordance with the OECD Principles of ***Good Laboratory Practice*** (GLP).

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



Unclassified

ENV/JM/MONO(2014)35

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

ENV/JM/MONO(2014)35
Unclassified

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"

- facilitate an assessment 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 and reports test method performance metrics (cv, z', s/b, ssmd). (Protocols to validate performance of reference chemical sets currently under development.)

- Component Endpoint Descriptions

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

- Description Documentation

  References, abbreviations and definitions

- Potential regulatory applications

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)

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.

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].

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.

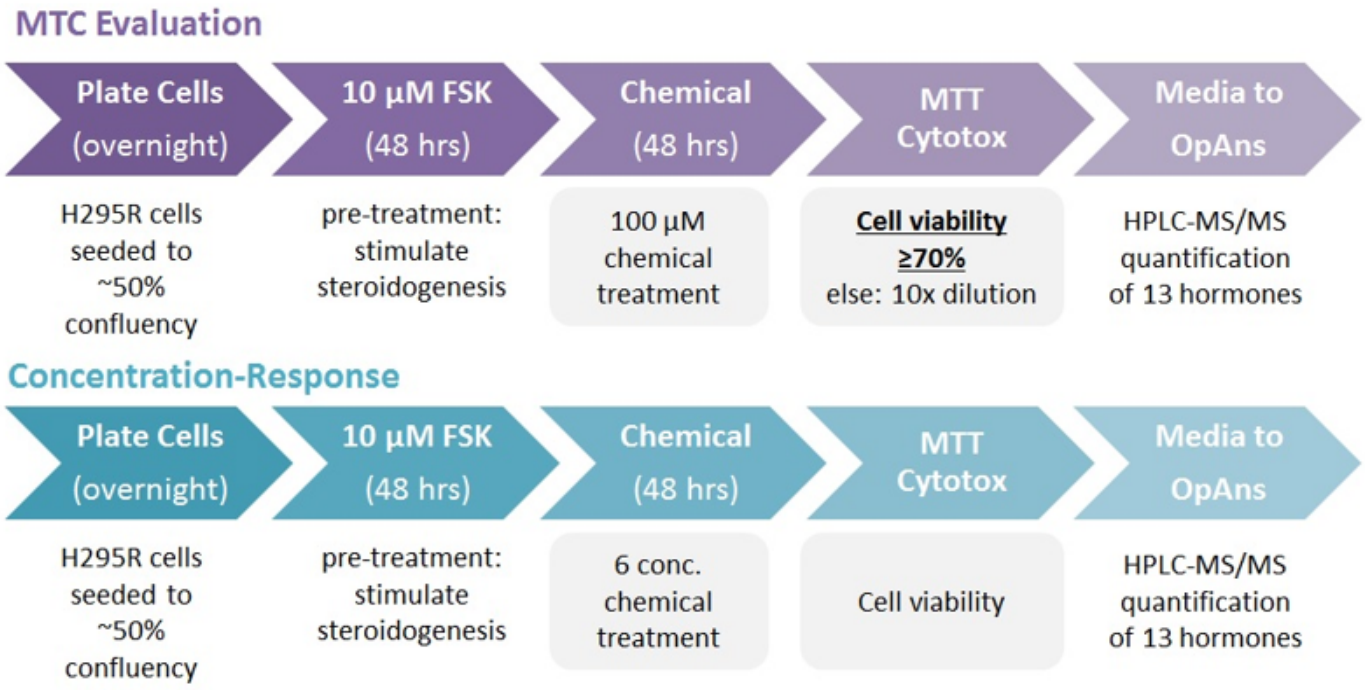


Figure 1. Schematic representation of general testing protocol for CeeTox Steroidogenesis assays. (Adapted from Karmaus et al. 2016.)

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.

1.3. Assay References

Assay Source Contact Information:

Cyprotex (Formerly CeeTox, Inc. in Kalamazoo, MI)
313 Pleasant St.
Watertown, MA 02472
1-888-297-7683
Fax: 1-617-812-0712
enquiries@cyprotex.com

Assay Publication Year:

2016

Assay Publication:

Karmaus AL, Toole CM, Filer DL, Lewis KC, Martin MT. 2016. High-throughput screening of chemical effects on steroidogenesis using H295R human adrenocortical carcinoma cells. Toxicological Sciences 150:323-332.

