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1. KIT CONTENTS

The LanthaScreen™ Estrogen Related Receptor gamma TR-FRET Coactivator Assay, catalog no. PV4408, contains the following:

Component	Composition	Amount	Storage Temp.	Individual Catalog no.
Fluorescein-PGC1a coactivator peptide (Puigserver et al, 1998)	100 µM in 50 mM HEPES, pH 7.5 Sequence: EAEEPSLLKLLLPANTQ	100 µl	-20 °C	PV4421
ERR gamma LBD, GST	ERR gamma ligand-binding domain in a buffer (pH 7.5) containing protein stabilizing reagents and glycerol. See Certificate of Analysis for the recommended molar concentration for this kit.	50 pmol	-80 °C	PV4416
LanthaScreen™ Tb-anti-GST antibody	Terbium labeled anti-GST antibody in HEPES buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM HEPES pH 7.5). See Certificate of Analysis for lot specific concentration	25 µg	-20 °C	PV3550
TR-FRET Coregulator Buffer B	Proprietary buffer (pH 7.5) including 20% glycerol	25 ml	4 °C	PV4418
DTT, 1 M	In water	1 ml	-20°C or -80°C	P2325

2. MATERIALS REQUIRED BUT NOT SUPPLIED

The following materials are required but not supplied in the kit:

- A fluorescence plate reader with excitation capabilities at 340 nm and with the appropriate filter sets installed for detecting the fluorescent emission signals of terbium at 495 nm and fluorescein at 520 nm (see Section 4).
- Pipetting devices for 1–1000-µl volumes, suitable repeater pipettors, or multi-channel pipettors.
- Black, 384-well assay plates. We recommend black Corning® 384-well, low-volume, round-bottom (non-binding surface) assay plates; Corning #3676. Other plate types may give satisfactory results as well.
- 96-well polypropylene plate that can accommodate a 400-µl volume per well. We recommend Nalgene Nunc #249944.
- A known ERR gamma inverse agonist, such as 4-hydroxytamoxifen, to serve as a positive control. We recommend Sigma catalog no. H7904.
- DMSO

3. INTRODUCTION

Invitrogen's LanthaScreen™ Estrogen Related Receptor gamma TR-FRET Coactivator Assay provides a sensitive and robust method for high-throughput screening of potential ERR gamma ligands as inverse agonists (sometimes referred to as antagonists) of coactivator displacement. The kit uses a terbium-labeled anti-GST antibody, a fluorescein-labeled

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coactivator peptide, and a ERR gamma ligand-binding domain that is tagged with glutathione-S-transferase (GST) in a homogenous mix-and-read assay format. This kit contains enough reagents for 800 assays of 20 µl each.

3.1 Principle of FRET and TR-FRET

For screening libraries of compounds, time-resolved FRET (TR-FRET) is a recognized method for overcoming interference from compound autofluorescence or light scatter from precipitated compounds. The premise of a TR-FRET assay is the same as that of a standard FRET assay: when a suitable pair of fluorophores is brought within close proximity of one another, excitation of the first fluorophore (the donor) can result in energy transfer to the second fluorophore (the acceptor). This energy transfer is detected by an increase in the fluorescence emission of the acceptor and a decrease in the fluorescence emission of the donor. In HTS assays, FRET is often expressed as a ratio of the intensities of the acceptor and donor fluorophores. The ratiometric nature of such a value corrects for differences in assay volumes between wells and corrects for quenching effects due to colored compounds.

In contrast to standard FRET assays, TR-FRET assays use a long-lifetime lanthanide chelate as the donor species. Lanthanide chelates are unique in that their excited-state lifetime (the average time that the molecule spends in the excited state after accepting a photon) can be on the order of a millisecond or longer. This is in sharp contrast to the lifetime of common fluorophores used in standard FRET assays, which are typically in the nanosecond range. Because interference from autofluorescent compounds or scattered light is also on the nanosecond timescale, these factors can negatively impact standard FRET assays. To overcome these interferences, TR-FRET assays are performed by measuring FRET after a suitable delay, typically 50 to 100 microseconds after excitation by a flashlamp excitation source in a microtiter plate reader. This delay not only overcomes interference from background fluorescence or light scatter, but also avoids interference from direct excitation due to the non-instantaneous nature of the flashlamp excitation source.

