Evaluating Perturbation of *In Vitro* Steroidogenesis Using a High-throughput H295R Assay

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Introduction

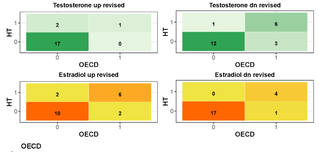
Background: Endocrine disruption is a toxicity of both physiological and regulatory importance; as steroid hormones regulate reproduction, development, and other biological processes, it is a priority to identify chemicals that may interact with production of these hormones. A high-throughput H295R assay (HT-H295R) was developed as part of the U.S. EPA's ToxCast program that includes measurement of 11 hormones across the steroid hormone biosynthesis pathway expressed in H295R cells, including progestagens, corticosteroids, androgens, and estrogens.

HT-H295R Screening: 2012 chemicals in single concentration screening, 656 in concentration-response.

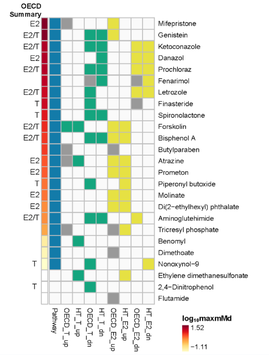
Objectives:

- Demonstrate that the HT-H295R assay may be used to predict testosterone (T) and estradiol (E2) production via comparison of the 25 reference chemicals in the OECD-validated H295R assay.
- Develop a statistical analysis that integrates data from 11 steroid hormones into a single numeric value that indicates the magnitude of effect on steroidogenesis in the HT-H295R assay.
- Begin development of a pathway-based kinetic model of steroidogenesis in HT-H295R.

HT-H295R vs. OECD Guideline Assay Results



Effect	Sensitivity	Specificity	Accuracy
Testosterone up	1.00	0.89	0.90
Testosterone dn	0.67	0.92	0.83
Estradiol up	0.75	0.83	0.80
Estradiol dn	0.80	1.00	0.95



HT-H295R results were compared to the OECD inter-laboratory validation study (Hecker et al., 2011) for chemical perturbation of T and E2 levels.

One-way ANOVA was used to determine hit calls in hormone measures between treated and controls.

- There was a high level of concordance between the testosterone and estradiol hormone responses between the HT-H295R and OECD-validated H295R assays.
- The maxmMd seemed to separate strong, moderate, and weak modulators of testosterone and estradiol production.
- The maxmMd identified chemicals (e.g. fenarimol, tricresyl phosphate, and benomyl) as pathway positives that were potential false negatives in the OECD-validated H295R assay.

References

Haggard DE, Karmaus AL, Martin MT, Judson RS, Setzer RW, Paul Friedman K (2017) High-throughput H295R steroidogenesis assay: utility as an alternative and a statistical approach to characterize effects on steroidogenesis. *Toxicol Sci*, ktx274

Hecker M, Hollert H, Cooper R, Vinggaard AM, Akahori Y, Murphy M, Nellemann C, Higley, E, Newsted J, Laskey J, Buckalew A, Grund S, Maletz S, Giesy J, Timm G (2011) The OECD validation program of the H295R steroidogenesis assay: Phase 3. Final inter-laboratory validation study. *Environ Sci Pollut Res Int*, 18(3) 503-515.

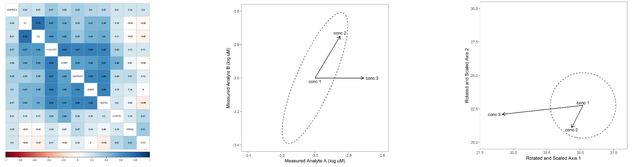
Karmaus AL, Toole CM, Filer DL, Lewis KC, Martin MT (2016) High-throughput screening of chemical effects on steroidogenesis using H295R human adrenocorticalcarcinoma cells. *Toxicol Sci* **150**(2) 323-32.

Saito R, Terasaki N, Yamazaki M, Masutomi N, Tsutsui N, and Okamoto M. (2016). Estimation of the mechanism of adrenal action of endocrine-disrupting compounds using a computational model of adrenal steroidogenesis in NCI-H295R cells. *J Toxicol* **2016**, 4041827

A Primer on Mahalanobis Distance

We use Mahalanobis distance to quantify disruption of steroidogenesis across 11 steroid hormone measurements.

Mahalanobis distance incorporates the effect size for each steroid hormone measure after adjusting for covariance between the steroid hormone measures.



Left: The residual covariances of many hormone measures in HT-H295R are highly correlated. **Middle:** Example of Euclidean distance for correlated hormone measures. Labels represent the mean concentrations two steroid hormone analytes, A and B, at three concentrations of a chemical. The ellipse represents the joint error distribution for both hormone analytes. Although the conc 3 response for analyte A is twice as large as conc 2, their Euclidean distances with respect to conc 1 are the same. **Right:** Mahalanobis corrects for correlations in hormone measures. Variables are changed by a rotation and rescaling so that the error distribution on the new axes is uncorrelated. Now, conc 3 is four times as far from conc 1 as is conc 2, more accurately representing the change in analyte concentrations.

The response at each concentration of a test chemical was considered as a point in 11-dimensional space, and a **mean Mahalanobis distance (mMd)** was computed for each concentration (Eq. 1 below).

