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1. Kit Contents

The LanthaScreen® TR-FRET Retinoid-Related Orphan Receptor (ROR) alpha Coactivator Assay, catalog no. PV5889, contains the following reagents:

Component	Composition	Amount	Storage Temp.	Individual Catalog no.
Fluorescein-D22 coactivator peptide (Chang *et al.*, 1999)	100 µM in 10 mM Na_2CO_3, pH 11.0 Sequence: LPYEGSLLLKLLRAPVEEV	100 µL	–20°C	PV4386
ROR alpha LBD, GST	ROR alpha 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.	10 µg	–80°C	PV5885
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 D	Proprietary buffer (pH 7.5) including 10% glycerol	2 × 25 mL	4°C	PV4420
DTT, 1 M	In water	1 mL	–20°C	P2325
LanthaScreen® Tb Instrument Control Kit	Reagents sufficient to test instrument performance to generate TR-FRET signal	1 kit	Various—see Control Kit protocol	PV5591

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. The ROR alpha assay was validated using black Corning® 384-well, low-volume, round-bottom (non-treated surface) assay plates; Corning #3677. See **Section 5.5** for important information on plate types.
- 96-well polypropylene plate that can accommodate a 300-µL volume per well and is tolerant of 100% DMSO. We recommend Nalgene Nunc #249944.
- DMSO to perform serial dilutions. We recommend Fluka 41647.

3. Introduction

Invitrogen's LanthaScreen® TR-FRET Retinoid-Related Orphan Receptor alpha (ROR alpha) Coactivator Assay provides a sensitive and robust method for high-throughput screening of potential ROR alpha ligands as inverse agonists of ligand-dependent coactivator displacement. The kit uses a terbium-labeled anti-GST antibody, a

fluorescein-labeled coactivator peptide, and a ROR alpha ligand-binding domain (ROR alpha-LBD) that is tagged with glutathione-S-transferase (GST) in a homogenous mix-and-read assay format. This kit contains enough reagents for 800 × 20-µL assays.

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 from precipitated compounds 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 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 (Tb) and europium (Eu). Terbium offers unique advantages over europium when used as the donor species in a TR-FRET assay. In contrast to europium-based systems that use the relatively large protein, APC, as the acceptor, terbium-based TR-FRET assays can use common small-molecule fluorophores such as fluorescein as the acceptor. Directly labeling a molecule such as a peptide with fluorescein is straightforward and inexpensive, as compared to indirect labeling via biotinylation and streptavidin-mediated recruitment of APC. Therefore, the use of directly labeled molecules (in this case the Fluorescein-D22 coactivator peptide) in a terbium-based TR-FRET assay simplifies assay development by reducing the number of components to optimize, avoids problems due to steric interactions involving large APC conjugates, reduces costs, and improves kinetics.

3.2 Assay Overview

Binding of the agonist to the nuclear receptor causes a conformational change around helix 12 in the ligand binding domain, resulting in higher affinity for the coactivator peptide (Figure 1, next page). Because ROR alpha is constitutively active, Fluorescein-D22 coactivator peptide is recruited in the absence of ligand. However, binding of an agonist may increase the recruitment of coactivator peptide, causing an increase in the TR-FRET signal compared to no compound. 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.

When an inverse agonist is bound to this nuclear receptor, helix 12 may adopt a conformation that decreases coactivator peptide binding, and the TR-FRET signal may be reduced compared to no compound (Figure 2, next page). Since there currently are no sufficiently potent commercially available ROR alpha agonists or inverse agonists, the displacement or additional recruitment of coactivator peptide has not been tested. The assay has been validated by detecting the ligand-independent recruitment of Fluorescein-D22 coactivator peptide in the presence of ROR alpha, and the assay window was determined as the fold change between the ligand independent recruitment of Fluorescein-D22 and wells containing no ROR alpha.

When running the LanthaScreen® TR-FRET ROR alpha Coactivator Assay, ROR alpha-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 at room temperature, the TR-FRET ratio of 520/495 is calculated and can be used to determine the EC_{50} from a dose response curve of the compound. Based on the biology of the ROR alpha-coactivator peptide interaction, this ligand EC_{50} is a composite value representing the amount of ligand required to bind to receptor, effect a conformational change, and either recruit or displace the coactivator peptide (see Figures 1 and 2) and result in a 50% change in TR-FRET signal.

The recommended concentrations of ROR alpha-LBD, GST and coactivator peptide Fluorescein-D22 have been optimized to produce a satisfactory assay window and Z' value for the "no receptor" and "receptor" controls. Please note that the assay window observed with these assays is highly dependent on the fluorescent plate reader—the most sensitive readers give the best window. Using ROR alpha with other coactivator peptides may require different optimal concentrations. For a list of available peptides, please visit the following web page:

http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Drug-Discovery/Target-and-Lead-Identification-and-Validation/Nuclear-Receptor-Biology/NRB-misc/Biochemical-Assays/Available-Fluorescent-Coregulator-Peptides.html

Please contact our Technical Support for additional information.