The most common lanthanides used in TR-FRET assays for HTS are terbium and europium. Terbium offers unique advantages over europium when used as the donor species in a TR-FRET assay. In contrast to europium-based systems that employ APC as the acceptor, terbium-based TR-FRET assays can use common fluorophores such as fluorescein as the acceptor. Because it is straightforward (and inexpensive) to label a molecule such as a peptide with fluorescein, directly labeled molecules may be used in terbium-based TR-FRET assays, rather than biotinylated molecules that must then be indirectly labeled via streptavidin-mediated recruitment of APC. The use of directly labeled molecules in a terbium-based TR-FRET assay reduces costs, improves kinetics, avoids problems due to steric interactions involving large APC conjugates, and simplifies assay development since there are fewer independent variables requiring optimization in a directly labeled system.

3.2 Assay Overview

ERR gamma constitutively binds coactivator peptide. Binding of inverse agonist to the nuclear receptor (Figure 1) causes a conformational change around helix 12 in the ligand binding domain, resulting in lower affinity for the coactivator peptide. When the terbium label on the anti-GST antibody is excited at 340 nm, energy is transferred to the fluorescein label on the coactivator peptide and detected as emission at 520 nm. Addition of ligand and displacement of the coactivator peptide is detected by a loss in FRET and ultimately a decrease in the emission at 520 nm.

When running the LanthaScreen™ Estrogen Related Receptor gamma TR-FRET Coactivator Assay, ERR gamma-LBD is added to ligand test compounds followed by addition of a mixture of the fluorescein-coactivator peptide and terbium anti-GST antibody. After an incubation period at room temperature, the TR-FRET ratio of 520:495 is calculated and can be used to determine the EC₅₀ from a dose response curve of the compound. Based on the biology of the ERR gamma-coactivator peptide interaction, this ligand EC₅₀ is a composite value representing the amount of ligand required to bind to receptor, effect a conformational change, and displace coactivator peptide (see Figure 1).

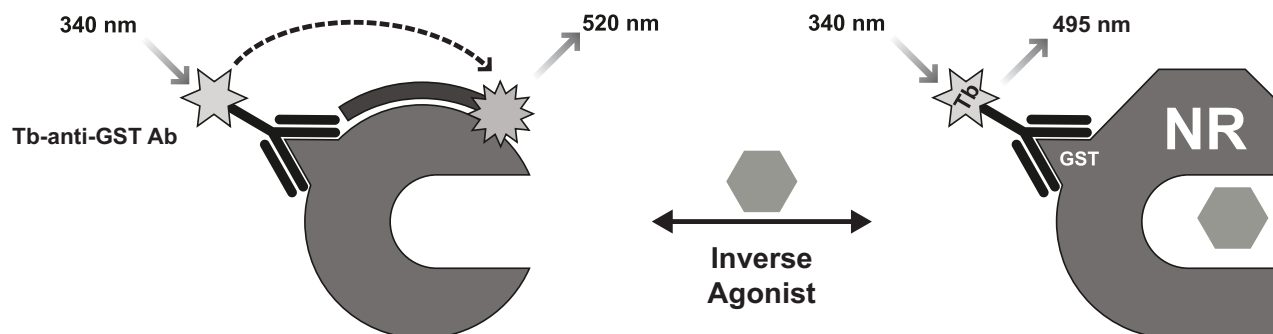


Figure 1. Principle of the nuclear receptor (NR) ligand dependent coactivator peptide displacement assay: Tb-anti-GST antibody indirectly labels the nuclear receptor by binding to the GST tag. Binding of the inverse agonist to the NR causes a conformational change that results in a decrease in the affinity of the NR for a coactivator peptide. The separation of the fluorescently labeled coactivator peptide from the NR with the terbium-labeled antibody causes a decrease in the TR-FRET signal.

