

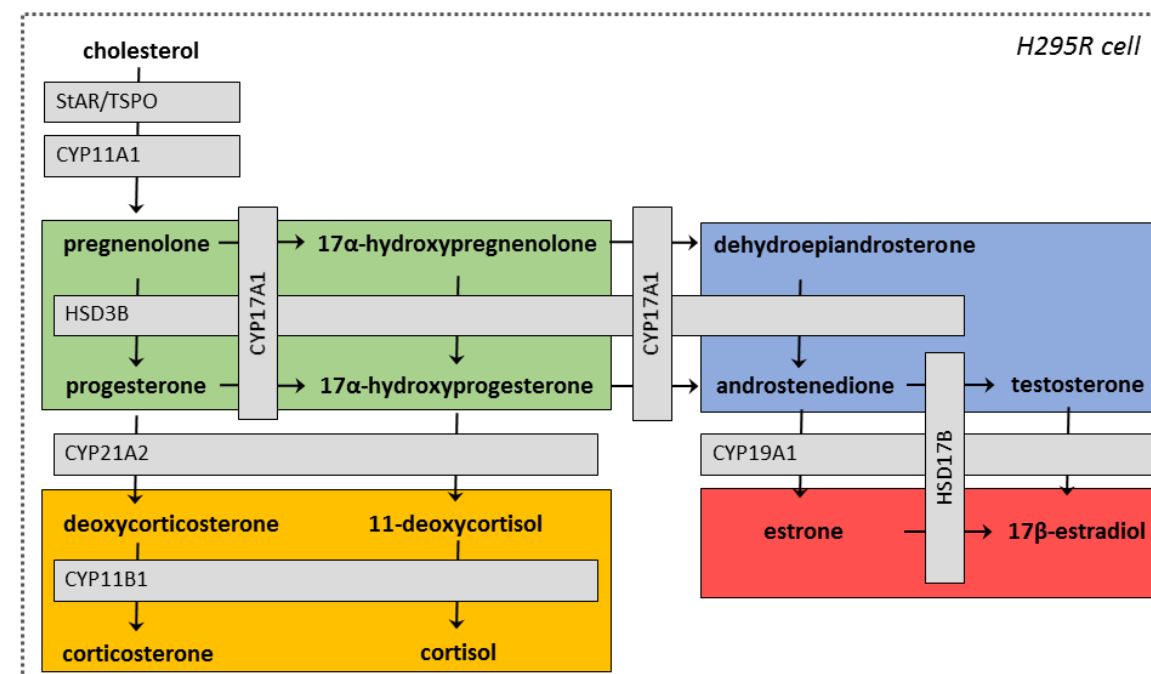


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**Background:** A high-throughput (HT) H295R assay was developed as part of the U.S. EPA's ToxCast program that includes measurement of 11 hormones across the steroid hormone biosynthesis pathway expressed in H295R cells, including progestagens, corticosteroids, androgens, and estrogens (Figure 1). To date, 2012 chemicals have been evaluated in the HT-H295R assay in single concentration screening, and of these, 656 were evaluated in concentration-response.

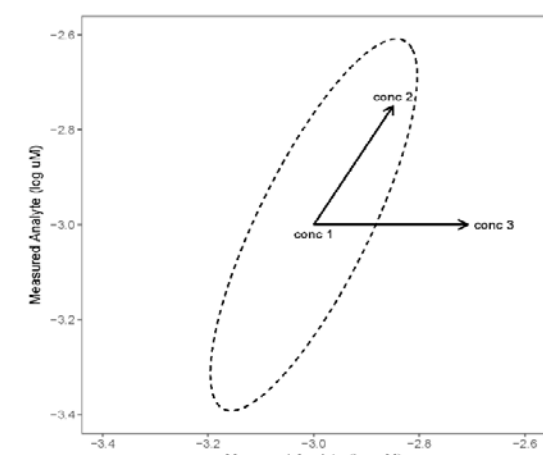


Representation of progestagen(green), glucocorticoid (yellow), androgen (blue), and estrogen (red) biosynthesis in H295R.

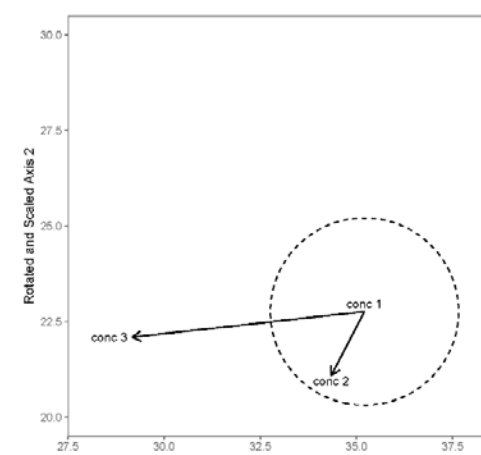
**Objectives:**

- ▶ 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
- ▶ Demonstrate that the 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

Mahalanobis distance was used as a basis for this novel approach due to the complexity of the 11 measured endpoints, and the expected correlation of the residuals of steroid hormone measures (all from the same treated well) in the HT-H295R assay.