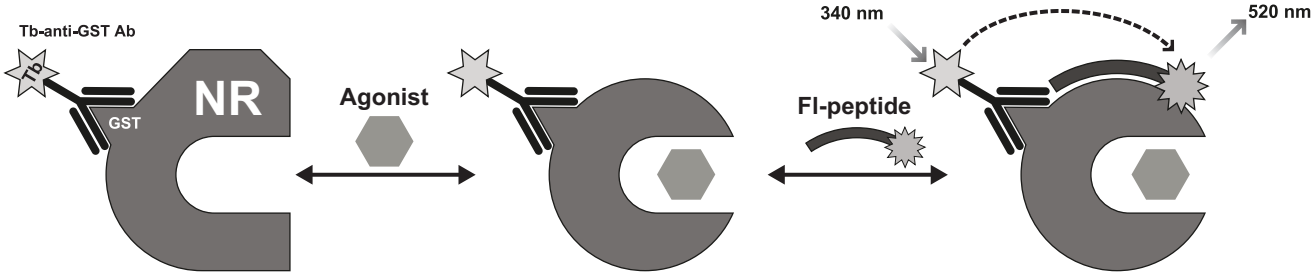


Figure 1. Principle of the nuclear receptor (NR) agonist-dependent coactivator peptide recruitment assay: Tb-anti-GST antibody indirectly labels the nuclear receptor (NR) by binding to the GST tag. Binding of the agonist to the NR causes a conformational change that results in an increase in the affinity of the NR for a coactivator peptide. The close proximity of the fluorescently labeled coactivator peptide to the terbium-labeled antibody causes an increase in the TR-FRET signal.

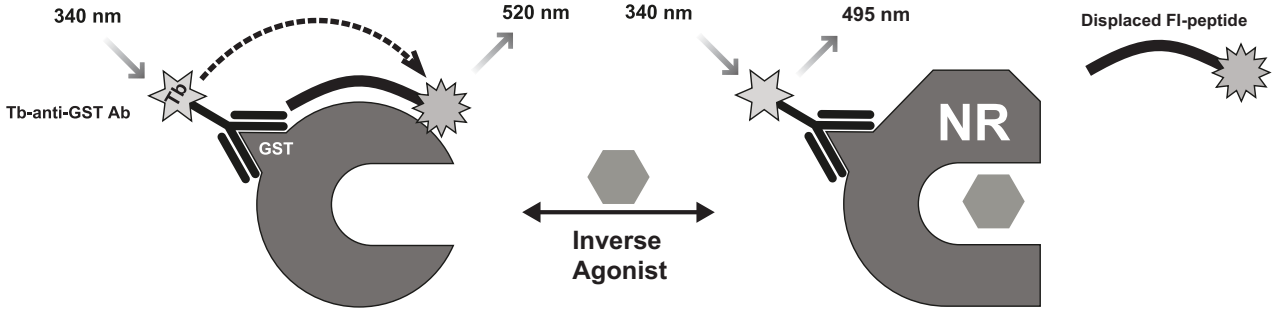


Figure 2. Principle of the nuclear receptor (NR) ligand dependent coactivator peptide displacement assay: Tb-anti-GST antibody indirectly labels the nuclear receptor by binding to the GST tag. Binding of the inverse agonist to the NR causes a conformational change that results in a decrease in the affinity 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

The LanthaScreen® Tb Instrument Control Kit has been included to facilitate instrument setup. We recommend using this kit to verify your instrument settings before proceeding with the LanthaScreen® TR-FRET assay. You can also visit www.invitrogen.com/instrumentsetup for instrument-specific information, including step-by-step instrument-specific guides for optimizing LanthaScreen® TR-FRET assays on your particular instrument. For additional help, contact Invitrogen Drug Discovery technical support at 800-955-6288 (select option 3 and enter 40266), or email drugdiscoverytech@invitrogen.com.

The LanthaScreen® technology has been tested on a variety of microplate readers. These include:

- Filter-based instruments (Tecan Ultra, GENiosPro™ and Infinite F500; BMG LABTECH PHERAstar; BioTek Synergy2 and Synergy4; PerkinElmer EnVison™ and Victor™; and Molecular Devices Analyst™)
- CCD-based imagers (PerkinElmer ViewLux™)
- Monochromator-based instruments (Tecan Safire^2 and Infinite M1000™)

4.1 General Considerations

The excitation and emission spectra of terbium and fluorescein are shown in Figure 3. To read a LanthaScreen® Tb assay, the instrument is configured to excite the terbium donor around 340 nm, and to separately read the terbium emission peak that is centered at approximately 490 nm and the fluorescein emission that is centered at approximately 520 nm.

Separation of the terbium emission signal from the fluorescein emission signal is critical for assay success. This separation is achieved with the proper selection of filter bandwidths. For this reason, a standard fluorescein emission filter cannot typically be used. After the measurements are taken, the signal from the fluorescein emission is divided (or "ratioed") by the terbium signal to provide a TR-FRET emission ratio.

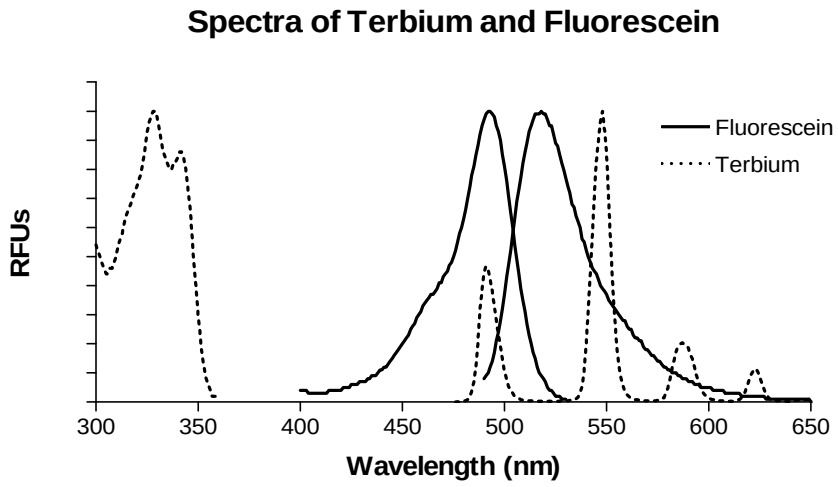


Figure 3. Excitation and emission spectra of fluorescein and terbium.