4. INSTRUMENT SETTINGS

General Settings:

Excitation	340 nm filter (30 nm bandwidth)
Emission	520 nm filter (25 nm bandwidth)
Emission	490 or 495 nm filter (10 nm bandwidth)
Delay Time	100 μ s
Integration Time	200 μ s

The excitation and emission spectra of terbium and fluorescein are shown in Figure 2. As with other TR-FRET systems, the terbium donor is excited using a 340-nm excitation filter with a 30-nm bandwidth. However, the exact specifications of the excitation filter are not critical, and filters with similar specifications will work well. In general, excitation filters that work with europium-based TR-FRET systems will perform well with the LanthaScreen™ terbium chelates.

Spectra of Terbium and Fluorescein

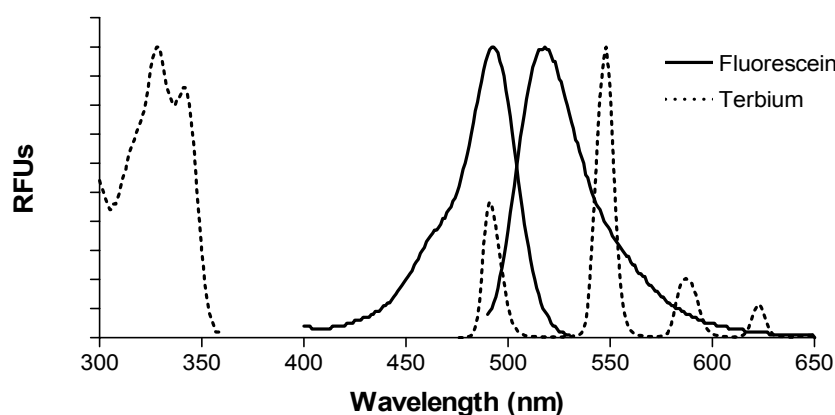


Figure 2: Excitation and Emission spectra of fluorescein and terbium.

As shown in the figure, the terbium emission spectrum is characterized by four sharp emission peaks, with silent regions between each peak. The first terbium emission peak (centered between approximately 485 and 505 nm) overlaps with the maximum excitation peak of fluorescein. Energy transfer to fluorescein is then measured in the silent region between the first two terbium emission peaks. Because it is important to measure energy transfer to fluorescein without interference from terbium, a filter centered at 520 nm with a 25 nm bandwidth is used for this purpose. The specifications of this filter are more critical than those of the excitation filter. In general, standard “fluorescein” filters may not be used, because such filters also pass light associated with the terbium spectra as well. The emission of fluorescein due to FRET is referenced (or “ratioed”) to the emission of the first terbium peak, using a filter that isolates this peak. This is typically accomplished with a filter centered at 490 or 495 nm, with a 10 nm bandwidth. In general, a 490 nm filter will reduce the amount of fluorescein emission that “bleeds through” into this measurement, although instrument dichroic mirror choices (such as those on the Tecan Ultra instrument) may necessitate the use of a 495 nm filter. The effect on the quality of the resulting measurements is minimal in either case. Filters suitable for LanthaScreen™ assays are available from Chroma (www.chroma.com) as filter set PV001, or from other vendors. A LanthaScreen™ filter module for the BMG PheraStar is available direct from BMG Instruments.

Aside from filter choices, instrument settings are similar to the settings used with europium-based technologies. In general, the guidelines provided by the instrument manufacturer can be used as a starting point for optimization. A delay time of 100 μ s followed by a 200- μ s integration time is typical for a LanthaScreen™ assay. The number of flashes or measurements per well is highly instrument dependent and should be set as advised by your instrument manufacturer. In general, LanthaScreen™ assays can be run on any filter-based instrument capable of time-resolved FRET, such as the Tecan Ultra, BMGLabTech PHERAStar, Molecular Devices Analyst, or PerkinElmer Envision. LanthaScreen™ assays have also been performed successfully on the Tecan Safire² and Molecular Devices M5 monochromator-based instrument. Contact Invitrogen Technical Services for instrument-specific setup guidelines.

