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Development of a Homogeneous Luminescent HTS Platform for Detection of Nuclear Receptor Modulators Using the AlphaScreen™ Technology

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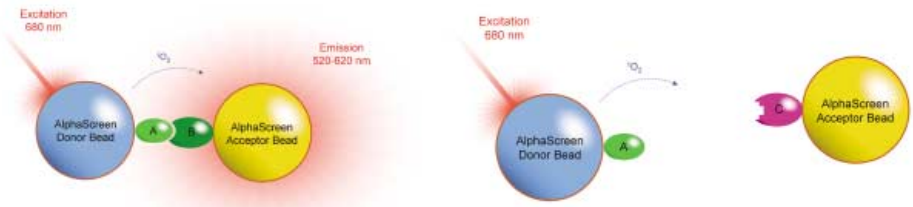
1

Abstract

The interaction between Nuclear Receptors (NR) and coactivators is a key step in signal transduction from the receptor to the transcriptional machinery. Using AlphaScreen, we have developed a highly sensitive assay to detect and characterize compounds modulating the heterodimerization of NR with the coactivator SRC-1. Estrogen (ERα) and retinoic acid (RARγ) receptors were chosen as representative members of the two major NR subfamilies, and for their importance as therapeutic targets. Our results show that AlphaScreen NR assays are highly sensitive and only require low nanomolar concentrations of reagents to produce a high quality signal. AlphaScreen NR assays enable precise pharmacodynamic characterization of agonists or antagonists targeting either the ligand binding site or the receptor-coactivator heterodimerization interface. The overall results presented here show the versatility, sensitivity, robustness and ease of execution of AlphaScreen NR assays which allow one to screen for NR modulators.

2 Principles of AlphaScreen

AlphaScreen is a bead-based Amplified Luminescent Proximity Homogeneous Assay. When biological interactions bring the Donor and Acceptor beads into close proximity, reactive oxygen, generated upon laser excitation of the Donor beads, initiates a luminescence/fluorescence cascade in the Acceptor beads. This process leads to a highly amplified signal with light output in the 520-620 nm range. When the Acceptor and Donor beads are not in proximity, the reactive oxygen decays and only a very low background signal is generated.



3 Models to Measure Nuclear Receptor Heterodimerization

Depending on receptor availability, AlphaScreen NR assays can be developed using native as well as epitope-tagged proteins. For capturing native receptors, it is crucial to use antibodies specific for epitopes located outside the ligand or the co-activator binding domains. Antibodies against either A or F domains are well suited.

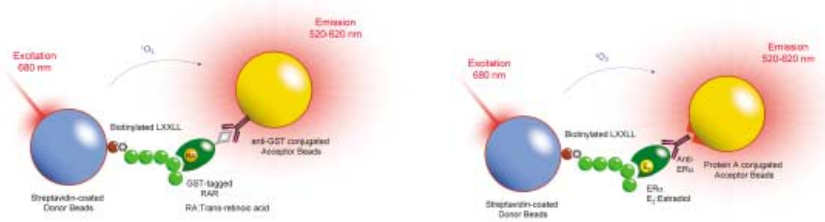
Model A: Epitope-Tagged Receptor

Anti-GST-coated Acceptor beads are used to capture the GST-fusion RAR-ligand binding domain whereas the biotinylated SRC-1 LXXLL motif is captured by the streptavidin Donor beads.

Model B: Native Receptor

Protein A-coated Acceptor beads serve to capture anti-ERα antibodies (C-terminus/F-region specific) binding to full length ERα. A biotinylated peptide derived from SRC-1 LXXLL motif is captured by the streptavidin Donor beads

In both models, agonist-induced changes in nuclear receptor ligand binding domain conformation triggers the interaction of the receptor with the coactivator sequence. This binding event brings the beads into proximity and leads to the generation of the AlphaScreen signal.