Aside from filter choices, other 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 µs followed by a 200-µs integration time is typical for a LanthaScreen® Tb assay. The number of flashes or measurements per well is highly instrument dependent and should be set as advised by your instrument manufacturer.

4.2 General Settings for Filter-Based Instruments

Excitation	340 nm filter (30 nm bandwidth)
Fluorescein Emission	520 nm filter (25 nm bandwidth)
Terbium Emission	490 or 495 nm filter (10 nm bandwidth)
Dichroic Mirror	Fluorescein (Tecan Ultra, GENios, Infinite™, GENiosPro™) LANCE/TRF (EnVision, VictorEnVison™, Victor™) 380 nm (preferred) or 400 nm (Analyst™) Built In (PHERAstar™)
Delay Time	100 µs
Integration Time	200 µs

Note that excitation filters with similar bandwidths will give satisfactory performance as long as the center wavelength falls at or between 330 nm and 340 nm. Excitation and emission filters from Chroma Technologies for many microplate readers are available directly from Invitrogen at www.invitrogen.com/LanthaScreen. A LanthaScreen® Tb filter optical module is available directly from BMG LABTECH for use on the PHERAstar. Perkin Elmer Envision™ users may require a specific filter holder which is available from PerkinElmer as part # 2100-8110 for a package of 10.

4.3 General Settings for Monochromator-Based Instruments

Excitation	332 nm (20 nm bandwidth)
Fluorescein Emission	515 nm (20 nm bandwidth)
Terbium Emission	486 nm (20 nm bandwidth)
Delay Time	100 µs
Integration Time	200 µs

Some monochromator-based instruments, such as the Tecan Infinity M1000™, allow each emission wavelength to be individually set. In this case, match the settings to those listed for the filter-based instruments above. When the bandwidths of the two emission wavelengths cannot be individually set, use the general settings for monochromator-based instruments listed in the table above as a guide.

Note that not all monochromator-based instruments are capable of a 20 nm bandwidth. Smaller bandwidth settings may be used, but with a decrease in assay performance. Additionally, we have found that while some monochromator-based instruments (e.g., the Tecan Safire^{2}™ and Tecan Infinity M1000™) give satisfactory performance in LanthaScreen® Tb assays, other monochromator-based instruments (e.g., the Tecan Safire™ and Molecular Devices Gemini™ series) may not be optimal for detection, although some change in signal and a ratio may still be observed. We have also found that with some assays, white plates give better assay performance when using monochromator-based instruments. With filter-based instruments, the difference between plate colors is typically negligible.

4.4 General Settings for CCD-Based Instruments

For a step-by-step guide to optimizing LanthaScreen® Tb assays on the PerkinElmer ViewLux™, visit www.invitrogen.com/instrumentsetup and click on the **ViewLux™** link.

5. Guidelines and Recommendations

5.1 Reagent Handling

5.1.1 ROR alpha-LBD

Store ROR alpha-LBD at –80°C. Thaw on ice, mix with pipetting or inversion of the tube.

We recommend aliquoting undiluted ROR alpha-LBD after the first thaw for subsequent experiments. Perform all dilutions while on ice. Never vortex the ROR alpha-LBD stock or dilutions.

5.1.2 TR-FRET Coregulator Buffer D

Thaw TR-FRET Coregulator Buffer D at room temperature upon receipt. Mix well before first use, because the buffer is viscous and may not have thawed evenly. Store the buffer at 4°C.

5.1.3 LanthaScreen® Tb anti-GST Antibody

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

Important: To help minimize the potential effect of spurious (random) donor emission spikes in your assay readout, centrifuge the stock vial of Tb anti-GST Antibody (~10,000 rpm for 10 minutes) prior to use. After centrifugation, pipet the quantity of antibody needed for your assays from the top of the liquid, thereby minimizing the potential mixing of any precipitate that has been spun to the bottom of the tube.

5.1.4 Fluorescein-D22 Coactivator peptide

Store fluorescein-D22 coactivator peptide at –20°C and thaw before use.

5.2 Assay Plate Types

Some ligands may stick to various plastics, making them difficult to handle and causing differences in the actual concentration of the ligand. For this reason, we recommend using the same plate type and same type of pipette tips from assay testing through screening. Depending on the type of plates and pipette tips used in assay setup, you may observe different EC_{50} values than reported here.

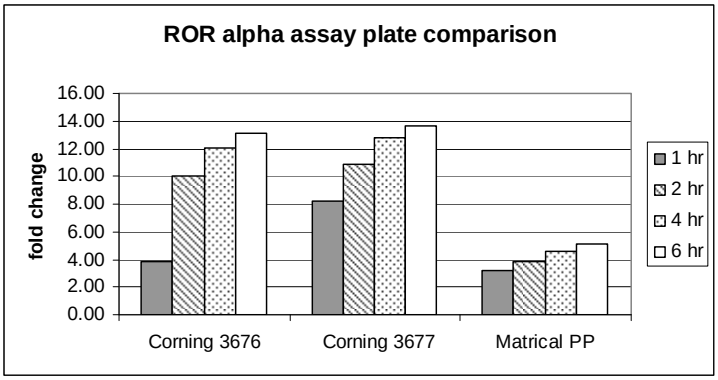


Figure 4. Representative experiments of LanthaScreen® TR-FRET ROR alpha Coactivator Assay run with different assay plate types: Assay composition: 2 nM ROR alpha-LBD, 150 nM Fluorescein D22, and 2 nM Tb anti-GST antibody in various 384-well low-volume assay plates. Results for 1, 2, 4, and 6 hour incubations are shown with the fold change between the receptor and no receptor control. The assay was validated using Corning 3677 plates.

