High-throughput Assay Adaptations for Androgen and Estrogen Steroidogenesis Screening

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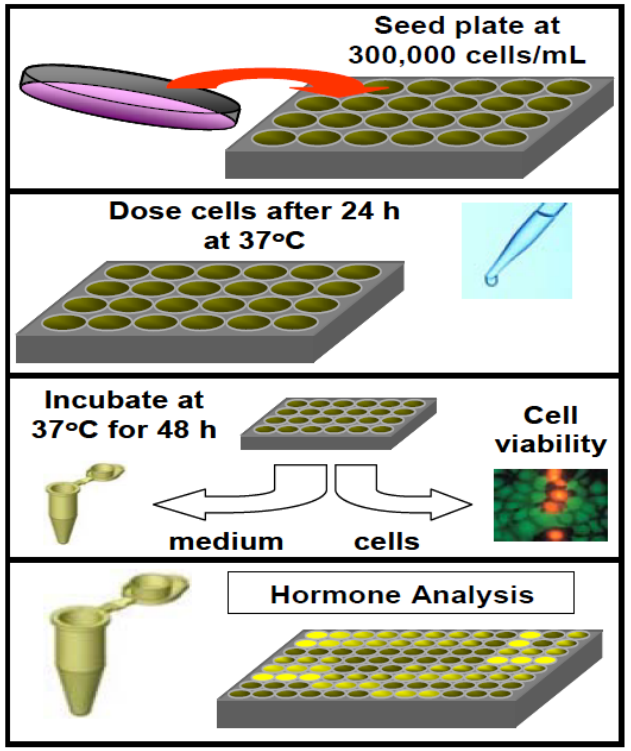
Outline

- **Part 1**: Adaptation of the H295R Assay for High-throughput Androgen and Estrogen Steroidogenesis Screening

- **Part 2**: Development of a High-throughput 5α-reductase Screening Assay for Androgen Steroidogenesis

Part 1: OECD TG 456 - H295R Steroidogenesis Assay



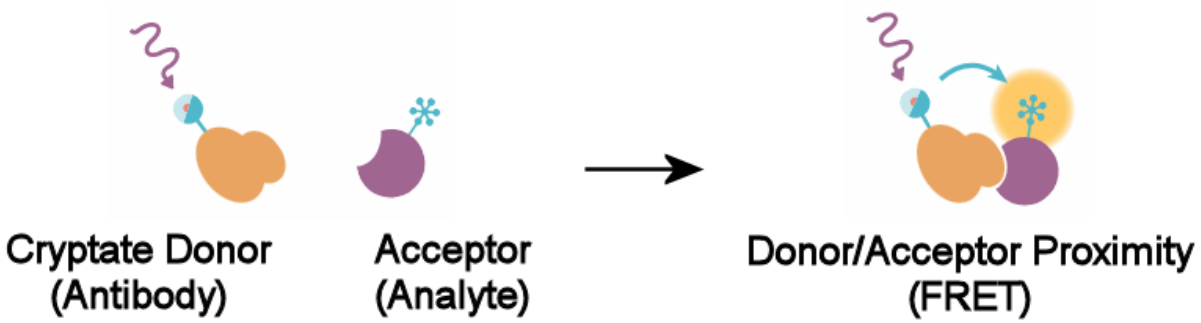
1. Begin H295R cell culture using an original ATCC batch
2. Culture cells for ≥ 5 passages (split cells when ~90% confluent)
3. Seed at a density of 300,000 cells/mL into 24 well plates, and incubate at 37°C and 5% CO_2 for 24h
4. Replace medium and dose cells
5. Incubate at 37°C and 5% CO_2 for 48h
6. Remove medium from cells and extract with ether
7. Conduct cell viability assay immediately after removal of medium
8. Measure hormone concentrations in medium extract

Figure 3.2: *H295R Steroidogenesis Assay to measure effects of chemicals on production of testosterone (T) and estradiol (E2).*

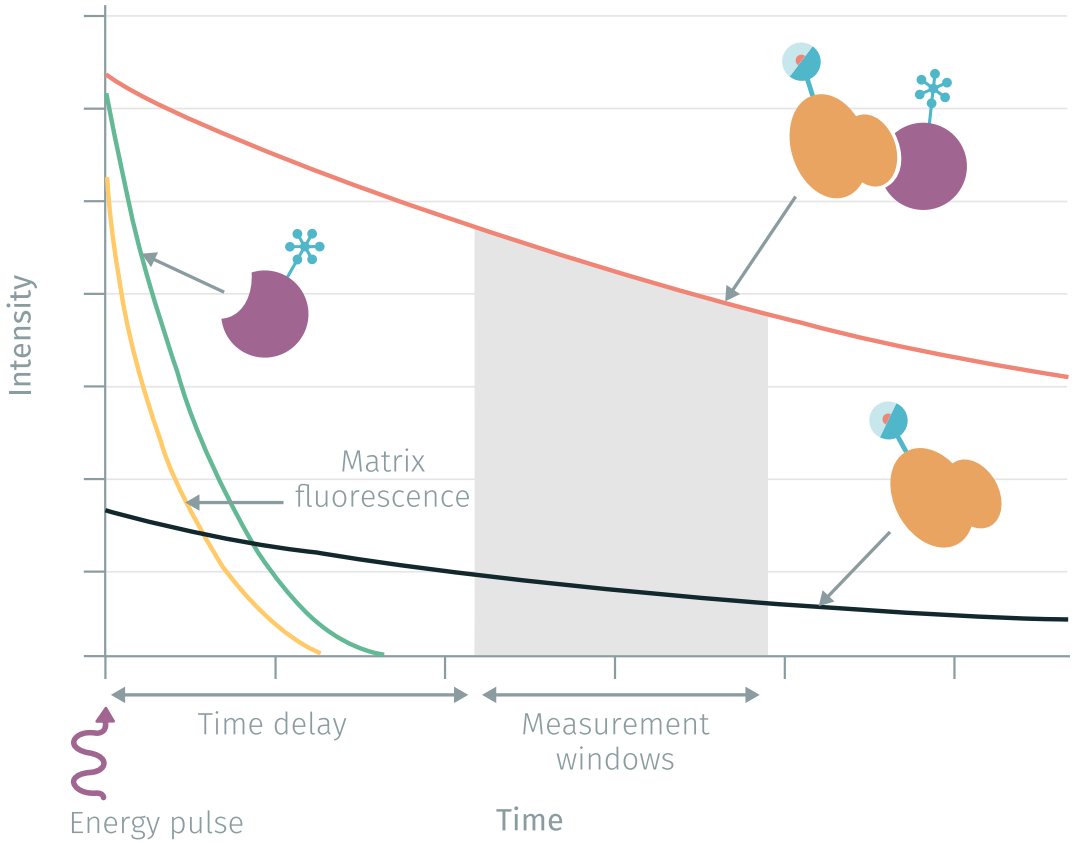
- **OECD TG 456:** Evaluates 17β-Estradiol (E2) and Testosterone (T) synthesis.
- **HT-H295R:** 96-well assay evaluates 13 hormones including androgens, estrogens, progestagens, and glucocorticoids.
- **Objective:** Adapt the H295R cell line to 384-well high-throughput format using Homogenous Time Resolved Fluorescence (HTRF) technology to evaluate E2 and T endpoints.