5. GUIDELINES AND RECOMMENDATIONS

5.1 Reagent Handling

ERR gamma-LBD

Store ERR gamma-LBD at -80°C . Thaw on ice before use and perform all dilutions while on ice. Never vortex the ERR gamma-LBD stock or dilutions. This reagent is stable up to at least 8 freeze/thaws. We recommend aliquotting the ERR gamma-LBD after the first thaw.

TR-FRET Coregulator Buffer B

Thaw TR-FRET Coregulator Buffer B at room temperature upon receipt. Mix well before first use, as the buffer is viscous and may not have thawed evenly. The buffer is stable at room temperature.

LanthaScreen™ Tb anti-GST Antibody

Store Tb anti-GST antibody at -20°C and thaw before use.

Fluorescein-PGC1a Coactivator Peptide

Store fluorescein-PGC1a coactivator peptide at -20°C and thaw before use.

5.2 Ligand Dilutions

The procedure described below involves diluting the ligand to 100X in DMSO followed by transfer into complete assay buffer, resulting in a 2X ligand, 2% DMSO dilution. This may be done in a DMSO-tolerant assay plate or polypropylene tubes. (Dilution in DMSO increases solubility of ligands and reduces sticking to plastics.) After addition of all reagents to the assay, the final concentration will be 1X ligand and 1% DMSO.

Note: Handling of some ligands can be problematic due to their tendency to stick to various plastics. These ligands may show varying degrees of stickiness, causing differences in the actual concentration of the ligand, depending on the type of pipette tips and plates used in the set up of the assay. Therefore, you may observe different EC_{50} values than reported here.

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5.3 Solvent Tolerance

The assay was validated in the presence of 1% DMSO. However, the assay has been performed with up to 8% DMSO, 8% ethanol, and 8% methanol (in addition to the 1% DMSO present from the ligand dilution) with good results.

5.4 Note on Reagent Order of Addition

The assay was validated using three additions per well in which ERR gamma-LBD was added to inverse agonist dilutions, followed by the addition of pre-mixed fluorescein-PGC1a coactivator peptide and Tb anti-GST antibody (inverse agonist, receptor, peptide/antibody). A pre-mixture of ERR gamma-LBD, fluorescein-PGC1a coactivator peptide, and Tb anti-GST antibody may also be added to the inverse agonist dilutions for a total of 2 additions per well, although the assay was not fully validated in this manner. However, it is important to consider the effect of time and temperature on this three- component pre-mixture when developing the assay.

5.5 Incubation Conditions

Incubation Time

The incubation time can be set by the user. As a guide, results for various time points using 4-hydroxytamoxifen (4-OHT) as the inverse agonist are shown in Table 1 below. The EC₅₀ and Z' are stable from 1 to 6 hours (Table 1).

Incubation Time (hours)	EC ₅₀ 4-OHT	Z'-Factor
1	130 nM	0.87
2	130 nM	0.87
4	140 nM	0.86
6	140 nM	0.81
24	280 nM	0.68

Table 1. Effect of Incubation Time on Assay Performance. Sample data represents mean values from 3 separate experiments (n = 24). EC₅₀ values were determined by fitting the data to a sigmoidal dose response (variable slope) equation in GraphPad Prism 4.0 (data not shown). Z'-factors were calculated using the method of Zhang *et al.* (Zhang *et al.*, 1999) on the 24 replicates of maximum inverse agonist and no inverse agonist. Z'-factor is an indication of the robustness of the assay, where values ≥ 0.5 indicate an excellent assay, while a value of 1 indicates a theoretically ideal assay with no variability.

