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Biochemical and cellular fluorescence-based nuclear receptor assays for high-throughput screening

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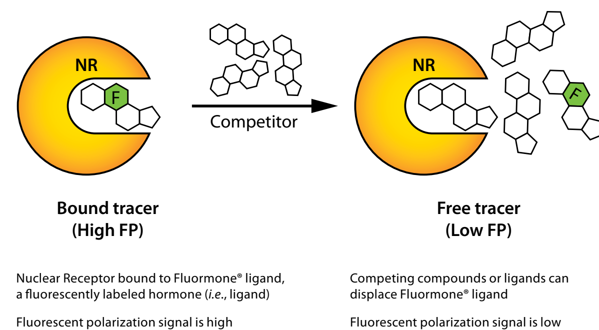
Abstract

We are developing a suite of cellular and biochemical nuclear hormone receptor assays using FP, FRET, and TR-FRET detection of receptor function to create assays whose Z′ reproducibility, cost, and throughput are well-suited to HTS for drug discovery. The availability of multiple assay formats spanning the range from purified components to intact cells has the ability to couple quantitative binding data with information on the more complex *in vivo* regulation of receptor function to streamline hit validation and lead optimization. Our PolarScreen™ FP nuclear receptor assays are built to provide robust, high-throughput assays for competitive binding studies. These assays measure the amount of labeled ligand (Fluormone™) bound to a purified nuclear receptor protein. PolarScreen™ competitive binding assays are available using green, red and far-red fluorescent ligands. Kits are available for 6 different nuclear receptors. Our versatile LanthaScreen™ toolbox reagents can be used to build cofactor recruitment assays for nuclear receptors, using a TR-FRET readout. The cellular assays are built using the GeneBLAzer® platform to measure nuclear hormone receptor activation of beta-lactamase (BLA) reporter genes in live cells. The superior data reproducibility needed for HTS is derived from using the ratio of FRET signals from the nontoxic cell-permeable BLA substrate to permit internally controlled monitoring of receptor activity in living cells. The whole-cell, addition-only assays are developed for primary screening in 384-well plates. Cellular assays have been launched for 9 nuclear receptors, and there is an active program in place to rapidly expand this approach to new targets.

Available assays and formats

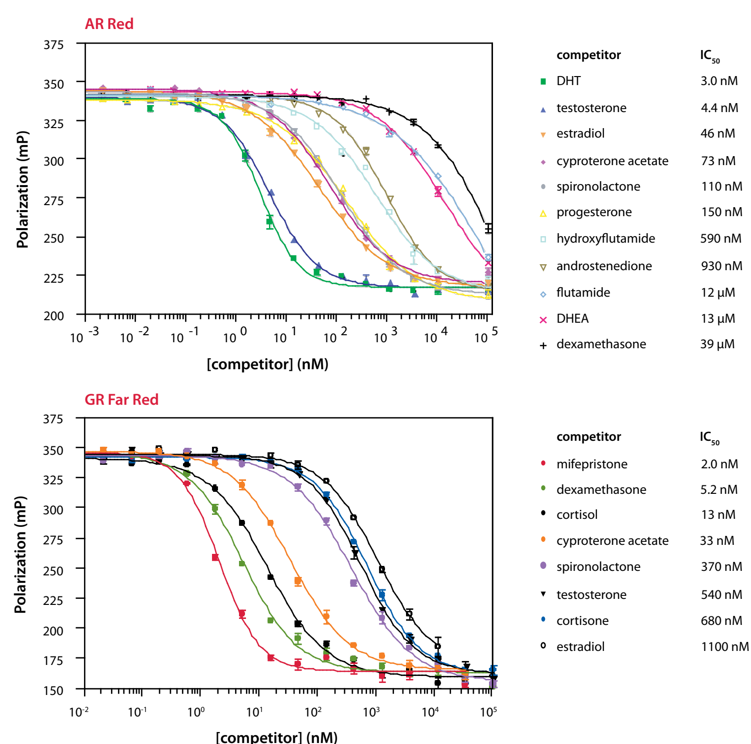
Nuclear Receptor	GeneBLAzer® Cell-based Assay	PolarScreen™ Fluormone® Assay
AR	●	Green, Red
ER α	●	Green, Red
ER β	●	Green, Red
GR	●	Green, Red, Far Red
PR	●	Green, Red, Far Red
MR	●	
VDR	●	
PPAR γ	●	Green, Red
LXR β	●	
UAS-bla 293T	Parental cell line	