Hecker, Giesy, Timm, 2008, U.S. EPA Report

Hormone Detection:
Homogenous Time-Resolved Fluorescence Technology



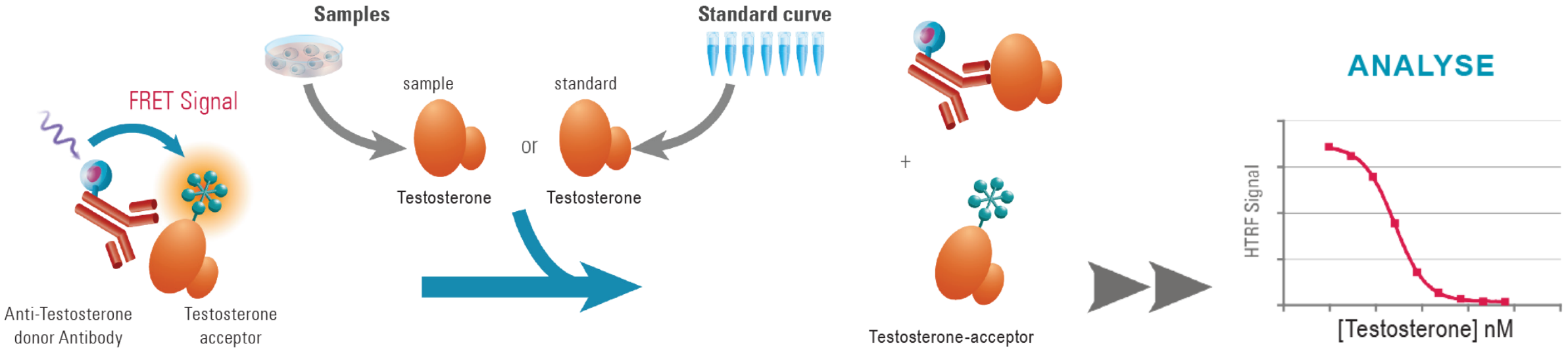
Principle of Fluorescence Resonance Energy Transfer (FRET) Immunoassay



Principle of Homogenous Time-Resolved Fluorescence (HTRF) Detection

https://www.cisbio.eu/content/htrf-technology/

Hormone Detection:
Homogenous Time-Resolved Fluorescence Technology

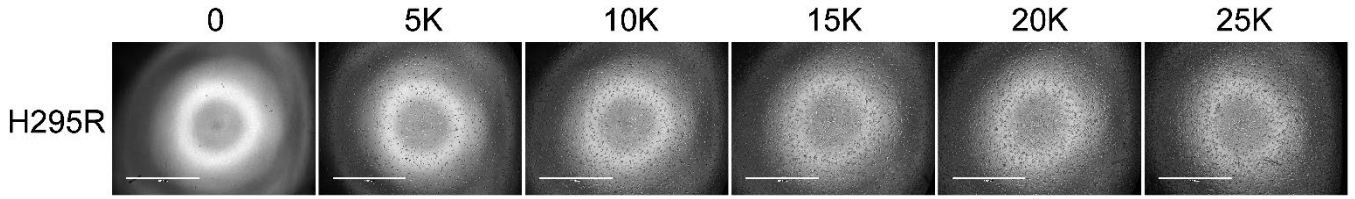


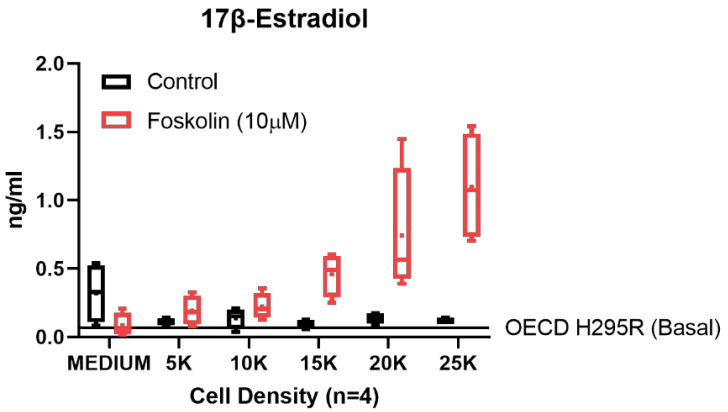
Homogenous Time-Resolved Fluorescence (HTRF) competitive immunoassay technology can rapidly measure E2 and T concentrations

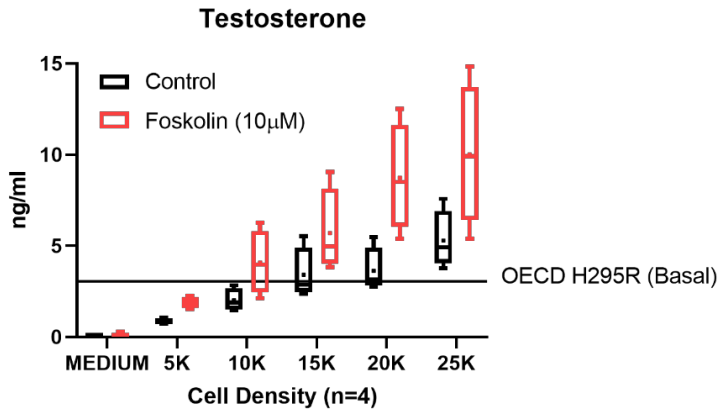
	Sensitivity: LLOQ (pg/ml)	
	TG 456	***HTRF***
Testosterone	100	100
17β- Estradiol	10	19

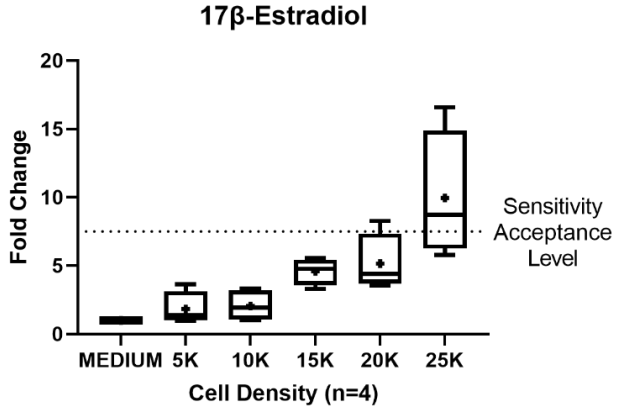
https://www.cisbio.eu/content/htrf-technology/

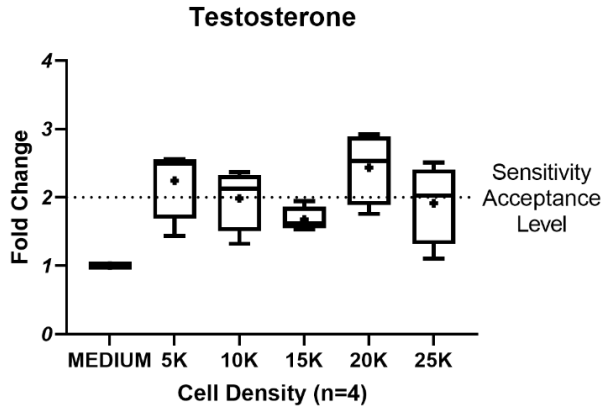
H295R Assay Optimization











Initial basal and induced analyte levels in 384-well format are consistent with guideline study parameters.