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The CeeTox H295R estradiol assay was used to screen a large chemical library for changes in estradiol levels resulting from interference with steroidogenesis in H295R human adrenocortical carcinoma cells. For an initial screening application, a three-tiered screening approach was employed. The first stage involved establishing the maximum tolerated concentration (MTC) for each test chemical, respectively, which was identified using the MTT assay as an indicator of cell viability. Once MTC was determined, cells were treated with that concentration to evaluate steroid hormone changes at the MTC. In this single concentration "MTC screening" assay, any response ≥ |1.5-fold| relative to DMSO controls was considered to represent significant effects on hormone production. Following the MTC single concentration screen, those chemicals with significant effects on at least 4 hormones were considered active and subsequently assessed in concentration-response format using a 6-concentration series. After chemical treatment, which consists of 48hr forskolin pre-stimulation followed by 48hr chemical treatment, medium was subjected to MBTE and dansyl chloride derivatization for estradiol quantification. HPLC-MS/MS spectra were resolved and the absolute quantity of estradiol from the medium sample was reported in ng/mL. Chemicals were tested in duplicate, and each plate included duplicated controls for stimulation (10µM forskolin) and inhibition (3 µM procholaz) of steroidogenesis. 4 replicates per plate of DMSO-only solvent controls served as neutral controls. The LLOQ and LLOD for estradiol were reported as 0.03 ng/mL and 6 ng/mL, respectively, using 0.4, 1, and 16 ng/mL standards, with precision of 6.3% and accuracy of 101.4% (Karmaus *et al.*, 2016, Table 1).

Scientific Principles:

The steroidogenic pathway is a series of hydroxylation and dehydrogenation steps carried out by CYP450 and hydroxysteroid dehydrogenase enzymes. Environmentally-relevant chemicals can elicit endocrine disruption by altering steroid hormone biosynthesis and metabolism (steroidogenesis), resulting in adverse reproductive and developmental effects. The ToxCast HTS program adapted the OECD-validated H295R steroidogenesis assay using human adrenocortical carcinoma cells (TG No. 456; which established performance-based testing guidelines for the quantification of two steroid hormones, testosterone and estradiol) to quantitatively assess the concentration-dependent effects of chemicals on 13 steroid hormones in 4 hormone classes including progestagens, androgens, estrogens and glucocorticoids.

To identify xenobiotics with the capacity to disrupt the steroid hormone biosynthetic pathway, this assay quantitatively assesses changes in steroid hormone levels. The CeeTox Steroidogenesis assay also demonstrates a novel implementation of H295R cells as a high-throughput and multiplexed screening platform for screening a diverse chemical library for xenobiotic interference with steroidogenesis.