5.3 Ligand Dilutions

The following procedure involves diluting the ligand to 100X in DMSO and then transferring it into complete assay buffer, resulting in a 2X ligand, 2% DMSO dilution. This may be done in a DMSO-tolerant assay plate or tubes such as those made from polypropylene or HDPE. Dilution in DMSO reduces compound precipitation and sticking to plastics, and improves IC_{50} reproducibility.

After all reagents have been added to the assay, the final concentration will be 1X ligand and 1% DMSO.

5.4 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, although a decrease in assay window was observed at higher concentrations of DMSO and ethanol.

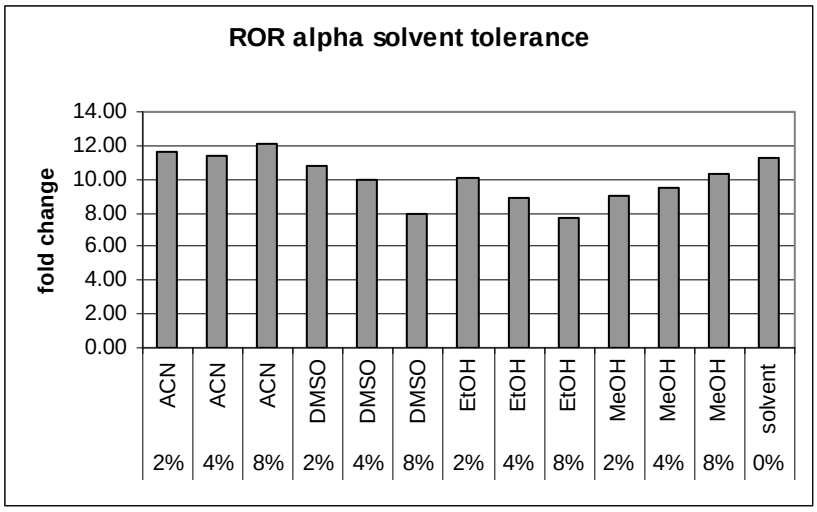


Figure 5. Representative LanthaScreen® TR-FRET ROR alpha Coactivator Assays run with different concentrations of various solvents: Assay composition: 2 nM ROR alpha-LBD, 150 nM Fluorescein D22, and 2 nM Tb anti-GST antibody in a 384-well low volume assay plate. Results for 2.5 hour incubations are shown with the fold change between the receptor and no-receptor control.

5.5 Note on Reagent Order of Addition

The assay was validated using three additions per well in the following order:

1. Compounds or solvent controls
2. Pre-mixed fluorescein-D22 peptide and Tb anti-GST antibody
3. ROR alpha-LBD

Note: A pre-mixture of ROR alpha-LBD, fluorescein-D22 peptide, and Tb anti-GST antibody may be added to the ligand dilutions for a total of two additions per well, although the assay was not fully validated in this manner and equilibration times may differ in this format. It is important to consider the effect of time and temperature on this three-component pre-mixture when developing the assay.

5.6 Incubation Conditions

5.6.1 Incubation Time

The incubation time can be set by the user. As a guide, results for various time points are shown in Table 1 below. The Z′ values for the receptor and no receptor control were stable from 1 to 6 hours. It is critical to maintain the same incubation time and temperature during testing and screening for consistent results.

Incubation Time (hours)	Z′-Factor	Assay Window (fold change)
1	0.65	8.0
2	0.69	10
4	0.72	12
6	0.72	13

Table 1. Effect of Incubation Time on Assay Performance. Sample data represents mean values from 3 separate experiments (n ≥ 4). Assays were measured on a BMG LABTECH PHERAstar fluorescent plate reader. Different plate readers may provide very different assay windows, because the assay window is highly dependent on fluorescent plate reader sensitivity. Z′-factors were calculated using the method of Zhang *et al*. (Zhang *et al.*, 1999) with 24 replicates of ″no ligand controls″ and ″no receptor controls.″ 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.

5.6.2 Temperature

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

6. Agonist/Inverse Agonist Assay

The procedure in this section describes a method for determining the EC_{50} of an agonist (n = 4), the Z′ factor for maximum agonist and no agonist controls (n = 24), and the Z′ factor for receptor and no receptor controls (n=24) using the potential agonist-induced recruitment of fluorescein D22 peptide to ROR alpha-LBD. The same procedure can be potentially used to detect inverse agonists that cause the displacement of the ligand-independent recruitment of fluorescein D22 peptide to ROR alpha-LBD. The only variable is the ligand concentration. All other assay components (ROR alpha-LBD, peptide, Tb anti-GST antibody) are fixed at concentrations optimized to produce a satisfactory assay window (Z′-factor >0.5) as calculated from the receptor and no receptor controls. Different plate readers may provide very different assay windows, because the assay window is highly dependent on the sensitivity of the plate reader.

Higher concentrations of nuclear receptor may result in a larger TR-FRET signal window; however, it will decrease the sensitivity of the assay with regard to differentiating tight-binding ligands. The recommended final concentrations are listed in the following table. If a component concentration is changed, the concentrations of the other components may need to be re-optimized.