Temperature

We recommend that assays be conducted at room temperature (20–23°C).

6. INVERSE AGONIST ASSAY

The procedure in this section describes a method for determining the EC₅₀ of an inverse agonist (n = 4) and the Z' factor for maximum inverse agonist and no inverse agonist controls (n = 24) using the ligand-induced displacement of fluorescein PGC1a coactivator peptide to ERR gamma-LBD. The only variable is the inverse agonist concentration. All other assay components (ERR gamma-LBD, peptide, Tb anti-GST antibody) are fixed at concentrations optimized to provide an assay with the lowest EC₅₀ with the control inverse agonist, while still maintaining a Z'-factor >0.5. Although a higher concentration of nuclear receptor may give a larger TR-FRET signal, it will compromise the sensitivity of the assay with regard to differentiating tight-binding ligands. The recommended final concentrations for optimal assay performance are listed in the following table. If component concentrations are changed, the assay must be re-optimized. The data in Figure 3 was generated using a method adapted from this protocol.

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Component	Final Assay Concentration
Fluorescein-PGC1a	500 nM
Tb anti-GST antibody	5 nM
ERR gamma LBD-GST	See Certificate of Analysis for the recommended molar concentration for this kit

6.1 Inverse agonist Assay—Procedure

Prepare Complete TR-FRET Coregulator Buffer B and Inverse Agonist Controls

1. Prepare Complete TR-FRET Coregulator Buffer B by adding 1 M DTT to TR-FRET Coregulator Buffer B for a final concentration of 5 mM DTT. Complete TR-FRET Coregulator Buffer B must be prepared fresh daily.

For example: Add 30 µl of 1 M DTT to 5.97 ml of TR-FRET Coregulator Buffer B.

Note: Keep Complete TR-FRET Coregulator Buffer B at room temperature for the preparation of all reagents except for 4X ERR gamma-LBD, which should be prepared with cold buffer. Reserve an appropriate volume of buffer **on ice** for preparation of 4X ERR gamma-LBD (see step 8 for concentrations and example volumes needed).

2. For the “no inverse agonist” controls, add DMSO to Complete TR-FRET Coregulator Buffer B for a final concentration of 2% DMSO. Add 10 µl of this solution to row C, columns 1–24 of a 384-well assay plate (see the plate layout in **Section 6.2**).

For example: Add 10 µl of DMSO to 490 µl of Complete TR-FRET Coregulator Buffer B.

3. Prepare a solution of control inverse agonist (we recommend 4-hydroxytamoxifen) at 100X of the final desired maximum starting concentration using DMSO.

For example: If the final desired maximum starting concentration of inverse agonist is 50 µM, prepare a solution of 5 mM inverse agonist in DMSO.

4. For the “maximum inverse agonist” controls, dilute the 100X inverse agonist solution from step 3 to 2X using Complete TR-FRET Coregulator Buffer B. Add 10 µl of this solution to row D, columns 1–24 in the 384-well assay plate (see the plate layout in **Section 6.2**).

For example: Add 10 µl of 100X inverse agonist solution to 490 µl of Complete TR-FRET Coregulator Buffer B.

Prepare 2X Inverse Agonist Dilution Series

Note: Although steps 5 and 6 below require more pipetting than other methods of preparing a serial dilution of inverse agonist, we have found that this approach provides a robust method for preparing the dilution series without problems due to inverse agonist solubility.

5. Prepare a 12-point 100X dilution series of inverse agonist in a 96-well plate by serially diluting the 100X inverse agonist solution from step 3 three-fold using DMSO.

For example: Add 20 µl of DMSO to wells A2–A12 in a 96-well polypropylene plate. To well A1, add 30 µl of the 100X inverse agonist solution prepared in step 3. Perform a three-fold serial dilution by transferring 10 µl of the 100X inverse agonist solution from well A1 to the 20 µl of DMSO in well A2. Mix by pipetting up and down. Repeat for wells A2–A12.