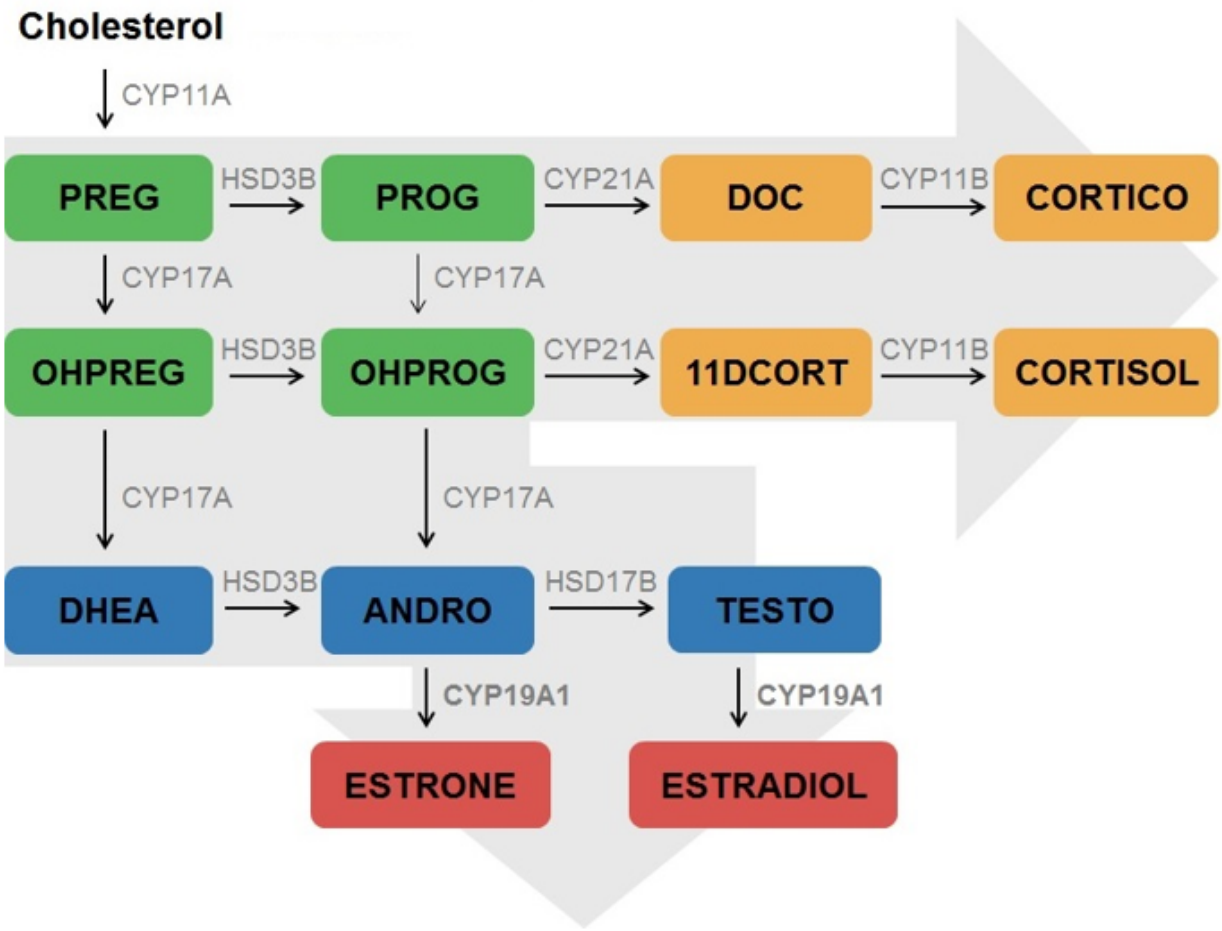


Figure 2. Subset of the steroidogenesis pathway targeted by the CeeTox H295R Steroidogenesis assays. (Modified from Karmaus *et al.* 2016.)

Method Development Reference:

Nielsen FK, Hansen CH, Fey JA, Hansen M, Jacobsen NW, Halling-Sørensen B, Björklund E, Styrishave B. (2012). "H295R cells as a model for steroidogenic disruption: a broader perspective using simultaneous chemical analysis of 7 key steroid hormones". Toxicology In Vitro 26:343-350.

OECD (2011). *Test No. 456 H295R Steroidogenesis Assay.* OECD Publishing, Paris

USEPA (2011). *Steroidogenesis (Human Cell Line – H295R)* OCSPP Guideline 890.1550. Standard Evaluation Procedure (SEP) Endocrine Disruptor Screening Program.

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Increased production of estradiol following interference with steroidogenesis as quantified by HPLC-MS/MS.

Analytical Elements:

CEETOX_H295R_ESTRADIOL_up readout data was analyzed in the positive (gain of signal) fitting direction using the DMSO controls as the baseline signal, and was reported as log2 fold-change increase in estradiol activity (compared between duplicate chemical treatment and plate-matched quadruplet DMSO controls). Data were log_2-transformed, 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 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 AIC value is considered the winning model and used in further analysis as the most appropriate predictor of assay effects. Estradiol stimulation was determined based on chemical-response data fulfilling the following criteria: the median of normalized response values at a single concentration exceeded the signal noise band (in this assay, any response over 6 times the baseline median absolute deviation in log_2 fold-change, which was approximately a 2.05 fold-change cut-off); the modeled top (modl_tp) of the curve was above the established response cutoff; and 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 publically available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

CEETOX_H295R_11DCORT_dn
CEETOX_H295R_11DCORT_up
CEETOX_H295R_ANDR_dn
CEETOX_H295R_ANDR_up
CEETOX_H295R_CORTIC_dn
CEETOX_H295R_CORTIC_up
CEETOX_H295R_CORTISOL_dn
CEETOX_H295R_CORTISOL_up
CEETOX_H295R_DHEA_dn
CEETOX_H295R_DHEA_up
CEETOX_H295R_DOC_dn
CEETOX_H295R_DOC_up
CEETOX_H295R_ESTRADIOL_dn
CEETOX_H295R_ESTRONE_dn
CEETOX_H295R_ESTRONE_up
CEETOX_H295R_MTT_Cytotoxicity_dn
CEETOX_H295R_MTT_Cytotoxicity_up
CEETOX_H295R_OHPREG_dn
CEETOX_H295R_OHPREG_up
CEETOX_H295R_OHPROG_dn
CEETOX_H295R_OHPROG_up
CEETOX_H295R_PREG_dn
CEETOX_H295R_PREG_up
CEETOX_H295R_PROG_dn
CEETOX_H295R_PROG_up
CEETOX_H295R_TESTO_dn
CEETOX_H295R_TESTO_up