Sample Experimental Design

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY
B	EMPTY	DMSO	PRO	99.9	18.7	6.24	0.562	0.125	0.0187	0.00624	0.000562	0.000125	DMSO	PRO	99.9	18.7	6.24	0.562	0.125	0.0187	0.00624	0.000562	0.000125	EMPTY
C	EMPTY	DMSO	PRO	99.9	18.7	6.24	0.562	0.125	0.0187	0.00624	0.000562	0.000125	DMSO	PRO	99.9	18.7	6.24	0.562	0.125	0.0187	0.00624	0.000562	0.000125	EMPTY
D	EMPTY	DMSO	PRO	99.9	18.7	6.24	0.562	0.125	0.0187	0.00624	0.000562	0.000125	DMSO	PRO	99.9	18.7	6.24	0.562	0.125	0.0187	0.00624	0.000562	0.000125	EMPTY
E	EMPTY	DMSO	PRO	99.9	18.7	6.24	0.562	0.125	0.0187	0.00624	0.000562	0.000125	DMSO	PRO	99.9	18.7	6.24	0.562	0.125	0.0187	0.00624	0.000562	0.000125	EMPTY
F	EMPTY	DMSO	PRO	99.9	18.7	6.24	0.562	0.125	0.0187	0.00624	0.000562	0.000125	DMSO	PRO	99.9	18.7	6.24	0.562	0.125	0.0187	0.00624	0.000562	0.000125	EMPTY
G	EMPTY	DMSO	PRO	99.9	18.7	6.24	0.562	0.125	0.0187	0.00624	0.000562	0.000125	DMSO	PRO	99.9	18.7	6.24	0.562	0.125	0.0187	0.00624	0.000562	0.000125	EMPTY
H	EMPTY	DMSO	PRO	99.9	18.7	6.24	0.562	0.125	0.0187	0.00624	0.000562	0.000125	DMSO	PRO	99.9	18.7	6.24	0.562	0.125	0.0187	0.00624	0.000562	0.000125	EMPTY
I	EMPTY	DMSO	PRO	99.9	18.7	6.24	0.562	0.125	0.0187	0.00624	0.000562	0.000125	DMSO	PRO	99.9	18.7	6.24	0.562	0.125	0.0187	0.00624	0.000562	0.000125	EMPTY
J	EMPTY	DMSO	PRO	99.9	18.7	6.24	0.562	0.125	0.0187	0.00624	0.000562	0.000125	DMSO	PRO	99.9	18.7	6.24	0.562	0.125	0.0187	0.00624	0.000562	0.000125	EMPTY
K	EMPTY	DMSO	PRO	99.9	18.7	6.24	0.562	0.125	0.0187	0.00624	0.000562	0.000125	DMSO	PRO	99.9	18.7	6.24	0.562	0.125	0.0187	0.00624	0.000562	0.000125	EMPTY
L	EMPTY	DMSO	PRO	99.9	18.7	6.24	0.562	0.125	0.0187	0.00624	0.000562	0.000125	DMSO	PRO	99.9	18.7	6.24	0.562	0.125	0.0187	0.00624	0.000562	0.000125	EMPTY
M	EMPTY	DMSO	PRO	99.9	18.7	6.24	0.562	0.125	0.0187	0.00624	0.000562	0.000125	DMSO	PRO	99.9	18.7	6.24	0.562	0.125	0.0187	0.00624	0.000562	0.000125	EMPTY
N	EMPTY	DMSO	PRO	99.9	18.7	6.24	0.562	0.125	0.0187	0.00624	0.000562	0.000125	DMSO	PRO	99.9	18.7	6.24	0.562	0.125	0.0187	0.00624	0.000562	0.000125	EMPTY
O	EMPTY	DMSO	PRO	99.9	18.7	6.24	0.562	0.125	0.0187	0.00624	0.000562	0.000125	DMSO	PRO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	EMPTY
P	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY
Medium	**No Forskolin**		**Forskolin (10 µM)**																					

Workflow – Add, Mix, Measure Principle			
Day 0	**Day 1**	**Day 2**	**Day 3**
Cell Seeding	Cell Treatment	No Treatment	Sample Collection and Viability
Seed cells at 25K per well in standard growth medium.	Supplement +/- Forskolin medium +/- test compound (no aspiration).	Continue incubation.	Collect sample from each well. Run Cell-titer Glo assay.

Controls

- Solvent Control: DMSO (0.2%)
- Positive Control: Prochloraz

Test compounds per plate: 9

Technical replicates per plate: 3

Cell Viability: ATP levels

Automated Assay Workflow for High-Throughput Screening

H295R 384-well Seeding.

Integra ViaFlo

- Rapid and uniform cell seeding.

Acoustic Chemical Dosing.

Labcyte Echo

- Small volume, high precision chemical dosing of assay plate.

Sample Collection and Viability Analysis.

Integra ViaFlo

- Consistent sample collection and storage.
- Multiplexed cell viability testing.

HTRF Analyte Immunoassay.

Certus Flex

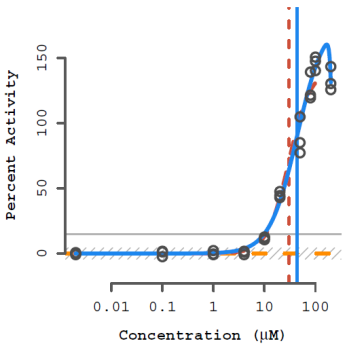
- Rapid and uniform small volume reagent dispensing.

Data Collection.