4 Methods

Assays were performed in white opaque 384-well microplates in a 25 µl final volume. Reagents were added as follows:

ERα assay

- 5 µl buffer, agonist or antagonist
- 5 µl ERα
- 5 µl biotinylated coactivator peptide
- 5 µl anti-ER / Protein A-Acceptor beads mix (preincubated 30 min)
- 5 µl Streptavidin (SA) Donor beads

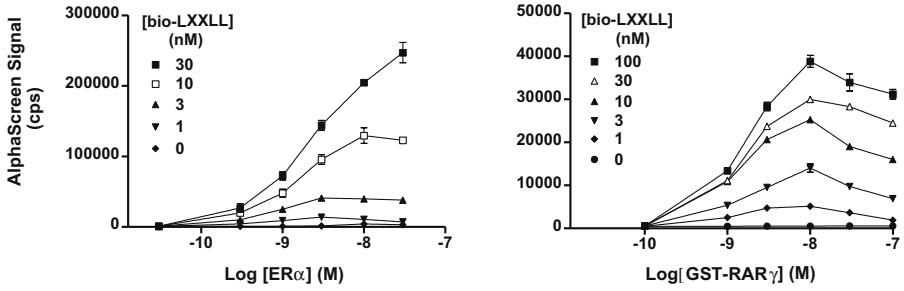
RARγ assay

- 5 µl buffer or non-biotinylated peptide
- 5 µl RARγ
- 5 µl biotinylated coactivator peptide
- 10 µl anti-GST Acceptor beads / SA Donor beads

5 Titration of NR and Biotin-LXXLL Containing Peptide

Biotin SRC-1 and receptor titration curves. Increasing concentrations of ERα (left panel) or GST-RARγ (right panel) were incubated along with increasing concentrations of biotinylated SRC-1 LXXLL peptide and a fixed concentration of agonist (10 nM). In both models, low nM concentrations of reagents are sufficient to produce robust signals. The signal decrease observed above 10 nM RARγ results from the saturation of the anti-GST Acceptor beads by GST-RARγ.

Plates were read following 1 hour incubation at room temperature. Data are the means ± SEM of triplicate determinations from a single experiment, which is representative of three independent experiments.



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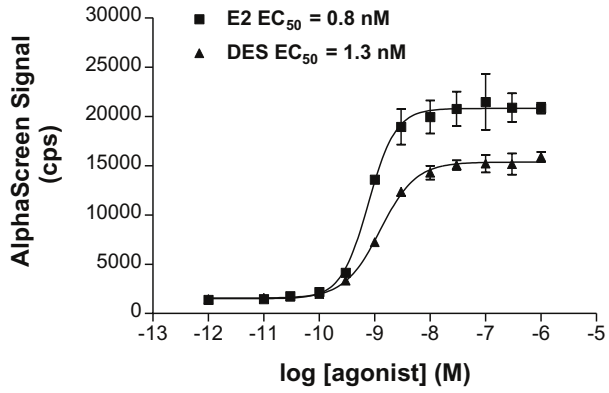
ERα Agonist Dose-Response Curves

ERα agonists dose-response curves. Increasing concentrations of Estradiol or diethylstilbestrol (DES) were incubated along with 3 nM ERα and 3 nM biotinylated-SRC-1 LXXLL peptide. Concentration-dependent signal increases were observed and EC50 values of 0.8 and 1.3 nM were measured with Estradiol and DES respectively.

EC_{50} values were estimated by non-linear regression analysis using GraphPad Prism software (San Diego, CA). Data are the means ± SEM of triplicate determinations from a single experiment, which is representative of three independent experiments.



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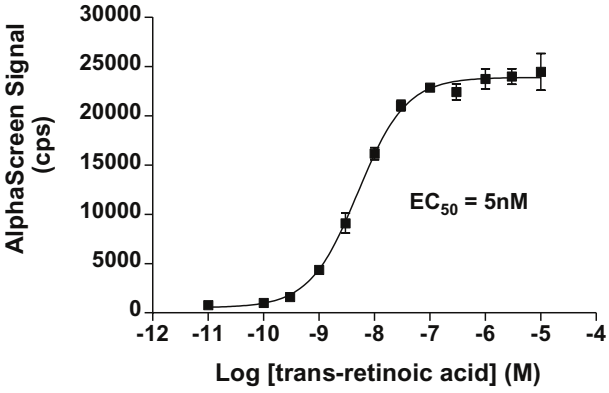
RARγ Agonist Dose-Response Curve

RARγ agonist dose-response curve. Increasing concentrations of trans-retinoic acid were incubated along with 3 nM RARγ and 30 nM biotinylated LXXLL peptide. Increasing concentrations of trans-retinoic acid lead to signal increase and an EC_{50} value of 5 nM was measured.