3.2. Assay Performance

Assay Performance Measures:

One measure of assay performance is reproducibility. For the initial screening exercise that employed single-concentration and concentration-response phases, a Pearson's linear correlation test, using the median $\log_2$-fold change results from the MTC, was performed to demonstrate the concordance between the single-concentration and multi-concentration phases of screening. The correlation of maximum fold-change (r^2) is presented in Karmaus et al. 2016, Table 2. The correlation coefficients for each hormone between the two phases of that study, conducted independently, are presented by an average $r^2 = 0.70$. For estradiol, the $r^2 = 0.64$. These values demonstrate that the assay is highly reproducible.

Assay Quality Statistics:

Robust Z-prime (Z') was calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and positive control (forskolin and prochloraz) wells. The Z' for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. Z'-forskolin ranged from 0.57-0.81, with a Z'-forskolin of 0.73 for estradiol. Z'-prochloraz ranged from 0.51-0.88, with a Z'-prochloraz of 0.72 for estradiol. Z' values ranging between 0.5 and 1 demonstrate that an assay signal can be reliably identified from variability around the baseline control.

The strictly standardized mean difference (SSMD) values were calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and positive control (forskolin and prochloraz) wells. The SSMD for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. SSMD-forskolin ranged from 1-18, with a SSMD-forskolin of 13 for estradiol. SSMD-prochloraz ranged from -27-7, with a SSMD-prochloraz of -11 for estradiol. SSMD is a measure of effect size and a means of demonstrating directionality, i.e. differences between positive and negative control response; values $\geq |7|$ demonstrate excellent dynamic range and robust responses with the positive and negative controls.

Reference Chemicals / Predictive Capacity:

(Ongoing project to develop reference chemical performance metrics for a wide variety of assays currently in progress)

3.3. Assay Scope and Limitations

Chemical Library:

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 [6].

4. Assay Documentation

4.1. References

[1] OECD (2011). Test No. 456 H295r Steroidogenesis Assay. Paris, OECD Publishing.
[2] Gracia, T., et al. (2006). Ecotoxicol Environ Saf 65(3): 293-305.
[3] Sanderson, J. T., et al. (2001). Toxicol Sci 61(1): 40-48. (PMID: 11294972)
[4] Jacobs, M., et al. (2008). Curr Drug Metab 9(8): 796-826. (PMID: 18855613)
[5] Mosmann, T. (1983). J Immunol Methods 65(1-2): 55-63.
[6] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
ATCC, American Type Culture Collection
DMSO, Dimethyl Sulfoxide
HPLC-MS/MS, High-Performance Liquid Chromatography, with tandem Mass Spectrometry
MTC, Maximum Tolerated Concentration
MTT, 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide
R^2, correlation coefficient, R-squared
SSMD, strictly standardized mean difference
Z', Robust Z-prime

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:

17 January 2017

Date of Revisions:

Author of Revisions:

5. Supporting Information:

ToxCast Assay Annotation Structure

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.