BMG CLARIOstar

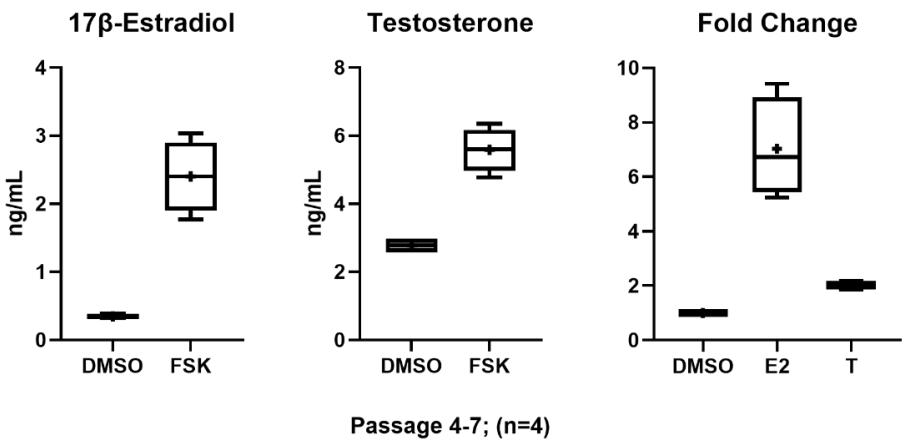
- Multi-mode plate spectrometer equipped with HTRF filter set.

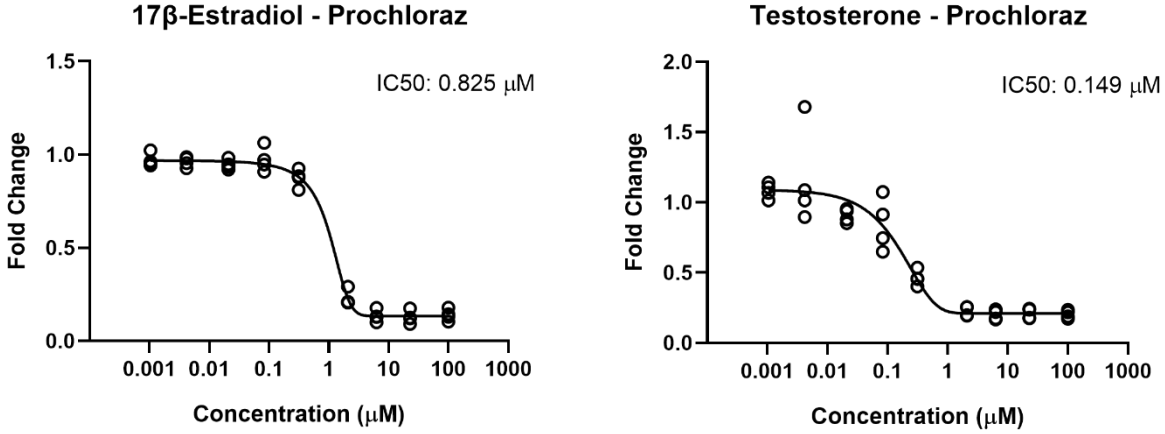
Data Analysis.



- ToxCast Pipeline.

Reference Chemical Performance





Absolute and relative analyte levels are within TG 456 specifications.

Prochloraz inhibition potency and effect size are consistent with interlaboratory validation study.

	Hormone (ng/ml)		Sensitivity - Induction (10 µM FSK fold-change)		Sensitivity - Inhibition (2* µM PRO fold-change)		Precision (%CV)	
	Basal	***FSK (10 µM)***	***TG 456***	***HTRF***	***TG 456***	***HTRF***	***TG 456***	***HTRF***
Testosterone	2.77 (± 0.20)	5.58 (± 0.56)	≥ 1.5	2.02 (± 0.15)	≤ 0.5	0.23 (± 0.03)	≤ 30	14.6 (± 5.0)
17β- Estradiol	0.34 (± 0.02)	2.40 (± 0.45)	≥ 7.5	7.03 (± 1.59)	≤ 0.5	0.23 (± 0.04)	≤ 30	12.2 (± 4.2)

*Values are mean ± SD. Passage 4-7 (n=4). *TG 456 tested at 1 µM.*

Chemical Training Set

Classification	CASRN	Chemical	Mode	17β-Estradiol	Testosterone
Core	125-84-8	Aminoglutethimide	Antagonist	1	1
Core	1912-24-9	Atrazine	Agonist	1	0
Core	17804-35-2	Benomyl	Agonist/Antagonist	1 (agonist)	1 (antagonist)
Core	94-26-8	Butylparaben	Agonist/Antagonist	1 (agonist)	1 (antagonist)
Core	66575-29-9	Forskolin	Agonist	1	1
Core	112809-51-5	Letrozole	Antagonist	1	0
Core	2212-67-1	Molinate	Agonist/Antagonist	1 (agonist)	1 (antagonist)
Core	67747-09-5	Prochloraz	Antagonist	1	1
Core	13647-35-3	Trilostane	Agonist	1	1
Supplemental	65277-42-1	Ketoconazole	Antagonist	1	1
Supplemental	446-72-0	Genistein	Agonist/Antagonist	1 (agonist)	1 (antagonist)
Supplemental	98319-26-7	Finasteride	Antagonist	1	1
Supplemental	80-05-7	Bisphenol A	Agonist/Antagonist	1 (agonist)	1 (antagonist)
Supplemental	51-03-6	Piperonyl butoxide	Antagonist	0	1
Supplemental	52-01-7	Spironolactone	Antagonist	0	1
Supplemental	60168-88-9	Fenarimol	Antagonist	1	1
Supplemental	17230-88-5	Danazol	Antagonist	1	0
Supplemental	117-81-7	Di(2-ethylhexyl) phthalate	Agonist	1	0
Supplemental	60-51-5	Dimethoate	Agonist	1	0
Supplemental	13311-84-7	Flutamide	Agonist	1	0
Supplemental	1610-18-0	Prometon	Agonist	1	0
Supplemental	84371-65-3	Mifepristone	Agonist	1	1
Azole	107534-96-3	Tebuconazole	Antagonist	1	1
Azole	85509-19-9	Flusilazole	Antagonist	1	0
Azole	94361-06-5	Cyproconazole	Antagonist	1	0
Azole	35554-44-0	Imazalil	Antagonist	0	1
Azole	68694-11-1	Triflumizole	Antagonist	0	1
Azole	88671-89-0	Myclobutanil	Antagonist	0	1
Azole	23593-75-1	Clotrimazole	Antagonist	1	1
Azole	55219-65-3	Triadimenol	Antagonist	1	1
Azole	43121-43-3	Triadimefon	Antagonist	1	1
Azole	133855-98-8	Epoxiconazole	Antagonist	1	1
Aromatase Inhibitor	27220-47-9	Econazole	Antagonist	1	0
Aromatase Inhibitor	1836-75-5	Nitrofen	Antagonist	1	0
Control	131-70-4	Monobutyl phthalate	Negative Control	0	0