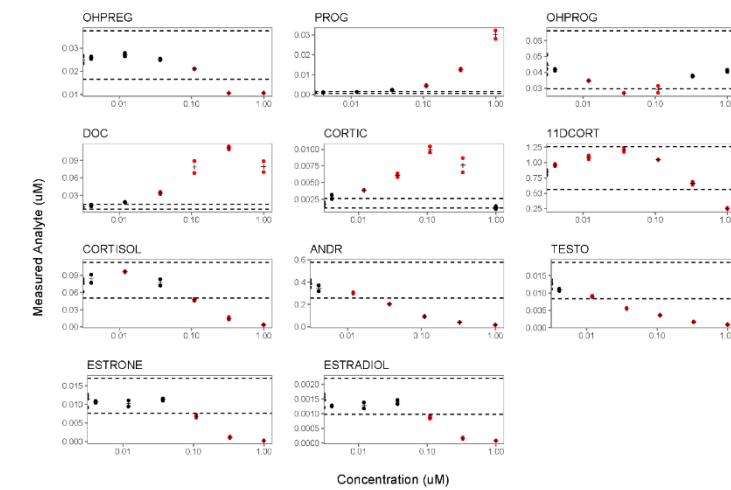
**Euclidean Distance (left):** Labels represent the mean (natural log) concentrations two steroid hormone analytes, A and B, at three concentrations of a test chemical. The ellipse represents the joint error distribution for both hormone analytes. Although the conc 3 response for analyte A is twice as large as conc 2, their Euclidean distances with respect to conc 1 are the same. **Mahalanobis Distance (right):** 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.



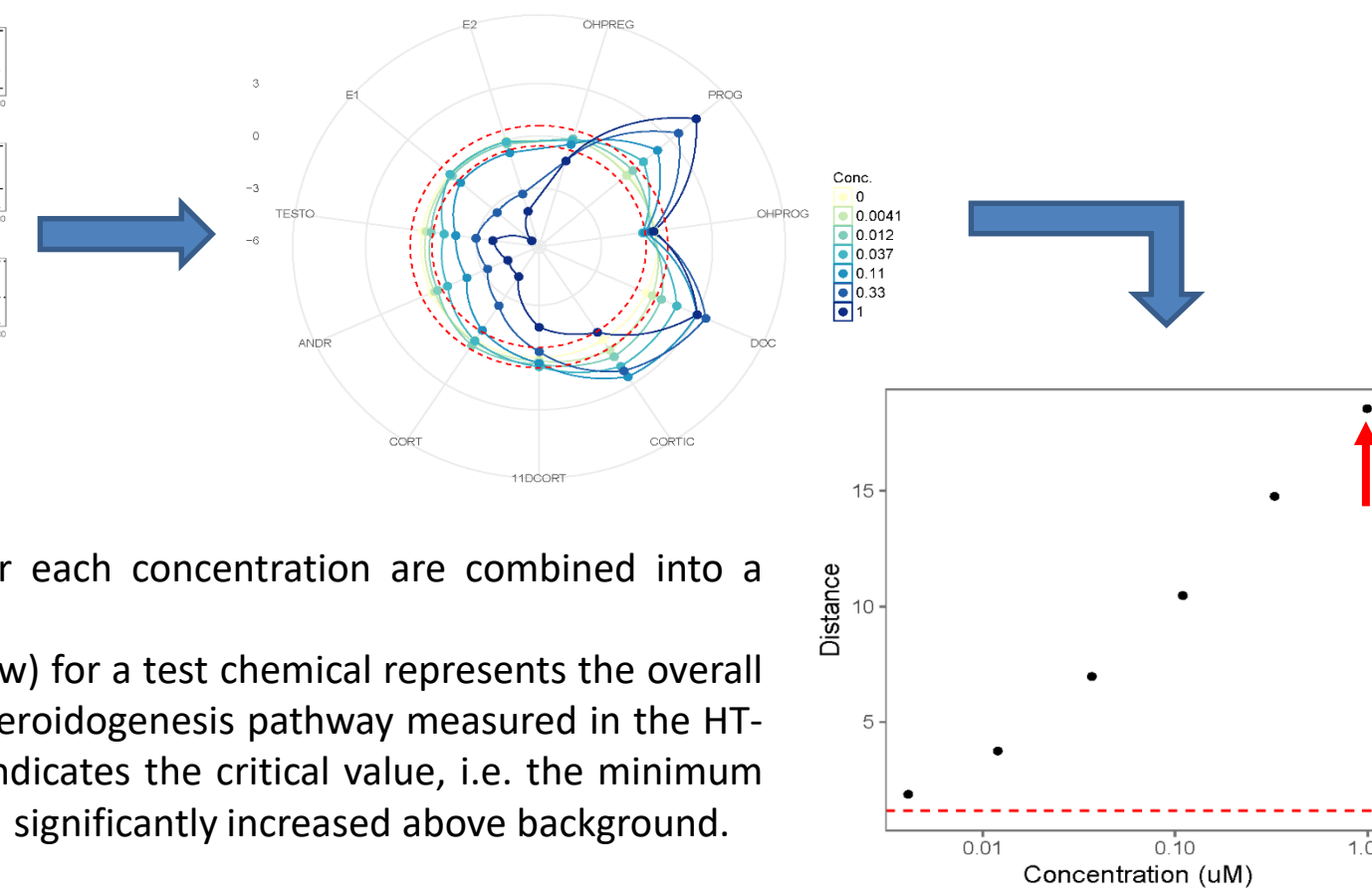
The general approach for the Mahalanobis distance for this analysis is as follows:

- ▶ For each chemical, the mean Mahalanobis distance (mMd) at each test concentration was calculated, i.e. the Mahalanobis distance scaled by the square root of the number of measured hormones for a test chemical (which ranged from 7 to 11).
- ▶ The maximum mMd (maxmMd) value for each chemical was selected to indicate the magnitude of steroidogenesis pathway perturbation.

HT-H295R profile for the steroidogenesis inhibitor, prochloraz

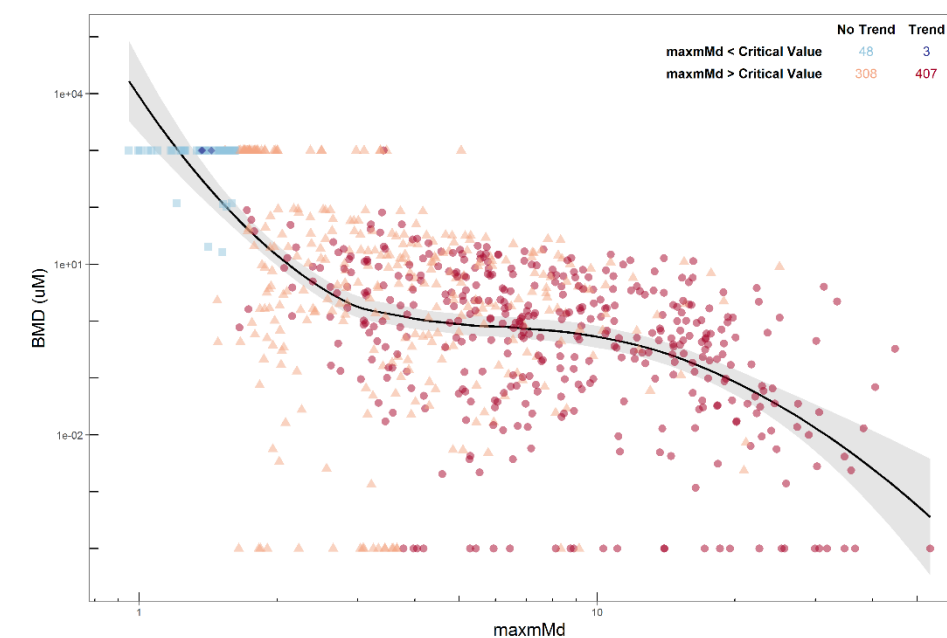


Radar visualization to better represent 11-dimensional space



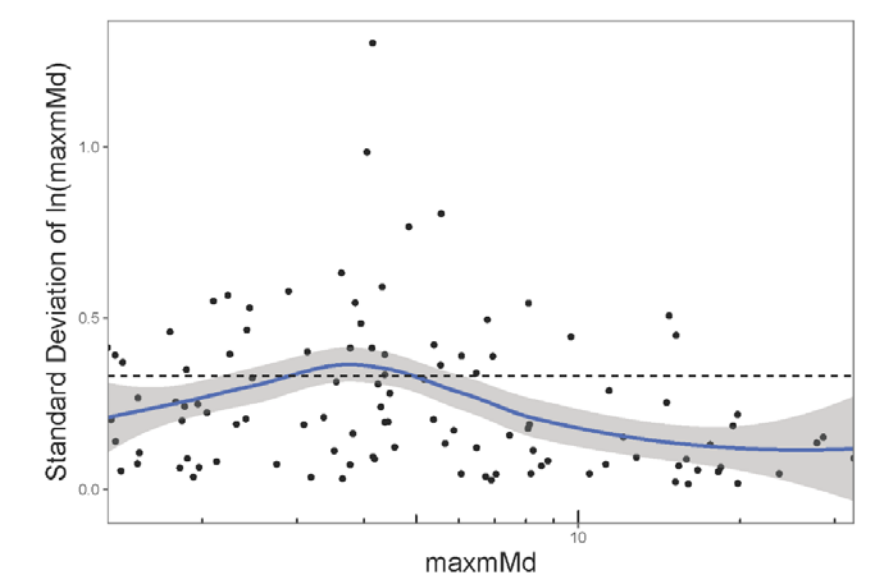
- ▶ Steroid hormone response data for each concentration are combined into a single value, the mMd.
- ▶ The maxmMd (indicated by red arrow) for a test chemical represents the overall magnitude of perturbation of the steroidogenesis pathway measured in the HT-H295R assay. The dashed red line indicates the critical value, i.e. the minimum mMd value needed to be considered significantly increased above background.

