Establishment of a High Throughput Endocrine Disruptor Screening Panel of Assays for Rapid Testing of Chemicals

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Abstract

The endocrine system is comprised of hormones released by internal glands and their target receptors on distant organs. Hormone receptors, including Androgen Receptor (AR), Estrogen Receptor (ER) and Thyroid Hormone Receptor (TR), are a wide family of proteins that bind to specific hormones and activate a broad range of signaling pathways, such as growth, metabolism, development, reproduction, and response to stress. Since these hormone receptors interact with their ligands with high specificity and high affinity, very low concentrations of hormone can induce significant cellular responses. Endocrine disruptors, either natural or man-made, can interfere with the normal actions of hormones and affect the endocrine system, causing serious health problems such as cancer, birth defects, and developmental disorders.

Traditional Endocrine Disruptor Screening Assays use radioactively labeled materials or animal models with high cost and low efficiency. Here we developed 384-well high throughput cell-based hormone activation assays and *in vitro* hormone receptor binding assays with luminescence or fluorescence polarization end points and 24-48 h turnaround time.

Our cell-based endocrine receptor activation assays utilize the Indigo Biosciences non-human mammalian cells engineered to express Human Androgen Receptor (AR), Human Estrogen Receptor (ERα or ERβ), or Human Thyroid Hormone Receptor (TRα or TRβ). Upon ligand activation, the hormone receptor will bind to the promotor sequence linked to a luciferase reporter gene, and the change in receptor activity is quantified by luminescence detection on a plate reader. The reporter cells are seeded in 384 well plates and dosed with increasing concentrations of test article, positive control or vehicle control and incubated at 37 °C for 24 hr. At the end of the incubation period, the cell viability is assessed fluorimetrically, and luciferase activity is measured using a plate reader. A fold increase in transcriptional activation of hormone receptor above vehicle control for each test article concentration measured is calculated, and EC50 value of activation is derived.

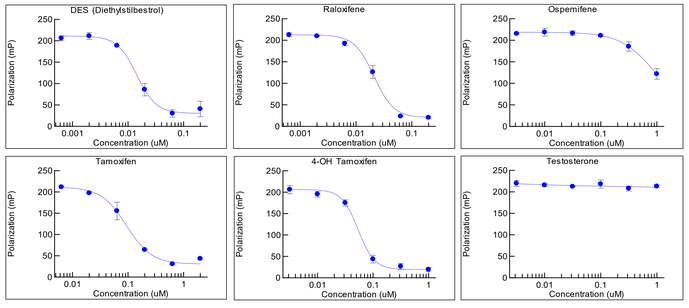
Our hormone receptor competitor binding assay utilizes the PolarScreen AR or ER Competitor Assay kit to determining the IC50 values of compounds that bind the AR-LBD. The assay provides a sensitive and efficient method for high-throughput, fluorescence polarization-based screening of potential AR or ER ligands. AR or ER is added to a fluorescently-tagged ligand in the presence of competitor test compounds in 384w plates. The presence of a competitor compound prevents the formation of a fluorescent ligand/receptor complex, resulting in a decrease of the polarization value due to ligand displacement. The shift in polarization value in the presence of test compounds is used to determine relative affinity of test compounds for the hormone receptor.

Estrogen Receptor (ER) Competitive Binding Assay

The PolarScreen Estrogen Receptor Alpha (ERα) Competitor Assay, Green was used to test the competitive binding of test compounds to ERα against a fluorescent ligand tracer. The ER-tracer complex was mixed with test compounds that had been serially diluted at a half log scale for a total of 6 doses. After 2-4 h incubation in dark , the fluorescence polarization (mP) signal was read a plate reader. Six compounds were tested and the change in polarization was determined. The positive control DES exhibited an EC50 of 12 nM and a delta mP of 160.

	Compound ID	IC50 (uM)
ERα	DES (Diethylstilbesterol)	0.012
	4-OH Tamoxifen	0.045
	Tamoxifen	0.086

	Compound ID	IC50 (uM)
ERα	Raloxifene	0.018
	Ospemifeme	1.09
	Testosterone	No Response

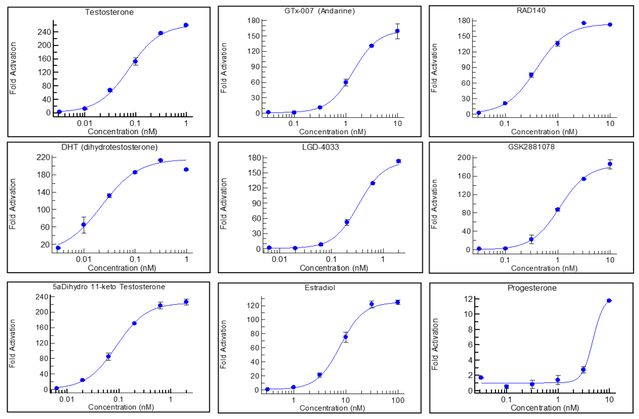


Androgen Receptor (AR) Activation

The Indigo Biosciences Human Androgen Receptor Reporter Assay kit was used to perform the cell-based AR activation assays. A total of 9 compounds and one positive control were tested. Eight of them showed a positive response and 1 showed a negative response. The positive control 5αDihydro 11-keto testosterone performed as expected with an EC50 of 0.086 nM and a maximum Fold Activation of 224.