6. Dilute each 100X inverse agonist serial dilution from step 5 to 2X using Complete TR-FRET Coregulator Buffer B.

For example: Transfer 5 µl of each of the 100X inverse agonist serial dilutions from row A of the 96-well plate (wells A1–A12) to row B (wells B1–B12). Add 245 µl of Complete TR-FRET Coregulator Buffer B to each well in row B of the 96-well plate. Mix by pipetting up and down.

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7. Transfer 10 µl of each of the 2X inverse agonist serial dilutions to duplicate columns of rows A and B of a 384-well assay plate as shown in the plate layout in **Section 6.2**. (n = 4).

For example: Columns 1 and 2 of rows A and B of the 384-well assay plate will receive 10-µl aliquots from well B1 of the 96-well assay plate, wells A and B in columns 3 and 4 of the 384-well assay plate will receive 10-µl aliquots from well B2 of the 96-well assay plate, and so on.

Prepare 4X ERR gamma-LBD

8. Prepare 4X ERR gamma-LBD using **cold** Complete TR-FRET Coregulator Buffer B from step 1. The recommended molar concentration of ERR gamma for this kit is listed on the Certificate of Analysis. **Never vortex the ERR gamma-LBD stock or dilutions.** Mix by pipetting or gentle inversion. Keep this solution on ice until needed for use in the assay.

For example: If the ERR gamma-LBD has a stock concentration of 5000 nM and the recommended concentration for this kit is 5 nM, prepare a 4X solution at 20 nM by adding 4 µl of ERR gamma-LBD stock to 996 µl of **cold** Complete TR-FRET Coregulator Buffer B.

9. Add 5 µl of 4X ERR gamma-LBD to rows A–D, columns 1–24 of the 384-well assay plate.

Prepare 4X Fluorescein-PGC1a/4X Tb anti-GST Antibody

10. Prepare a solution containing 2 µM fluorescein-PGC1a (4X) and 20 nM Tb anti-GST antibody (4X) using Complete TR-FRET Coregulator Buffer B at room temperature. The stock concentration of fluorescein-PGC1a is 100 µM and the concentration of Tb anti-GST antibody is indicated on both the vial label and the Certificate of Analysis (1 mg/ml = ~6.7 µM antibody).

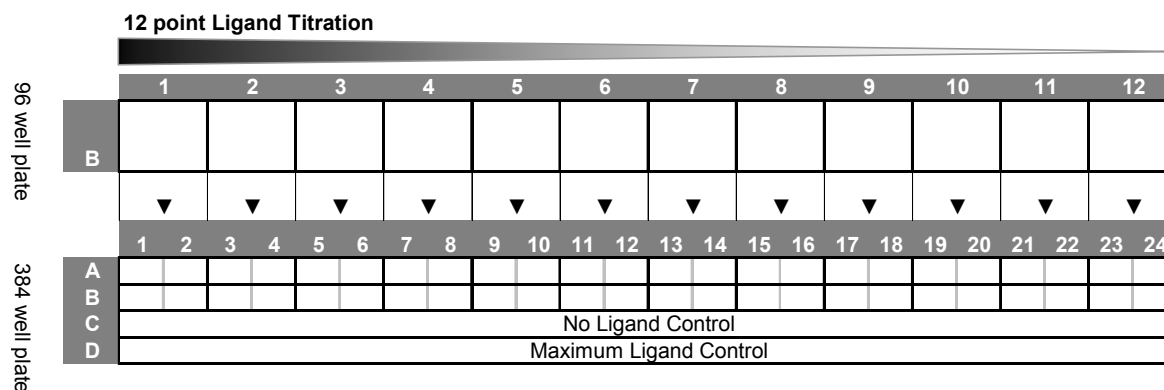
For example: Add 20 µl of 100 µM fluorescein-PGC1a and 3 µl of 6.7 µM Tb anti-GST antibody to 977 µl of Complete TR-FRET Coregulator Buffer B.