The **maximum mMd (maxmMd)** value for each chemical was selected to indicate the magnitude of steroidogenesis pathway perturbation.

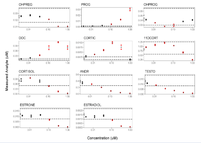
Eq. 1

$$mMD = \sqrt{(\mathbf{y_c} - \mathbf{y_1})' \mathbf{\Sigma}^{-1} (\mathbf{y_c} - \mathbf{y_1})} / N_h$$

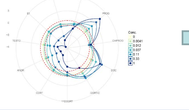
Where y_j is the vector of log-transformed hormone concentrations for the j^{th} concentration, N_h is the number of hormones with measurements for this chemical, and $\sum$ is the estimate of the covariance matrix

An example of the mMd and maxmMd computation for the steroidogenesis inhibitor, prochloraz:

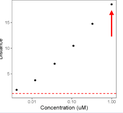
HT-H295R concentration response profile



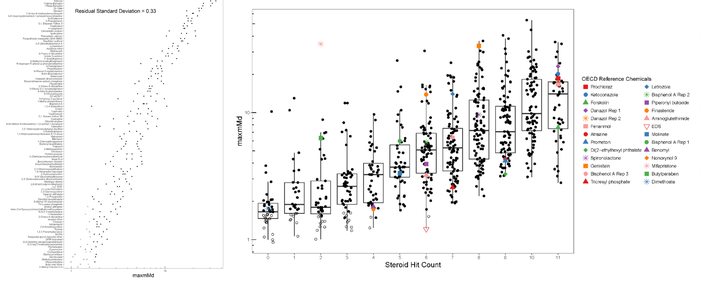
Radar visualization to better represent 11-dimensional nature of the data



mMd plot with red arrow indicating the maxmMd and the dashed line indicating the critical value

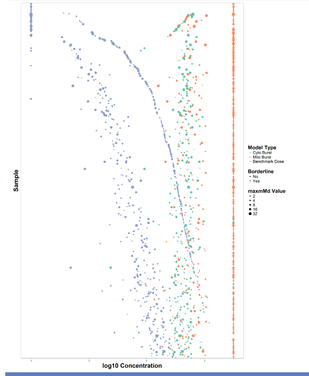


Reproducibility and Quantitative Value of the maxmMd Metric



- A total of 107 chemicals were replicated across multiple blocks; of these, 94 chemicals (87.9%) had concordant maxmMd pathway responses.
- maxmMd values were compared to the sum of steroid hormone hit counts, as measured by ANOVA.
- The maxmMd provides a quantitative indicator of activity that can distinguish chemicals that exert different magnitudes of effect on steroidogenesis but have the same hit count.

Filtering HT-H295R Positives Using More Data



For each mMd concentration-response curve, we calculated the minimum concentration needed to elicit a mMd equal to the critical value (benchmark dose, or BMD).

Ranking pathway positive hits was explored by controlling the BMD for cytotoxicity or mitochondrial toxicity (Eq. 2).

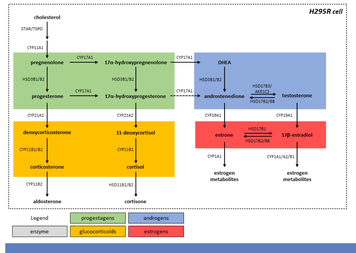
Eq. 2

$$selectivity = \min\left(burst_{cyto}, burst_{mito}\right) - BMD_{HT-H295R}$$

Where $burst_{cyto}$ and $burst_{mito}$ are the median $\log_{10}$ AC_{50} value for the 36 cytotoxicity or 15 mitochondrial toxicity assays in ToxCast/Tox21 and $BMD_{HT\text{-}H295R}$ is the calculated $\log_{10}$ BMD value for a test chemical.

- 657 out of 766 chemical samples had adequate cytotoxicity and mitochondrial toxicity assay data to calculate the selectivity score; of these, 547 (83%) passed the selectivity filter (≥ 0.5).
- This selectivity metric based on Mahalanobis distance could be used to prioritize chemicals for further screening in orthogonal or *in vivo* steroidogenesis assays.

Pathway-based Kinetic Modeling in HT-H295R



- To account for the inherent complexity of the steroidogenesis pathway in H295R, we have begun adapting a previously published dynamic model (Saito et al., 2016) with isozyme-specific kinetics.
- Current work includes analyzing time-course experiments to derive new enzyme kinetic parameters for the model, based on the stimulation conditions used in the HT-H295R assay.

Summary and Future Directions

- Comparison of the HT-H295R screening data analyzed by ANOVA with the summary results available from the OECD validation study suggests that the HT-H295R assay predicted T and E2 effects well.
- The maxmMd is a reproducible and quantitative metric to determine the magnitude of chemical perturbation of steroidogenesis in HT-H295R, representing a data-driven option for evaluating lists of chemicals.
- The number of steroid hormone significantly perturbed in the HT-H295R assay does not indicate the magnitude of effect on steroidogenesis, whereas the maxmMd can differentiate strong and weak modulators of steroidogenesis.
- Selectivity scoring is a useful method to filter HT-H295R pathway positives for additional screening.
- Future work will include more comprehensive benchmarking of the maxmMd metric, and further adapting the HT-H295R kinetic model based on time-course experiments.

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