Part 1: Summary

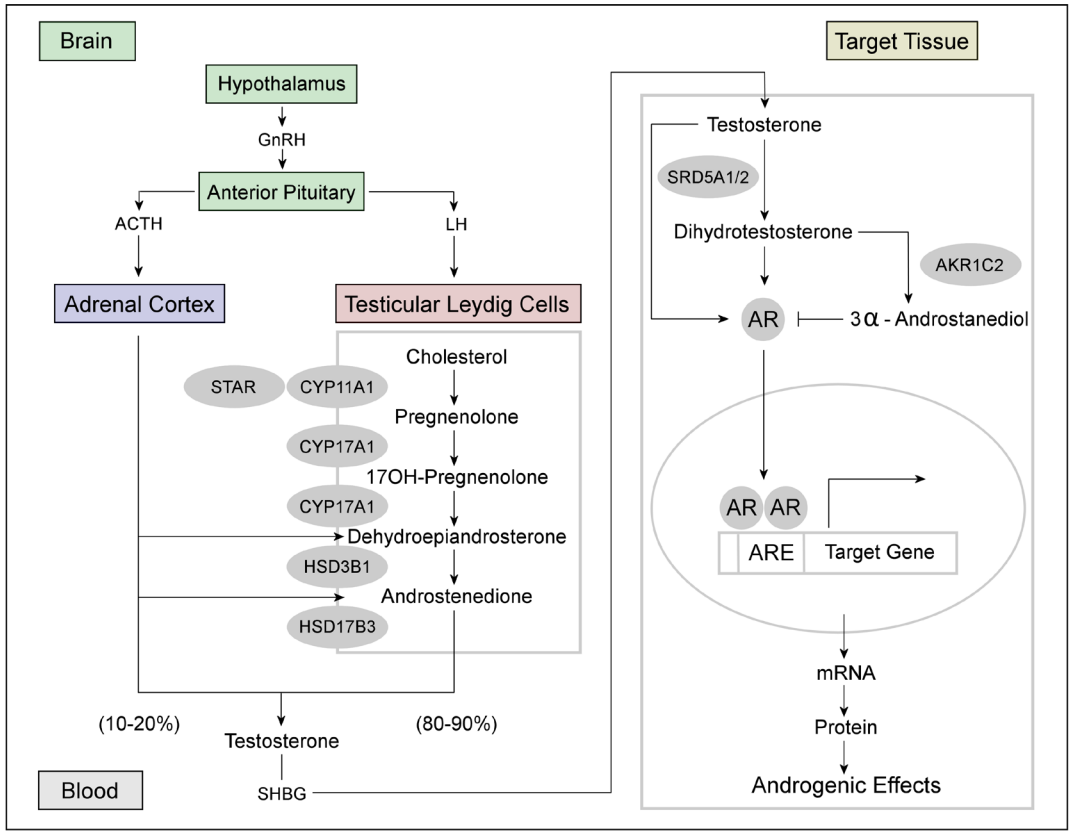
Strengths

- Adaptation of the H295R Assay to High-throughout Format.
  - Increased throughput to 384-well format with addition of automation to the workflow.
  - Multiplexed with rapid viability measurement.
  - Co-exposure of forskolin with test chemical enhances dynamic range and maintains the same 72-hour timeline as TG 456.
  - Reference chemicals (Forskolin and Prochloraz) exhibit acceptable potency and efficacy.
  - Requires considerably less test chemical and sample volumes.
  - Assay performance against a chemical training set is a work in progress.
- HTRF Immunoassay Technology
  - Rapid and safer detection method that can be used in place of RIA and ELISA.
  - Data generated in less time that eliminates the need for sample extraction.
  - Commercially available technology accessible to most modern laboratories.
  - Detects E2 and T within TG 456 specifications.

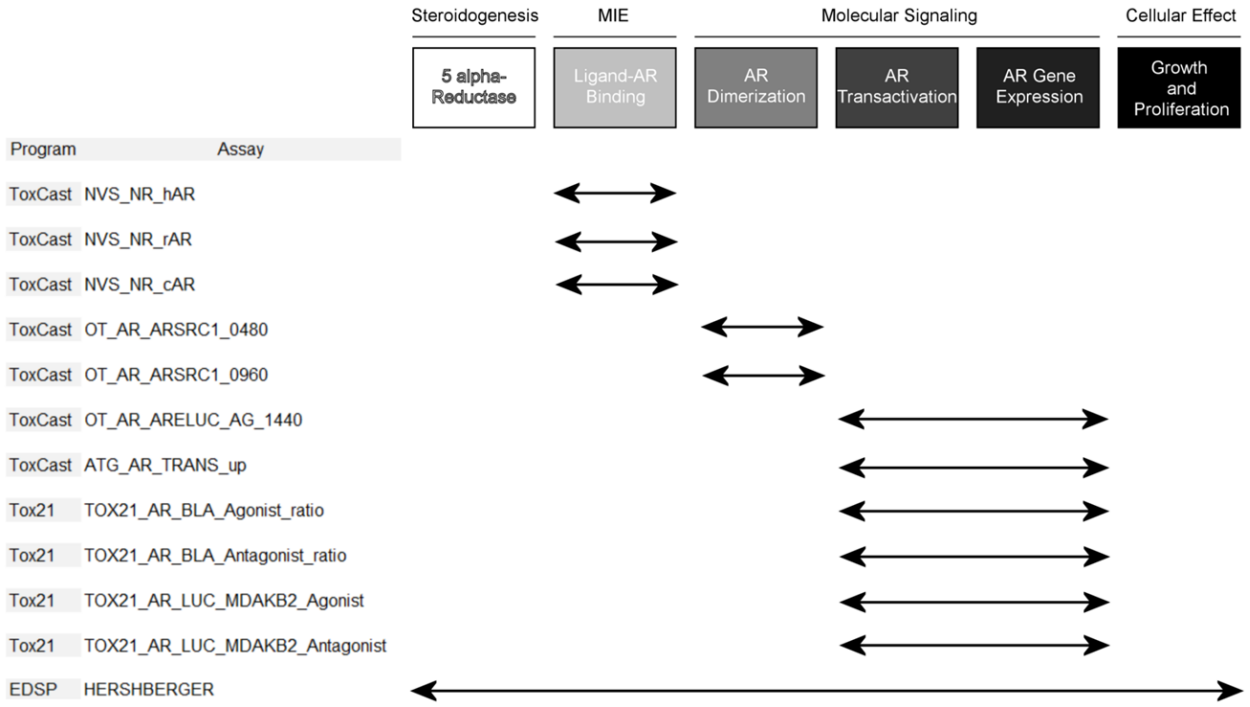
Limitations

- HTRF Immunoassay Technology
  - Supplied by a single vendor and currently restricted to T and E2.
  - Cross-reactivity of antibodies may limit detection of specific chemical classes.
  - Autofluorescence interference may be an issue for specific chemical classes.
  - Requires HTRF compatible plate spectrometer.