Figure 1—PolarScreen™ Fluormone® Assays



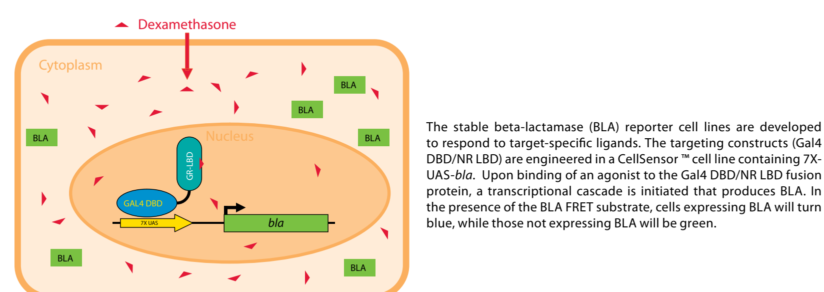
Receptor is added to a fluorescent ligand (Fluormone® ligand) in the presence of a test compound in a microwell plate. Test compounds capable of binding to the receptor will prevent the formation of a receptor/Fluormone® complex, causing the Fluormone® ligand to be free in solution. When the Fluormone® ligand is free in solution, its rotational mobility is greater than when bound to the receptor, and this results in a low polarization value. Test compounds that do not bind to the receptor will have no effect on formation of the receptor/Fluormone® complex, and the measured polarization value will remain high.

Figure 2—Red and Far Red PolarScreen™ Competition Assays

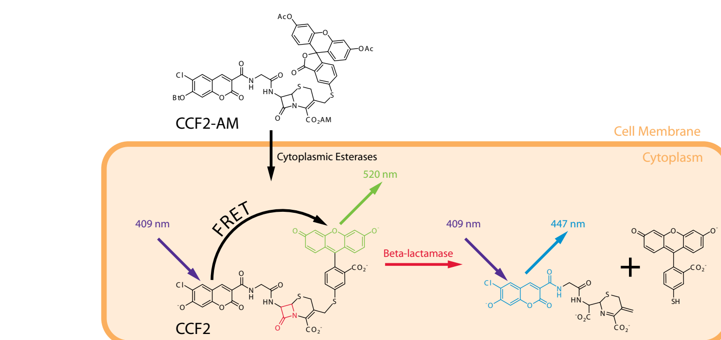


The affinities of a panel of ligands were determined for each receptor in the Red and Far Red competition assays. Fluormone® ligand and receptor were added sequentially to a dilution series of each ligand. Assay plates were incubated until binding equilibrium was reached prior to measuring polarization values. Data points represent the average ± 1 standard deviation of four replicate wells.

Figure 3—Principle of GeneBLAzer® Cell-based Assays



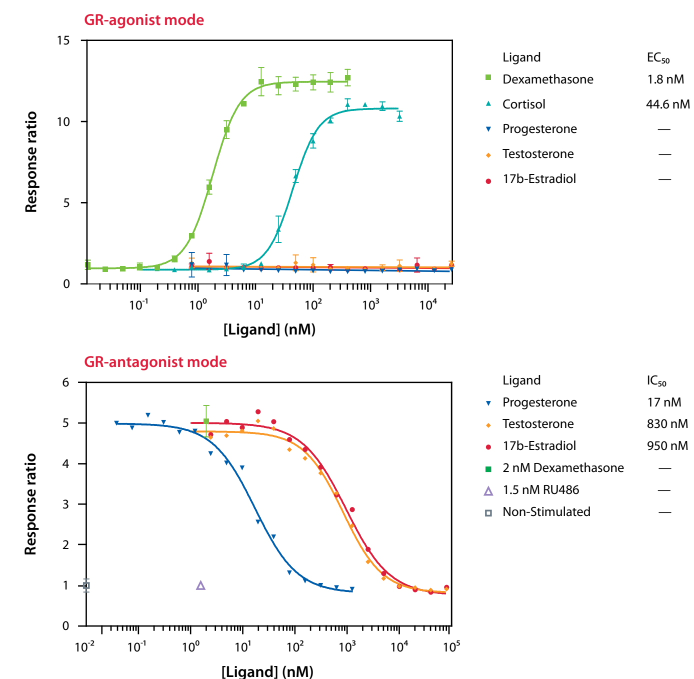
The stable beta-lactamase (BLA) reporter cell lines are developed to respond to target-specific ligands. The targeting constructs (Gal4 DBD/NR LBD) are engineered in a CellSensor™ cell line containing 7X-UAS-*bla*. Upon binding of an agonist to the Gal4 DBD/NR LBD fusion protein, a transcriptional cascade is initiated that produces BLA. In the presence of the BLA FRET substrate, cells expressing BLA will turn blue, while those not expressing BLA will be green.