Component	Final Assay Concentration
Fluorescein-D22	150 nM
Tb anti-GST antibody	2 nM
ROR alpha LBD-GST	See Certificate of Analysis for the recommended molar concentration for this kit

6.1 Agonist/Inverse Agonist Assay—Procedure

6.1.1 Prepare Complete TR-FRET Coregulator Buffer D and Agonist/Inverse Agonist Controls

Note: Thaw ROR alpha-LBD on ice just prior to use. Equilibrate all other assay components to room temperature.

1. Prepare Complete TR-FRET Coregulator Buffer D by adding 1 M DTT to TR-FRET Coregulator Buffer D (provided in the kit) for a final concentration of 5 mM DTT. Complete TR-FRET Coregulator Buffer D must be prepared fresh daily.

   Example: Add 30 µL of 1 M DTT to 5.97 mL of TR-FRET Coregulator Buffer D.

   Note: Buffer D is a specially formulated buffer that has been optimized to work with this kit. To ensure performance of this assay, we highly recommend using Buffer D.

2. For the "no receptor" controls, add 5 µL of Complete TR-FRET Coregulator Buffer D to row E, columns 1–24 of a 384-well assay plate (see the plate layout in **Section 6.2**)

3. For the "no ligand" controls, add DMSO to Complete TR-FRET Coregulator Buffer D for a final concentration of 2% DMSO. Add 10 µL of this solution to rows C and E, columns 1–24 of a 384-well assay plate (see the plate layout in **Section 6.2**).

   Example: Add 10 µL of DMSO to 490 µL of Complete TR-FRET Coregulator Buffer D.

4. Prepare a solution of control agonist or inverse agonist at 100X of the final desired maximum starting concentration using DMSO. At the time of this writing, no sufficiently potent commercial ROR alpha ligands were available.

   Example: If the final desired maximum starting concentration of ligand is 1 µM, prepare a solution of 100 µM ligand in DMSO.

5. For the "maximum ligand" controls, dilute the 100X ligand solution from Step 4 to 2X using Complete TR-FRET Coregulator Buffer D. 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**).

   Example: Add 10 µL of 100X ligand solution to 490 µL of Complete TR-FRET Coregulator Buffer D.

6.1.2 Prepare 2X Agonist Dilution Series

Note: Although the steps below require more pipetting than other methods of preparing a serial dilution of agonist, we have found that this approach provides a robust method for preparing the dilution series without problems due to ligand solubility. Dilution with 100% DMSO facilitates compound solubility during serial dilutions, which is important for obtaining consistent EC_{50} values.

1. Prepare a 12-point 100X dilution series of ligand in a 96-well polypropylene plate (DMSO tolerant) by serially diluting the 100X ligand in 100% DMSO. We recommend a three-fold dilution series.

   Example: Add 20 µL of 100% DMSO to wells A2–A12 in a 96-well polypropylene plate. To well A1, add 30 µL of the 100X ligand solution prepared in Step 4, previous section. Perform a three-fold serial dilution by transferring 10 µL of the 100X ligand solution from well A1 to the 20 µL of DMSO in well A2. Mix by pipetting up and down. Repeat for wells A2–A12.

2. Dilute each 100X ligand serial dilution from the previous step to 2X using Complete TR-FRET Coregulator Buffer D.

   Example: Transfer 5 µL of each of the 100X ligand 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 D to each well in row B of the 96-well plate. Mix by pipetting up and down.

3. To assay 4 replicates of each ligand concentration, transfer 10 µL of each of the 2X ligand serial dilutions to the 384-well assay plate according to the plate layout in **Section 6.2**.

   Example 1: Using a 12-channel pipette, transfer 10 µL aliquots from row B of the 96-well plate to alternate columns across row A of the 384-well plate, A1, A3, A5, A7, etc . Continue this process for Row A, this time pipetting into A2, A4, A6, etc. Repeat this process for row B of the 384-well plate.

   Example 2: Alternatively, use a 16-channel pipette where two tips fit into one well of the 96-well plate, then transfer to individual wells of the 384-well assay plate in rows A and B. Repeat to complete all of the columns (1–24).

With either method, wells A1, A2, B1, B2 all contain the highest concentration of ligand from B1 of the 96-well plate; wells A3, A4, B3, B4 contain the next concentration of ligand from B2 of the 96-well plate; and so forth for 4 replicates of each concentration with constant percent DMSO.

6.1.3 Prepare 4X ROR alpha-LBD

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

   Example: If the ROR alpha-LBD has a stock concentration of 4000 nM and the recommended concentration for this kit is 2 nM, prepare a 4X solution at 8 nM by adding 2 µL of ROR alpha-LBD stock to 998 µL of Complete TR-FRET Coregulator Buffer D.

2. Add 5 µL of 4X ROR alpha-LBD to rows A–D, columns 1–24 of the 384-well assay plate (see the plate layout in **Section 6.2**). We recommend working from low to high concentration of ligand to prevent ligand carry over.

6.1.4 Prepare 4X Fluorescein-D22/4X Tb anti-GST Antibody

1. Prepare a solution containing 0.6 µM Fluorescein-D22 (4X) and 8 nM Tb anti-GST antibody (4X) using Complete TR-FRET Coregulator Buffer D at room temperature. The stock concentration of Fluorescein-D22 is 100 µM and the concentration of Tb anti-GST antibody is indicated on both the vial label and the Certificate of Analysis (0.5 mg/mL = ~3.4 µM antibody).