Part 2: Development of a High-throughput 5α-reductase Screening Assay for Androgen Steroidogenesis

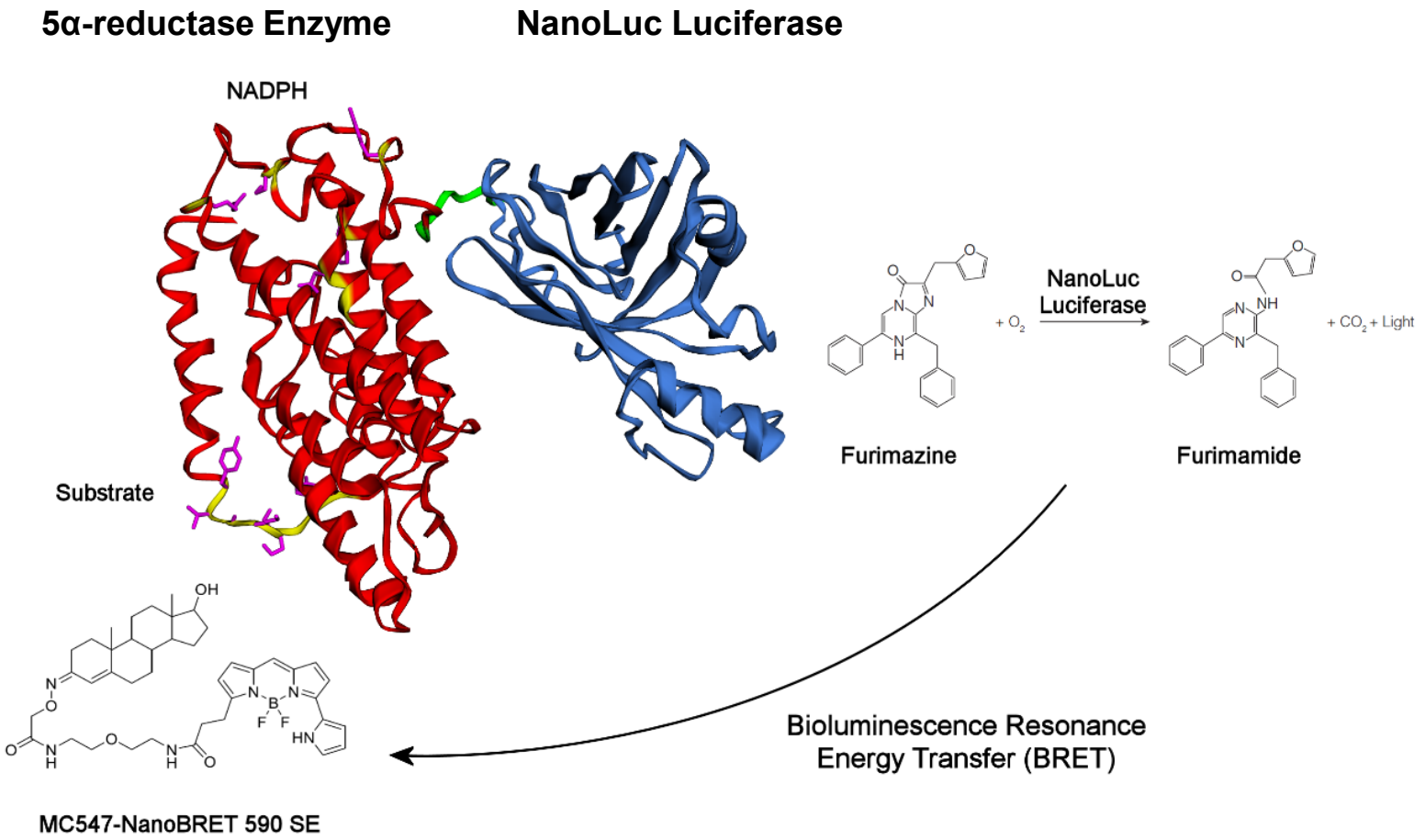


Androgen Steroidogenesis and Target Tissue Activity



Androgen *In Vitro* Assay Battery vs Hershberger Assay

5α-reductase NanoBRET Target Engagement Assay

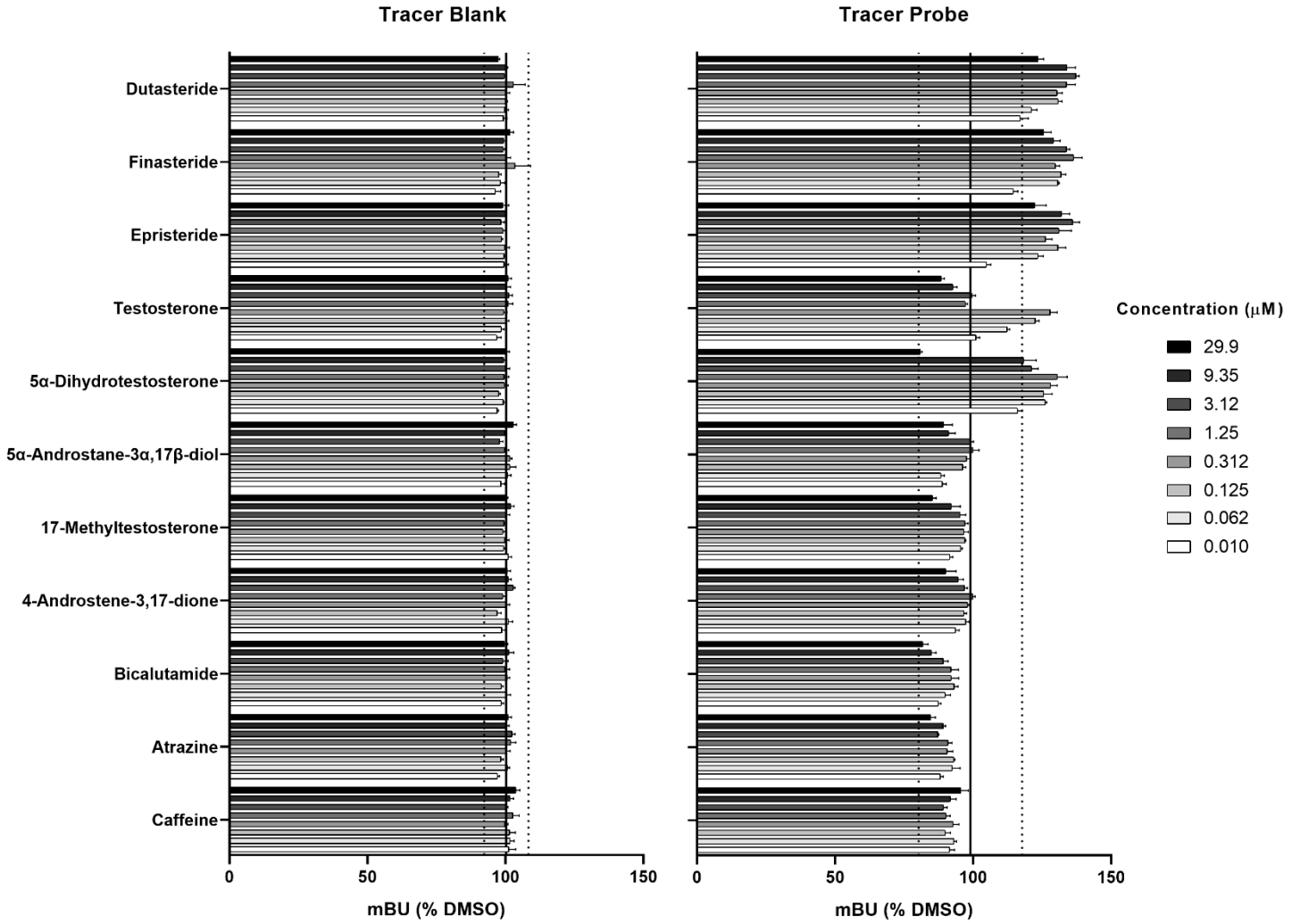


Cell-based assay: Places enzyme in native context, overcoming limitations to recombinant 5AR enzymes.