11. Add 5 µl of 4X peptide/4X antibody solution to rows A–D, columns 1–24 of the 384-well assay plate.

Plate Incubation and Reads

12. Gently mix the 384-well plate on a plate shaker and incubate at room temperature protected from light. The plate may be sealed with a cover to minimize evaporation.
13. Read the plate between 1 and 6 hours at wavelengths of 520 nm and 495 nm, using the instrument settings described in **Section 4**. If using a plate seal, spin the sealed plate in a centrifuge with an appropriate balance to spin down any condensation on the bottom of the seal, shake the plate gently, and read. Then proceed to data analysis as described in section 6.3.

6.2 Plate Layout



6.3 Inverse agonist Assay—Data Analysis

Calculate the TR-FRET ratio by dividing the emission signal at 520 nm by the emission signal at 495 nm. Generate a binding curve by plotting the emission ratio vs. the log [ligand]. To determine the EC₅₀ value, fit the data using

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an equation for a sigmoidal dose response (varying slope), as provided by GraphPad™ Prism 4.0 or another comparable graphing program.

The “maximum inverse agonist” and “no inverse agonist” control data can be used to calculate Z'-factor based on the equation of Zhang et al (Zhang *et al.*, 1999).

Note: The ligand EC₅₀ determined in the assay is a composite of multiple equilibria, including ligand binding to receptor and peptide binding to ligand/receptor complex.

6.4 Inverse agonist Assay—Reagent Volumes and Controls

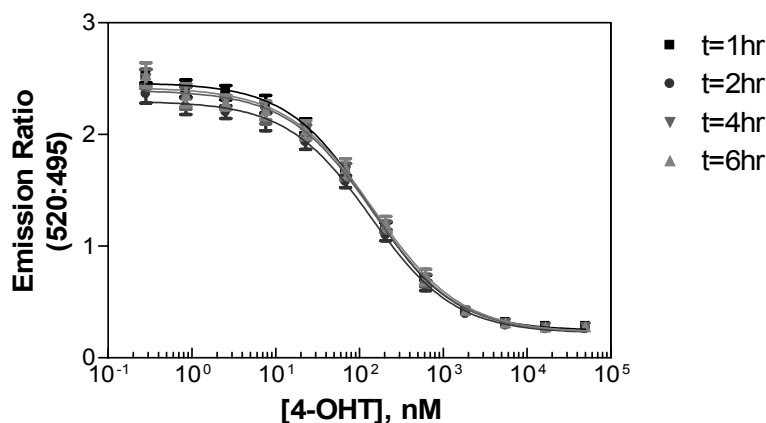
The following table summarizes the reagent volumes, order of addition, and potential controls for developing an inverse agonist mode assay. The protocol in **Section 6.1** may be adapted to screen test compounds using the following summary table as a guide.

Assay	Reagent Additions	Purpose
Test Compound	1. 10 µl 2X Test Compound (or dilution series) 2. 5 µl 4X ERR gamma-LBD 3. 5 µl 4X Fl-PGC1a/Tb anti-GST Ab	Assess coactivator displacement upon binding of test compound.
Positive Control	1. 10 µl 2X inverse agonist (or dilution series) 2. 5 µl 4X ERR gamma-LBD 3. 5 µl 4X Fl-PGC1a/Tb anti-GST Ab	Assess coactivator displacement upon binding of a known ERR gamma inverse agonist.
Negative Control	1. 10 µl 2X Compound solvent 2. 5 µl 4X ERR gamma-LBD 3. 5 µl 4X Fl-PGC1a/Tb anti-GST Ab	Provides indication of basal signal and accounts for possible interference from a compound's vehicle solvent.
No ERR gamma-LBD Control	1. 10 µl 2X compound solvent 2. 5 µl Complete TR-FRET Coregulator Buffer B 3. 5 µl 4X Fl-PGC1a/Tb anti-GST Ab	Provides absolute baseline for assay, not accounting for diffusion-enhanced FRET.