	Compound ID	EC50 (nM)
AR	Testosterone	0.071
	DHT (dihydrotestosterone)	0.026
	Progesterone	4.771
	Flutamide	No response
	GTx-007 (Andarine)	1.294

	Compound ID	EC50 (nM)
AR	LGD-4033	0.335
	RAD140	0.472
	GSK2881078	0.903
	Estradiol	7.437
	5αDihydro 11-keto Testosterone	0.086

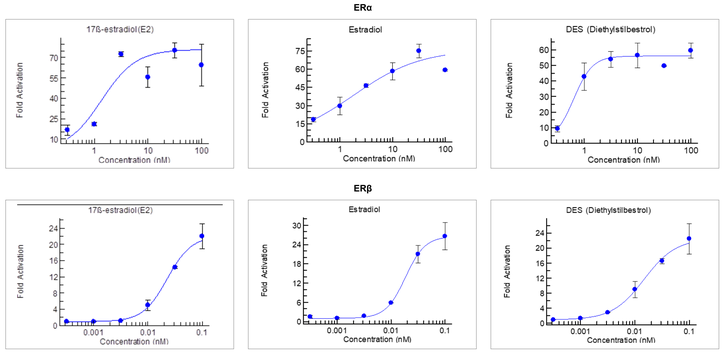


Estrogen Receptor (ER) Activation

The Indigo Biosciences Human Estrogen Receptors Reporter Assay PANEL kit was used to perform the cell-based ERα and Erβ activation assays. Eight compounds and one positive control were tested. Of these, 4 showed a positive response and 4 showed a negative response on ERα; 2 showed a positive response and 6 showed a negative response on ERβ. The positive control 17β-estradiol (E2) performed as expected with an EC50 of 1.36 nM and a maximum Fold Activation of 64 for ERα and an EC50 of 0.023 nM and a maximum Fold Activation of 22 for ERβ.

	Compound ID	EC50 (nM)
ERα	Estradiol	1.74
	DES (Diethylstilbesterol)	0.87
	17β-estradiol (E2)	1.36

	Compound ID	EC50 (nM)
ERβ	Estradiol	0.019
	DES (Diethylstilbesterol)	0.016
	17β-estradiol (E2)	0.023



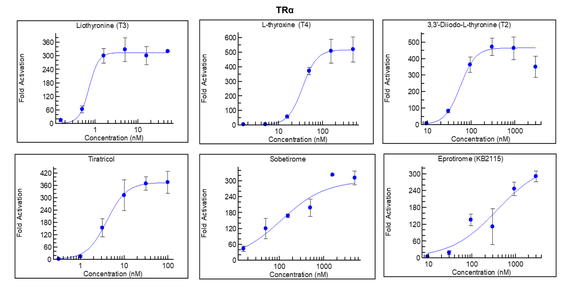
Thyroid Hormone Receptor (TR) Activation

The Indigo Biosciences Human Thyroid Hormone Receptor PANEL kit was used to perform the TRα and TRβ activation assays. Cells from the respective kits were seeded in 384 well plates and incubated for 4 hours. They were then dosed with test compounds that had been serially diluted at a half log scale for a total of 6 doses. After 24 h incubation, the TR activation (luminescence) and cell viability (fluorescence) were detected on a plate reader.

Eight compounds and one positive control were tested as described. All of them showed a positive response for TRα and six showed a positive response for TRβ. The positive control liothyronine (T3) performed as expected with an EC50 of 0.66 nM and a maximum Fold Activation of 369 for TRα and an EC50 of 4.72 nM and a maximum Fold Activation of 265 for TRβ.

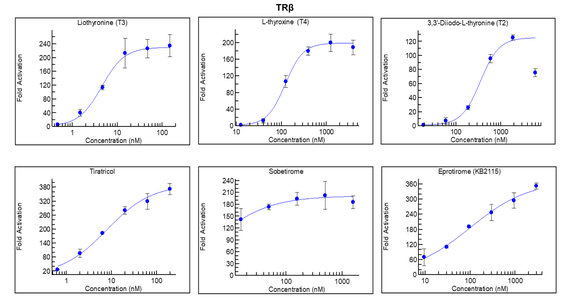
	Compound ID	EC50 (nm)
TRα	Liothyronine (T3)	0.66
	3,3'-Diiodo-L-thyronine (T2)	70.76
	L-thyroxine (T4)	48.03
	Eprotirome (KB2115)	246.25

	Compound ID	EC50 (nm)
TRα	Tiratricol	4.53
	Sobetirome	111.06
	KB-130015	3,861.66
	Amiodarone	1,016.98



	Compound ID	EC50 (nm)
TRβ	Liothyronine (T3)	4.72
	3,3'-Diiodo-L-thyronine (T2)	369.81
	L-thyroxine (T4)	261.67
	Eprotirome (KB2115)	60.43

	Compound ID	EC50 (nm)
TRβ	Tiratricol	4.96
	Sobetirome	6.14
	KB-130015	No response
	Amiodarone	No response



Conclusions

We validated the responses from a group of chemicals for both the *in vitro* hormone receptor binding assays and the cell-based hormone receptor activation assays. The results are consistent with literature report and correspond well between the two assays. Overall, our hormone receptor modulation assays were very effective at predicting chemicals with endocrine disrupting effect. Additional steroidogenesis assays will be developed to detect the hormone levels of AR and ER, in the presence of endocrine disruptors, from H295R cell culture supernatant with LC-MS. Combination of these three assays will identify more environmental and therapeutic articles with endocrine disrupting liability.