High specificity: Cell-permeable energy transfer probe will only resonate in close proximity to bioluminescence.

Can disruption of active metabolic steroid hormone-enzyme interactions be evaluated with NanoBRET Technology?

Reference Chemical Evaluation



Name	Classification	Active	IC50 (nM)	mEFF (%)
Dutasteride	5ARI	Yes	279	142
Finasteride	5ARI	Yes	122	134
Epristeride	5ARI	Yes	307	132
Testosterone	Androgen	Yes	719	129
5α-Dihydrotestosterone	Androgen	Yes	8.3	131
5α-Androstane-3α,17β-diol	Androgen	No	NA	NA
17-Methyltestosterone	Androgen	No	NA	NA
4-Androstene-3,17-dione	Androgen	No	NA	NA
Bicalutamide	Anti-Androgen	No	NA	NA
Atrazine	Negative	No	NA	NA
Caffeine	Negative	No	NA	NA

- Compounds do not exhibit non-specific fluorescence.
- 5ARIs, natural substrate (testosterone), and metabolite (5^{α}-DHT) exhibit gain-of-signal.

Primary Screen Results

Design

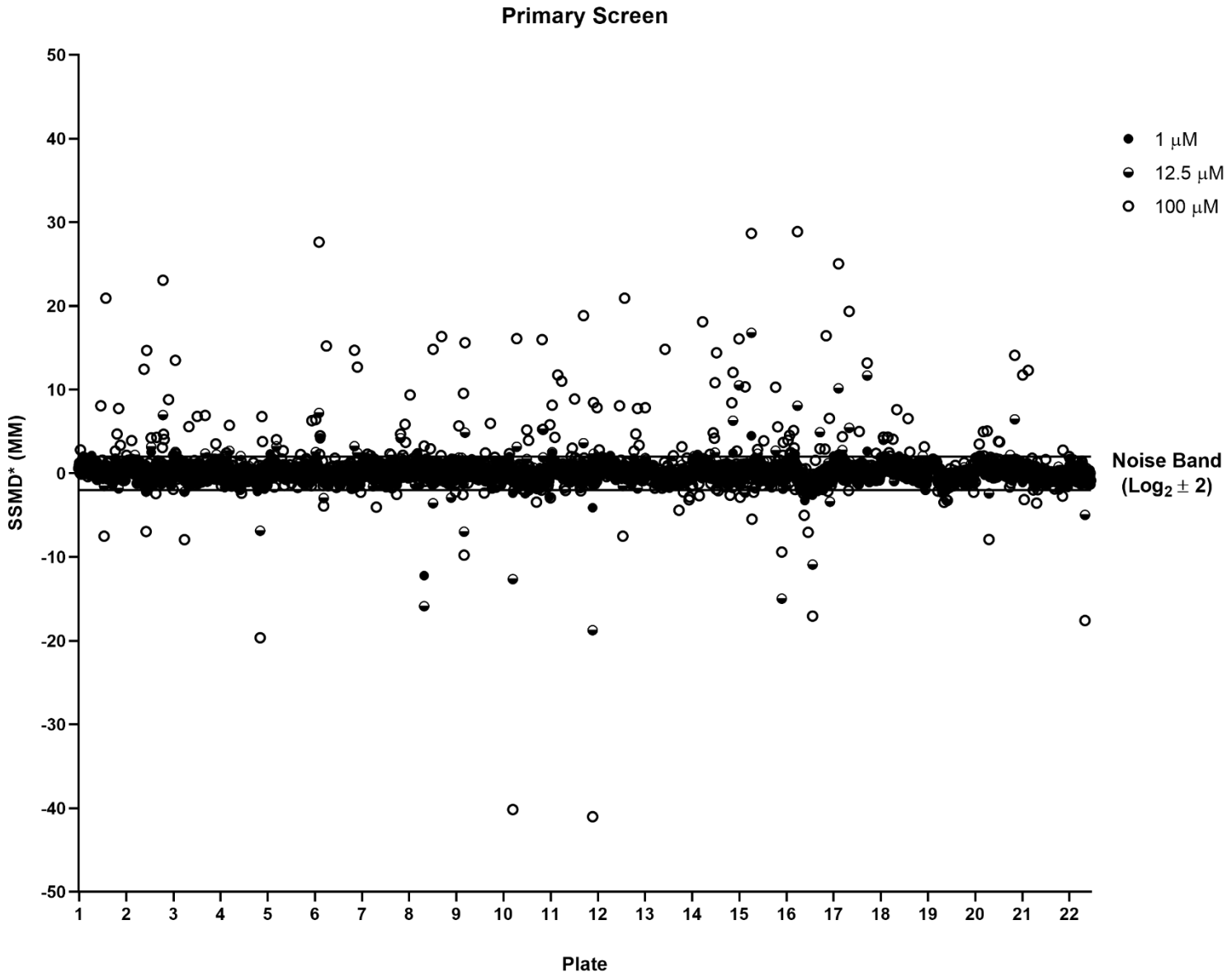
- 1803 blinded chemical library screened at three concentrations (1, 12.5, 100uM).