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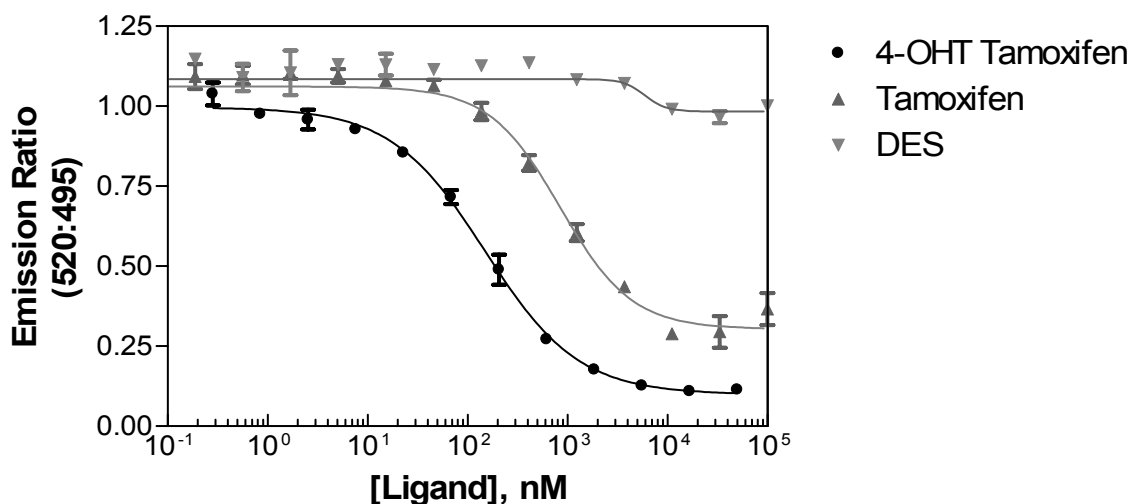
6.5 Inverse agonist Assay—Representative Data



Time Point	EC ₅₀ (nM)
1 hour	130
2 hour	130
4 hour	140
6 hour	150

Figure 3. Representative experiment of LanthaScreen™ TR-FRET ERR gamma-LBD TR-FRET assay. Assay composition: serial dilution of inverse agonist 4-hydroxytamoxifen (4-OHT), 5 nM ERR gamma-LBD, 500 nM Fluorescein PGC1a, and 5 nM Tb anti-GST antibody. Results for 1 hr, 2 hr, 4 hr, and 6 hr incubations are shown with the corresponding EC₅₀ values. The curves were generated using a sigmoidal dose response (variable slope) equation in GraphPad™ Prism® 4.0.

7. ASSAY PHARMACOLOGY



Ligand	EC ₅₀ (nM)
4-OH-tamoxifen	150
DES	ND
Tamoxifen	850

Figure 4: ERR gamma-LBD in the LanthaScreen™ TR-FRET ERR gamma Coactivator Assay, Inverse agonist Mode. Serial dilutions of various test compounds (1% final DMSO concentration) were assayed in inverse agonist mode (n = 4). Curves were fit using a sigmoidal dose-response equation (variable slope) in GraphPad™ Prism® 4.0. ND, not determined.

Note: Binding of different ligands may result in different conformations in the nuclear receptor and thus different affinities for the coregulator peptide. A lower peptide affinity will result in a decreased TR-FRET signal and a lower plateau in the dose response curve.

Note: The ligand EC₅₀ determined in the assay is a composite of multiple equilibria, including ligand binding to receptor and peptide binding to ligand/receptor complex.

8. REFERENCES

Puigserver P., Wu Z., Park C.W., Graves R., Wright M., and Spiegelman B.M. (1998) A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. *Cell* 92: 829-839.

Zhang, J. H., Chung, T. D., and Oldenburg, K. R. (1999) A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *J. Biomol. Screen.*, 4, 67-73.

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