After the BLA FRET substrate (CCF2/4-AM) enters a cell, endogenous esterases convert it to CCF2/4. Exciting CCF2/4 at 409 nm leads to efficient FRET from the coumarin moiety to the fluorescein derivative and produces green fluorescence detectable at 530 nm. Cleavage of CCF2/4 by beta-lactamase results in the separation of the two fluorophores and the loss of FRET; blue fluorescence, detectable at 460 nm, is now observed with excitation at 409 nm.

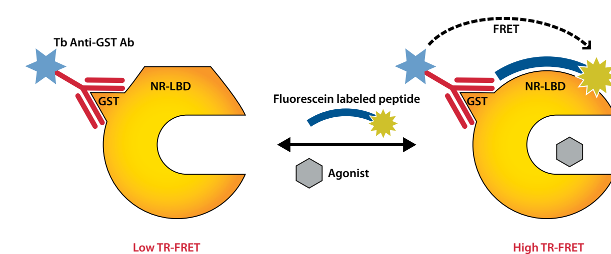
LBD = ligand binding domain; DBD = DNA binding domain; UAS = upstream activating sequence.

Figure4—Agonist and antagonist GeneBLAzer® Cell-based Assays



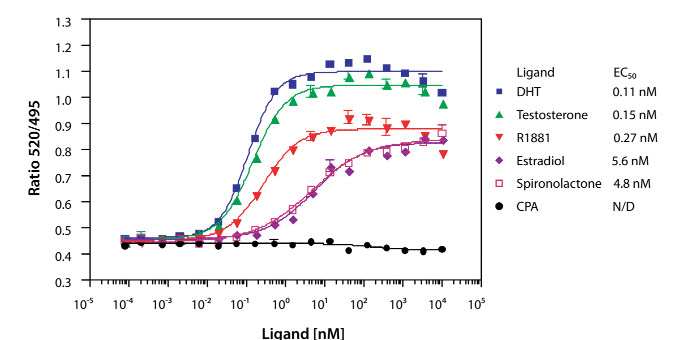
The concentration-dependent effect of potential GR ligands were tested in the GR cell-based agonist and antagonist assays. All ligand concentrations were tested in triplicate. In agonist mode, cells were treated with increasing concentrations of ligands in the presence of 0.5% DMSO for 16 hours. In antagonist mode, cells were treated in the presence of 2 nM dexamethasone and 0.5% DMSO.

Figure 5—LanthaScreen™ Co-Recruitment Assays



- Tb-anti-GST Ab binds to the NR-LBD via the GST tag
- Binding of the agonist to the NR-LBD causes a conformational change which increases its affinity for the co-activator peptide
- Close proximity of the fluorescein labeled co-activator peptide and Tb labeled Ab causes an increase in the TR-FRET signal

Figure 6—LanthaScreen™ AR Agonist Assay



Increasing concentrations of ligand were mixed with 5 nM AR LBD-GST, 500 nM Fl-D11FxxLF peptide, and 5 nM Terbium anti-GST antibody. TR-FRET was measured 2 hours after incubation at room temperature. All AR agonists gave expected rank potency.

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