- ▶ maxmMd values were compared to the sum of steroid hormone hit counts, as measured by ANOVA.
- ▶ Though for many chemicals higher maxmMd corresponded to higher hit counts, this was not always the case.
- ▶ The maxmMd provides a quantitative indicator of activity that can distinguish between chemicals that exert different magnitudes of effect on steroidogenesis but have the same hit count.



- ▶ The minimum concentration (benchmark dose, or BMD) needed to elicit a mMd equal to the critical value was calculated for each chemical.
- ▶ In general, smaller BMDs, i.e. greater potency, corresponds to higher maxmMd value, with the strongest relationship evident for the very weak or negative chemicals and the strongest maxmMd positive values.

- ▶ The maximum mean Mahalanobis distance (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 maxmMd appears to increase for more potent 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.

[illegible]

- ▶ A total of 107 chemicals were replicated across multiple blocks; of these, 94 chemicals (87.9%) had concordant maxmMd pathway responses.
- ▶ The standard deviation of the block-replicated maxmMd values decreases as the mean maxmMd increases.
- ▶ The estimated 95% confidence interval (maxmMd  $\times/\div$  1.93) is likely to overestimate the maxmMd variability for chemicals with high maxmMd values.

Heatmap showing the log<sub>10</sub>(maxM/Md) values for various pesticides across different pathways. The color scale ranges from -1.11 (red) to 1.52 (blue).

Pesticide	Pathway	log <sub>10</sub> (maxM/Md)
Mifepristone	HT_E2_dn	1.52
	HT_E2_up	1.52
Genistein	HT_E2_dn	1.52
	HT_E2_up	1.52
Ketoconazole	HT_E2_dn	1.52
	HT_E2_up	1.52
Danazol	HT_E2_dn	1.52
	HT_E2_up	1.52
Prochloraz	HT_E2_dn	1.52
	HT_E2_up	1.52
Fenarimol	HT_E2_dn	1.52
	HT_E2_up	1.52
Letrozole	HT_E2_dn	1.52
	HT_E2_up	1.52
Finasteride	HT_E2_dn	1.52
	HT_E2_up	1.52
Spironolactone	HT_E2_dn	1.52
	HT_E2_up	1.52
Forskolin	HT_E2_dn	1.52
	HT_E2_up	1.52
Bisphenol A	HT_E2_dn	1.52
	HT_E2_up	1.52
Butylparaben	HT_E2_dn	1.52
	HT_E2_up	1.52
Atrazine	HT_E2_dn	1.52
	HT_E2_up	1.52
Prometon	HT_E2_dn	1.52
	HT_E2_up	1.52
Piperonyl butoxide	HT_E2_dn	1.52
	HT_E2_up	1.52
Molinate	HT_E2_dn	1.52
	HT_E2_up	1.52
Di(2-ethylhexyl) phthalate	HT_E2_dn	1.52
	HT_E2_up	1.52
Aminoglutethimide	HT_E2_dn	1.52
	HT_E2_up	1.52
Tricresyl phosphate	HT_E2_dn	1.52
	HT_E2_up	1.52
Benomyl	HT_E2_dn	1.52
	HT_E2_up	1.52
Dimethoate	HT_E2_dn	1.52
	HT_E2_up	1.52
Nonoxynol-9	HT_E2_dn	1.52
	HT_E2_up	1.52
Ethylene dimethanesulfonate	HT_E2_dn	1.52
	HT_E2_up	1.52
2,4-Dinitrophenol	HT_E2_dn	1.52
	HT_E2_up	1.52
Flutamide	HT_E2_dn	1.52
	HT_E2_up	1.52

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

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

## Summary and Future Directions

- ▶ The maximum mean Mahalanobis distance (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 maxmMd appears to increase for more potent 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.

- ▶ 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.
- ▶ Future efforts will consist of incorporating additional assay data (nuclear receptors, CYP assays) as well as developing a pathway-based kinetic model of steroidogenesis in the HT-H295R assay.

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