   Example: Add 6 µL of 100 µM Fluorescein-D22 and 2.4 µL of 3.4 µM Tb anti-GST antibody to 992 µL of Complete TR-FRET Coregulator Buffer D.

2. Add 5 µL of 4X peptide/4X antibody solution to rows A–E, columns 1–24 of the 384-well assay plate (see the plate layout in **Section 6.2**).

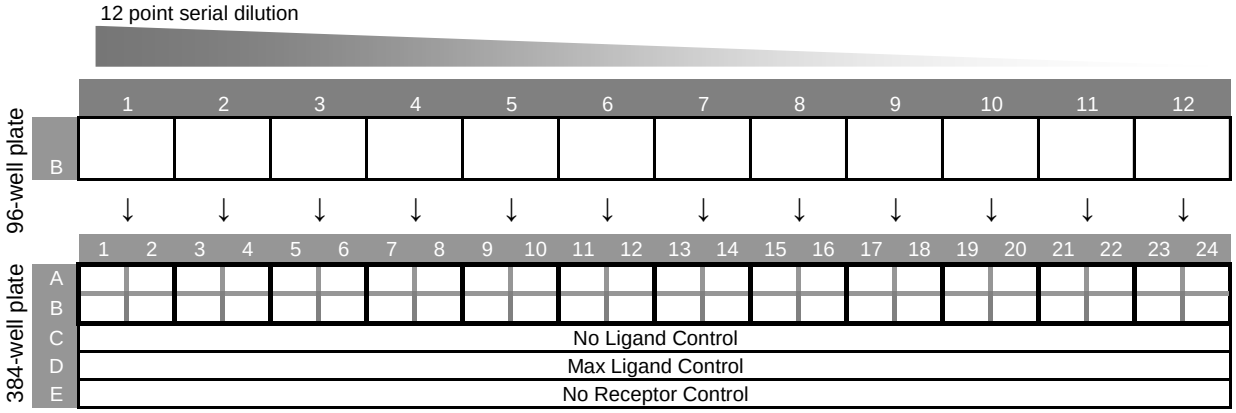
6.1.5 Plate Incubation and Reads

1. Briefly and 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.

   Note: When using assay plates that are **not** coated, such as the Corning 3677 plates, drops may cling to the side of the wells during additions to the plate. It is critical that the plates be gently tapped or centrifuged to ensure that all reagents reach the bottom of the well.

2. Read the plate between 1 and 6 hours at your chosen equilibration time 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 the next section.

6.2 Plate Layout



6.3 Agonist/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 EC_{50} value, fit the data using an equation for a sigmoidal dose response (varying slope), as provided by GraphPad™ Prism 4.0 or another comparable graphing program. To calculate the EC_{80} value for performing an antagonist assay, see **Section 7.1**. To determine the fold change (or assay window) without ligand, divide the average ratio of the "No Ligand Control" by the average ratio calculated for the "No Receptor Control" wells.

The "maximum ligand" and "no ligand" control data or the "no ligand" and "no receptor" control data (if no control ligand is available) can be used to calculate Z´-factor based on the equation of Zhang et al (Zhang *et al.*, 1999).

Note: The ligand EC_{50} determined in the assay is a composite of multiple equilibria, including ligand binding to receptor and peptide binding to ligand/receptor complex.

6.4 Agonist/Inverse Agonist Assay—Summary of Reagent Volumes and Controls

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

***Note*:** All controls should contain the same percentage of solvent as the wells containing test compound.

Assay	Reagent Additions	Purpose
Test Compound	1. 10 µL 2X Test Compound (or dilution series) 2. 5 µL 4X Fl-D22/Tb anti-GST Ab 3. 5 µL 4X ROR alpha-LBD	Assess coactivator recruitment (agonist) or displacement (inverse agonist) upon binding of test compound.
Positive Control	1. 10 µL 2X control ligand (or dilution series) 2. 5 µL 4X Fl-D22/Tb anti-GST Ab 3. 5 µL 4X ROR alpha-LBD	Assess coactivator recruitment (agonist) or displacement (inverse agonist) upon binding of a known ROR alpha ligand.
Negative Control	1. 10 µL 2X compound solvent in Coregulator Buffer D 2. 5 µL 4X Fl-D22/Tb anti-GST Ab 3. 5 µL 4X ROR alpha-LBD	Generate baseline signal for the assay, including possible ligand-independent coactivator recruitment.
No ROR alpha-LBD Control (recommended if no Positive Control is available)	1. 10 µL 2X compound solvent in Coregulator Buffer D 2. 5 µL 4X Fl-D22/Tb anti-GST Ab 3. 5 µL Complete TR-FRET Coregulator Buffer D	Can be compared to the negative control to determine ligand-independent coactivator recruitment. May also be used to determine diffusion-enhanced TR-FRET.*
* Diffusion-enhanced TR-FRET occurs when the donor (Tb-anti-GST Antibody) passes by the acceptor (Fl-TRAP220) in solution during the excited state lifetime of the donor. The resulting signal is not related to a binding event. At higher concentrations of donor or acceptor, the probability of this occurrence increases and results in a larger background signal due to diffusion enhanced TR-FRET.		