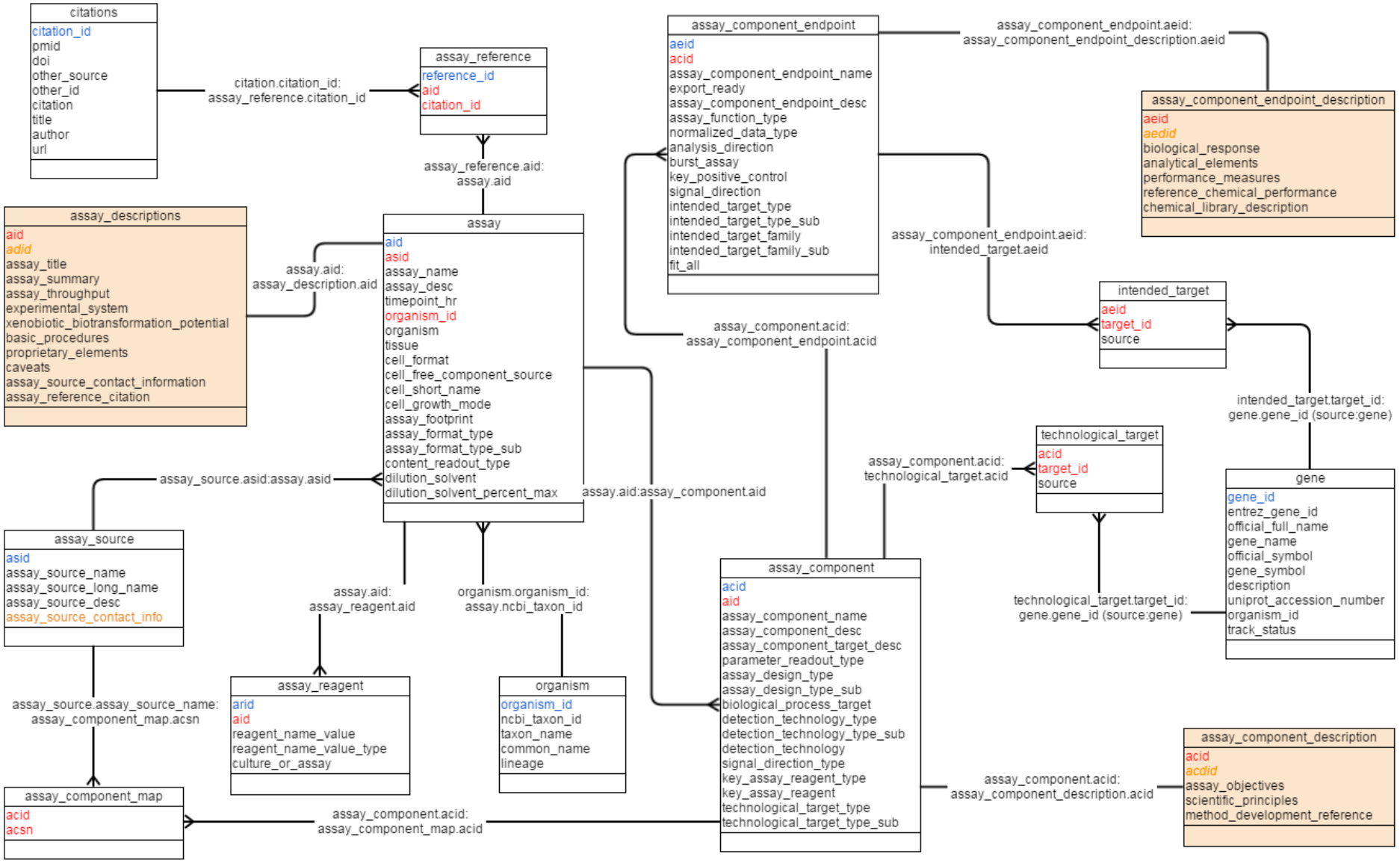
Caveats:

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.

ToxCast Assay Description Document Database;
Integration into *invitrodb*



European Commission

JOINT RESEARCH CENTRE

EURL ECVAM DataBase service on ALternative Methods to animal experimentation (DB-ALM)

About the DB-ALM

Welcome to the DB-ALM version with an entirely revised data retrieval approach

The DB-ALM is a **public, factual** database service that provides **evaluated information** on development and applications of advanced and alternative methods to animal experimentation in biomedical sciences and toxicology, both in research and for regulatory purposes.

Starting from 2015, method summaries are provided in a format compliant with the new OECD Guidance. Read more

News

Updated statistics of DB-ALM usage available. More

Comprehensive DB-ALM Activity Report can be accessed from here

EURL ECVAM Status Report 2017 on alternative methods now available online

The service is operated by the **European Commission's Joint Research Centre** and implements a mandate of the EURL ECVAM (EU Reference Laboratory on Alternatives to Animal Testing) in relation to Directive 2010/63/EU[1,2] on the protection of animals used for scientific purposes

The current version of the DB-ALM covers the following Data Sectors:

Topic Summaries: Thematic review data sheets in a form of an executive summary on alternative methods, available in the DB-ALM for an entire topic area (e.g. Percutaneous Absorption, Eye Irritation).

Method Descriptions: Two levels of detail: i) method summaries covering the scientific principle, the needs addressed, main applications, and current status of the development, validation or acceptance; ii) protocols include detailed technical instructions to enable the transfer of a method to a laboratory.