Results

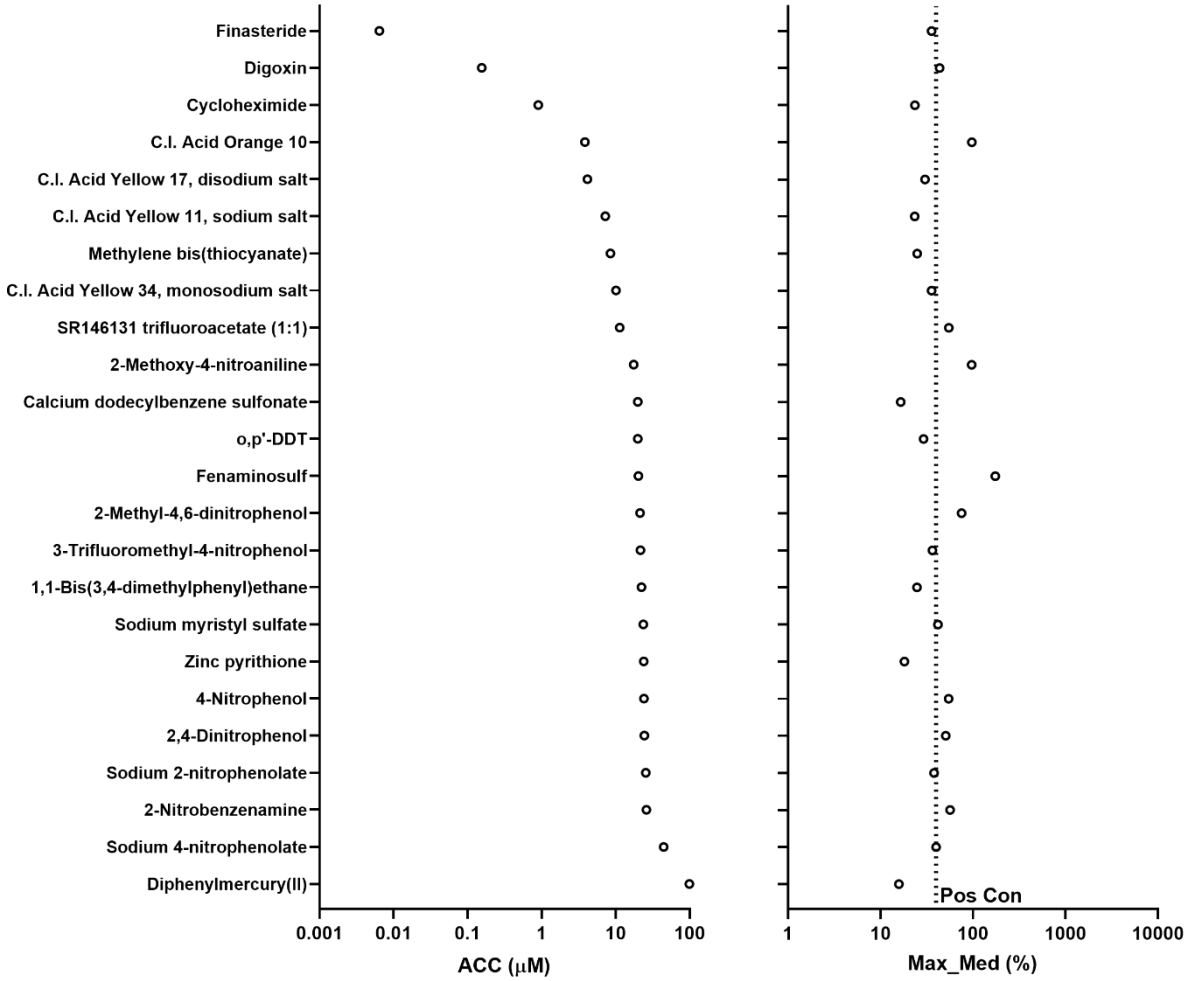
- 228/1769 (13%) identified for loss and gain of signal.

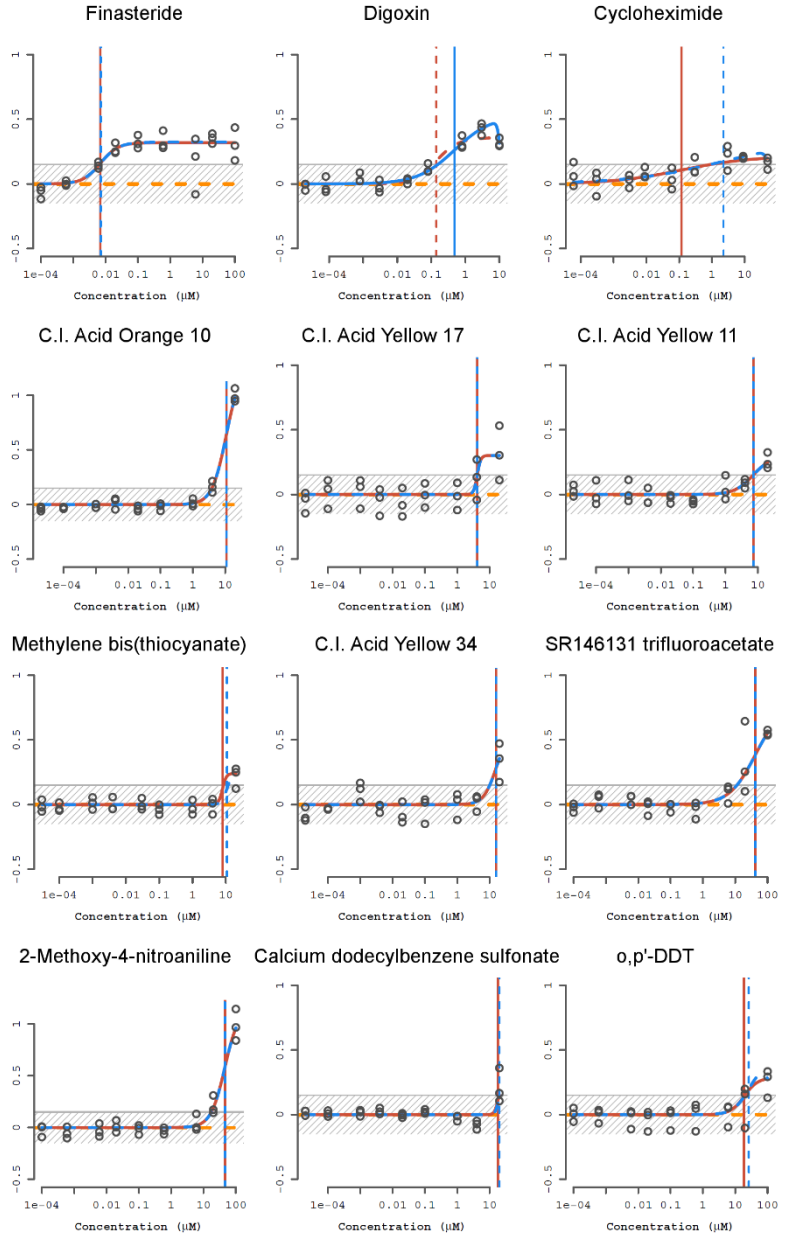
Next Step

- 228 chemicals triaged for multiple-concentration secondary screening.



Secondary Screen Results





Results

- Screen hits filtered for cytotoxicity and autofluorescence.
- 24 chemicals identified as putative inhibitors.

Part 2: Summary

Strengths

- Development of a 5α-reductase high-throughput assay.
  - Coverage of key in vitro data gap for androgen steroidogenesis.
  - Screening completed across a large chemical library.
  - Inclusion of autofluorescence and cytotoxicity filters for hazard identification.
  - Sufficient sensitivity for identifying validated 5α-reductase inhibitors.
- NanoBRET Target Engagement Technology
  - Cell-based assay format enables evaluation of direct chemical-protein interactions.

Limitations

- NanoBRET Target Engagement Technology
  - Application to a dynamic metabolic system increases variability.
  - Pharmacological mechanisms for inhibition of 5α-reductase enzyme may be inconsistent with intended loss-of-function assay design.
  - Low assay dynamic range in this application.

Acknowledgements

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