6.5 Agonist Assay—Representative Data

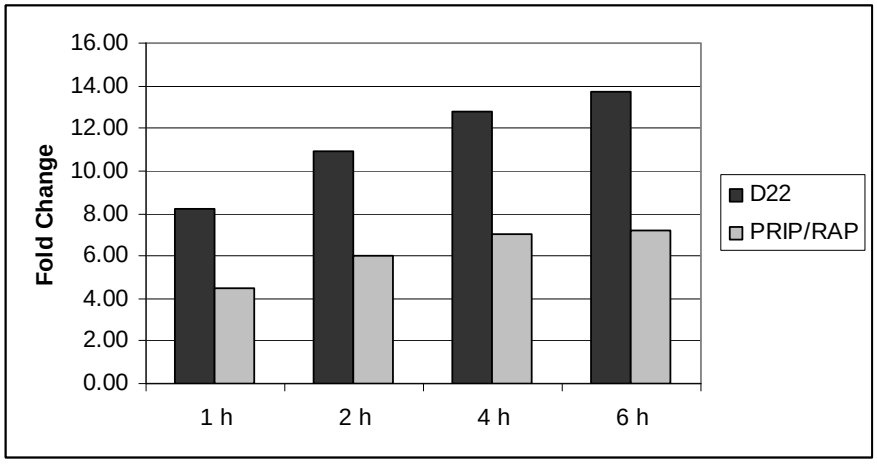


Figure 6. Representative experiments of LanthaScreen® TR-FRET ROR alpha Coactivator assay run with D22 and PRIP/RAP coactivator peptides. Assay composition: 2 nM ROR alpha-LBD, 150 nM Fluorescein D22 or Prip/Rap, and 2 nM Tb anti-GST antibody in Corning 3677 384-well low-volume assay plates. Results for 1, 2, 4, and 6 hour incubations are shown with the fold change of the TR-FRET ratio between the receptor and no receptor control.

7. Antagonist Assay

Although no commercial antagonist for ROR alpha was available at the time this assay was developed, the procedure described below describes a general method that could be performed to assess antagonist activity. For this approach, we define "antagonist" as a compound that does not necessarily recruit or displace coregulator peptides by itself, but does competitively displace an agonist or possibly an inverse agonist, which can then result in recruitment or displacement of coregulator peptides relative to the agonist or inverse agonist.

Component	Final Assay Concentration
Fluorescein-D22	150 nM
Tb anti-GST antibody	2 nM
Agonist concentration	EC_{80} calculated from assay performed in agonist/inverse agonist mode
ROR alpha LBD-GST	See Certificate of Analysis for the recommended molar concentration for this kit

Note: We recommend determining the EC_{80} of the agonist/inverse agonist by performing the assay described in **Section 6**. Although a concentration greater than the EC_{80} will give a larger assay window, the sensitivity of the assay as defined by the ability to identify antagonists will be compromised.

7.1 Calculating the EC_{80} from the Agonist Assay

Calculate the EC_{80} from the agonist assay, using the EC_{50} and Hill Slope determined from the curve fit of the sigmoidal dose response (variable slope) equation:

$EC_{80} = 10\text{^}(\log EC_{50} + ((1/\text{Hill Slope}) \times \log(80/(100 - 80)))$

7.2 Antagonist Assay

7.2.1 Prepare Complete TR-FRET Coregulator Buffer D and Antagonist Controls

1. Prepare Complete TR-FRET Coregulator Buffer D by adding 1 M DTT to TR-FRET Coregulator Buffer D for a final concentration of 5 mM DTT. Complete TR-FRET Coregulator Buffer D must be prepared fresh daily.
2. For the "no antagonist" controls (Negative Control) in the presence of EC_{80} agonist, add DMSO to Complete TR-FRET Coregulator Buffer D 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 7.3**)
3. Prepare a solution of control antagonist at 100X of the final desired maximum starting concentration using DMSO.
4. For the "maximum antagonist" controls (Positive Control), dilute the 100X antagonist solution from the previous step to 2X using Complete TR-FRET Coregulator Buffer D. Add 10 µL of this solution to row D, columns 1–24 in a 384-well assay plate (see the plate layout in **Section 7.3**).
5. For the no agonist, no antagonist controls (Alternative Positive Control), add DMSO to Complete TR-FRET Coregulator Buffer D for a final concentration of 2% DMSO. Add 10 µL of this solution to row E, columns 1–24 of a 384-well assay plate (see the plate layout in **Section 7.3**).

7.2.2 Prepare 2X Antagonist Titration

Prepare the serial dilution of antagonist as described for the agonist or inverse agonist in **Section 6.1.2**.

7.2.3 Prepare 4X ROR alpha-LBD

1. Prepare 4X ROR alpha LBD as described in **Section 6.1.3**.
2. Add 5 µL of 4X ROR alpha-LBD to rows A–E, columns 1–24 of the 384-well assay plate. We recommend adding ligand in the order of low to high concentration (right to left) to prevent ligand carryover.

7.2.4 Prepare 4X Fluorescein-D22/4X Tb anti-GST Antibody

1. Prepare 4X Fluorescein-D22/4X Tb anti-GST Antibody as described in **Section 6.1.4**.
2. Add 5 µL of 4X peptide/4X antibody solution to row E ONLY, columns 1–24 of the 384-well assay plate (see the plate layout in **Section 7.3**).

7.2.5 D22/4X Tb anti-GST Antibody/4X EC_{80} Agonist

1. Prepare a 100X solution of the EC_{80} of the agonist in Complete Buffer D.

   Example: If the EC_{80} of the agonist is 8 nM, prepare a 100X solution at 800 nM in Complete Buffer D.