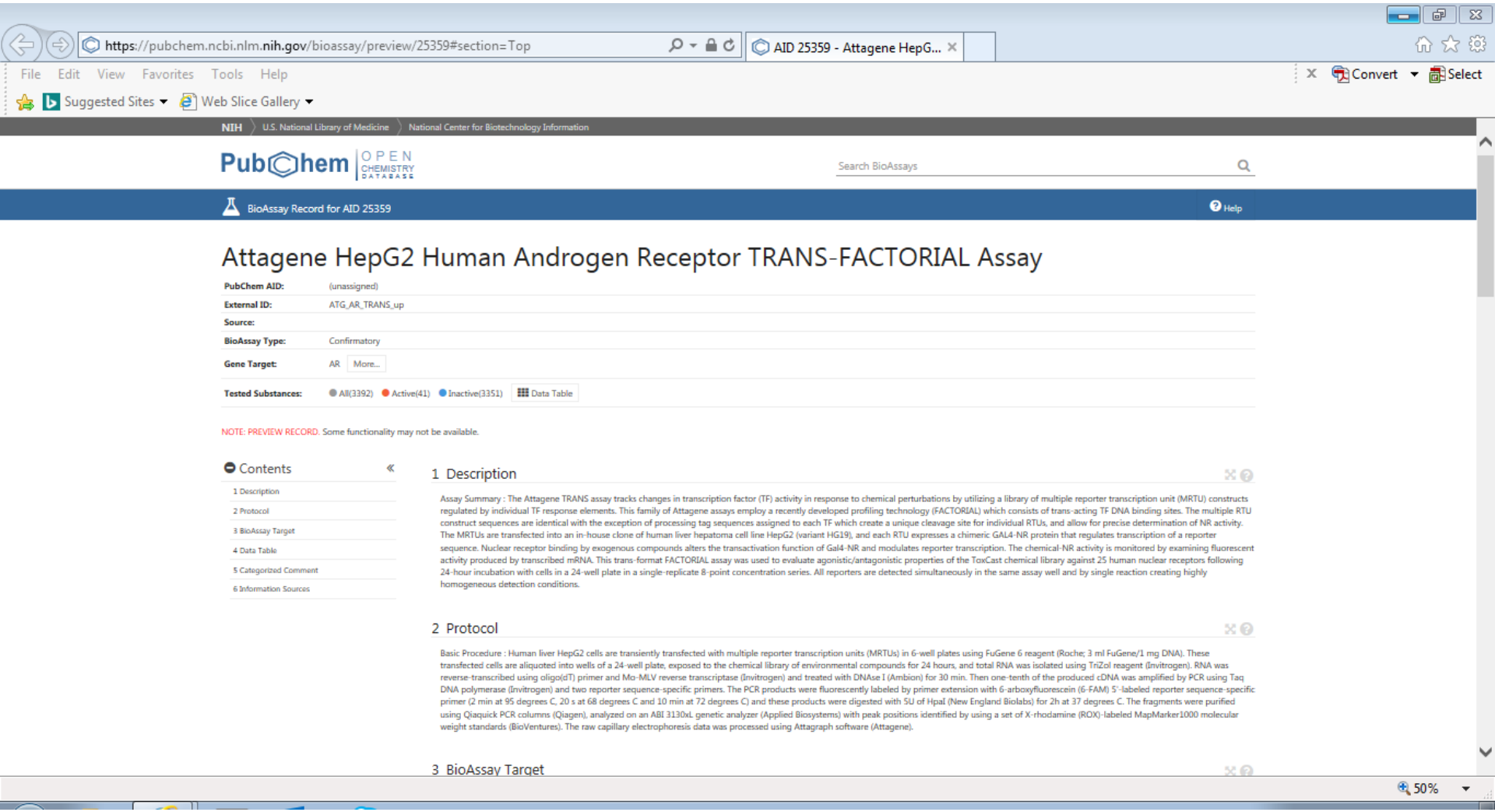
Project & Study Descriptions: Evaluations of methods, including EU integrated projects and selected formal validation studies as summary records, cross-referenced with related data sectors.

Compounds & Test Results: Lists of substances and selected individual investigations performed with methods included in the DB-ALM.

People & Institutions: Information on people and institutions active in the field of alternative methods is provided based on voluntary participation.

Bibliography: All references analysed for the compilation of the data sheets.

Other public repositories of published Methods :



Acknowledgments

National Center for Computational Toxicology, US EPA