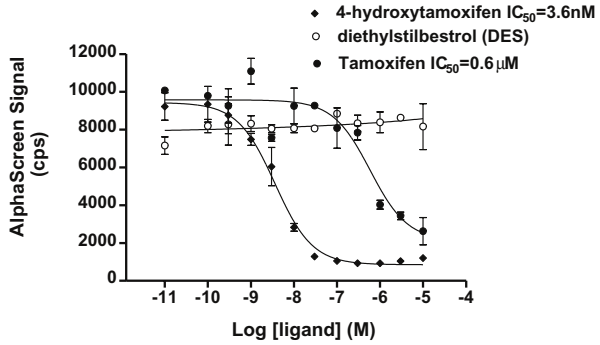
EC_{50} value was estimated by non-linear regression analysis using GraphPad Prism software (San Diego, CA). Data are the means ± SEM of triplicate determinations from a single experiment, which is representative of three independent experiments.



8 Inhibition of Agonist-Induced Heterodimerization by Antagonists

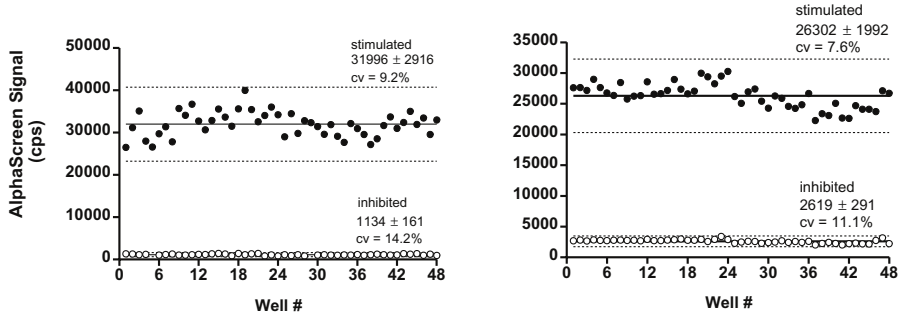
ERα antagonist dose-response curves. Increasing concentrations (0 to 10 μM) of antagonists tamoxifen (l) and 4-hydroxy-tamoxifen (u), as well as the agonist DES (m) used as a negative control, were incubated along with 3 nM SRC-1 peptide and 1 nM full length ERα. Both antagonists inhibited the agonist-induced ERα heterodimerization as observed by the concentration-dependent signal decrease. As expected, the agonist DES had no effect up to 10 μM. These results demonstrate that the assay discriminates between agonist and antagonist responses and allows one to establish genuine rank order of antagonist potency.

IC_{50} values were estimated for each antagonist by non-linear regression analysis using Graph Pad Prism software (San Diego, CA). Data are the means ± SEM of triplicate determinations from a single experiment.



9 Intra-Plate Assay Variability

Intraplate variability for AlphaScreen NR assay. The intraplate variability was studied on two distinct data populations: 48 wells were used to measure agonist-induced heterodimerization (stimulated) and 48 different wells were used to measure antagonist inhibition of agonist-induce heterodimerization (inhibited). Left panel: CV values measured with the ERα were 9.2% and 14.2% for stimulated and inhibited signals respectively. Right panel: for RARγ, CV values were 7.6% for the stimulated signal and 11.1% for the inhibited signal obtained in the presence of an excess of unlabeled LXXLL peptide. Z' values of 0.7 were obtained with both assays which indicates that AlphaScreen NR assays are suitable for HTS.



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Conclusion

AlphaScreen technology was used to develop Nuclear Receptor functional assays allowing one to:

- Discriminate nuclear receptor ligands on a functional basis.
- Assess both agonist and antagonist potencies.
- Reduce reagent consumption (high sensitivity and large S/B ratio).
- Versatile assay development (native receptor or tagged recombinant receptor).
- Obtain robust screening parameters (Z' > 0.5).
- Screen for antagonists targeting either the ligand binding site or the receptor-coactivator heterodimerization interface.

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