2. Prepare a solution containing 0.6 µM Fluorescein-D22 (4X), 8 nM Tb anti-GST antibody (4X), and 4X of the agonist EC_{80} using Complete TR-FRET Coregulator Buffer D. The concentration of Fluorescein-D22 as supplied is 100 µM and the concentration of Tb anti-GST antibody is indicated on both the vial label and the Certificate of Analysis (0.5 mg/mL = ~3.4 µM antibody using a Molecular Weight of 150kDa).

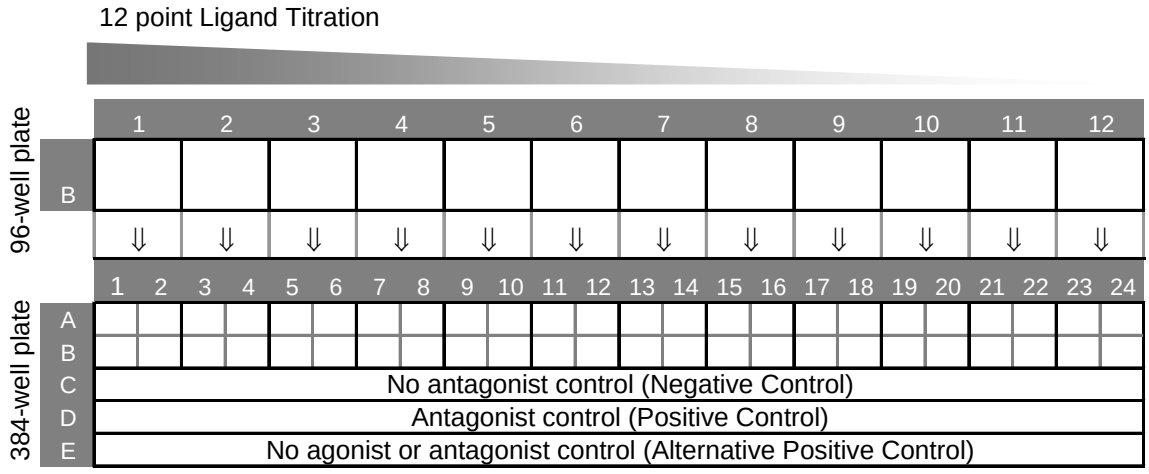
   Example: Add 40 µL of the 100X solution of the EC_{80} agonist, 6 µL of 100 µM fluorescein-D22, and 2.4 µL of 3.4 µM Tb anti-GST antibody to 949 µL of Complete TR-FRET Coregulator Buffer D.

3. Add 5 µL of 4X peptide/4X antibody/4X EC_{80} agonist solution to rows A–D, columns 1–24 of the 384-well assay plate (see the plate layout in **Section 7.3**).

7.2.6 Plate Incubation and Reads

For plate incubations, follow the steps in **Section 6.1.5**.

7.3 Plate Layout



7.4 Antagonist Assay—Data Analysis

Calculate the TR-FRET ratio by dividing the emission at 520 nm by the emission at 495 nm. Generate a binding curve by plotting the emission ratio vs. the log [antagonist]. To determine the IC_{50} value, fit the data using an equation for a sigmoidal dose response (varying slope), as provided by GraphPad™ Prism 4.0 or other comparable graphing program.

The "no antagonist" and "maximum antagonist" control data (rows C and D) can be used to calculate Z´-factor based on the equation of Zhang *et al* (Zhang *et al.*, 1999). If no control antagonist is available, the "no agonist/no antagonist" control (row E) can be substituted for the positive control in the Z'-factor calculation.

7.5 Antagonist Assay—Summary of Reagent Volumes and Controls

The following table summarizes the reagent volumes and order of addition, along with potential controls for developing an antagonist mode assay. The protocol in **Section 7.2** may be adapted, using the summary table below as a guide, to screen test compounds as needed by the user.

Assay	Reagent Additions	Purpose
Test Compound	1. 10 µL 2X Test Compound (or dilution series) 2. 5 µL 4X Fl-D22/Tb anti-GST Ab/ EC_{80} agonist 3. 5 µL 4X ROR alpha-LBD	Assess disruption of coactivator recruitment by competition of test compound and a known ROR alpha agonist at the EC_{80} for binding to ROR alpha.
No antagonist (Negative control)	1. 10 µL 2X Compound Solvent 2. 5 µL 4X Fl-D22/Tb anti-GST Ab/ EC_{80} agonist 3. 5 µL 4X ROR alpha-LBD t	Provides maximum FRET ratio for the antagonist assay.
Max Antagonist (Positive Control)	1. 10 µL 2X known ROR alpha antagonist 2. 5 µL 4X Fl-D22/Tb anti-GST Ab/ EC_{80} agonist 3. 5 µL 4X ROR alpha-LBD	Assess disruption of coactivator recruitment by competition of a known ROR alpha antagonist at the EC_{80} of a known agonist for binding to ROR alpha.
No Agonist or Antagonist (Alternate Positive Control)	1. 10 µL 2X Compound Solvent 2. 5 µL 4X Fl-D22/Tb anti-GST Ab 3. 5 µL Complete TR-FRET Coregulator Buffer D	Recommended since antagonists are not established for ROR alpha at this time and may stand in for the positive control. Provides baseline for assay and can be used to assess diffusion-enhanced TR-FRET.*
* Diffusion-enhanced TR-FRET occurs when the donor (Tb-anti-GST Antibody) passes by the acceptor (Fl-TRAP220) in solution during the excited state lifetime of the donor. The resulting signal is not related to a binding event. At higher concentrations of donor or acceptor, the probability of this occurrence increases and results in a larger background signal due to diffusion enhanced TR-FRET.		

8. References

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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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