LanthaScreen® TR-FRET Peroxisome Proliferator Activated Receptor gamma Coactivator Assay

Catalog no. PV4548 **Shipping: Dry Ice** **Initial Storage: Varies**

Protocol part no. PV4548.pps Rev. date: 2 February 2010

TABLE OF CONTENTS

1. Kit Contents

The LanthaScreen® TR-FRET Peroxisome Proliferator Activated Receptor gamma Coactivator Assay, catalog no. PV4548, contains the following:

COMPONENT	COMPOSITION	AMOUNT	STORAGE TEMP.	INDIVIDUAL CATALOG NO.
Fluorescein-TRAP220/DRIP-2 peptide (Rachez *et al.*, 2000)	100 µM in 50 mM HEPES buffer, pH 7.5 Sequence: NTKNHPMLMNLLKDNPAQD	100 µL	–20°C	PV4549
PPAR gamma LBD, GST	PPAR gamma ligand-binding domain in a buffer (pH 8.0) containing protein stabilizing reagents and glycerol. See Certificate of Analysis for the recommended molar concentration for this kit.	10 µg	–80°C	PV4545
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
Nuclear Receptor Buffer F	Proprietary buffer (pH 7.5) including 10% glycerol	2 × 25 mL	20°C–30°C	PV4547
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 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. 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 but have not been fully tested.
- 96-well polypropylene plates that can accommodate a 300-µL volume per well for the ligand serial dilutions in 100% DMSO. Polypropylene plates are needed since they are tolerant to DMSO. We recommend Nalgene® Nunc™ #249944.
- A known PPAR gamma agonist, such as GW1929, to serve as a positive control in agonist mode. We recommend Tocris catalog no. 1664.
- A known PPAR gamma antagonist, such as GW9662, to serve as positive control in antagonist mode. We recommend Cayman Chemical catalog no. 70785.
- DMSO to perform serial dilutions. We recommend Fluka 41647.

3. Introduction

The LanthaScreen® TR-FRET Peroxisome Proliferator Activated Receptor gamma Coactivator Assay provides a sensitive and robust method for high-throughput screening of potential PPAR gamma ligands as agonists of coactivator recruitment. The kit uses a terbium-labeled anti-GST antibody, a fluorescein-labeled coactivator peptide, and a PPAR 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 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 employ 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. It is straightforward (and inexpensive) to directly label a molecule such as a peptide with fluorescein for use in terbium-based TR-FRET assays. In addition, the assay format is simpler than using a biotinylated peptide that would then be indirectly labeled via streptavidin-mediated recruitment of APC. Therefore, the use of directly labeled molecules (in this case the Fluorescein-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 agonist to the nuclear receptor (Figure 1) causes a conformational change around helix 12 in the ligand binding domain, resulting in higher 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.

When running the LanthaScreen® TR-FRET Peroxisome Proliferator Activated Receptor gamma Coactivator Assay, PPAR 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 emissions 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 PPAR gamma-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 recruit coactivator peptide (see Figure 1).

The recommended concentration of PPAR gamma-LBD GST, fluorescein-coactivator peptide and Tb anti-GST antibody have been optimized to produce a satisfactory assay window ($Z' > 0.5$) while using the lowest receptor

concentration to achieve the best sensitivity to tight binding ligands. The assay window is highly dependent on the sensitivity of the fluorescent plate reader. Different plate readers may provide different assay windows and Z' factors. These assays were optimized using a BMG LABTECH PHERAstar fluorescent plate reader, which is considered very sensitive for these assays. If the assay window is not acceptable on your instrument, additional receptor or receptor and antibody may be added. Please note that this will decrease the sensitivity of the assay to tight binders. Also, using PPAR gamma with other coactivator peptides may require different optimal peptide concentrations. For a list of available peptides, visit:

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.

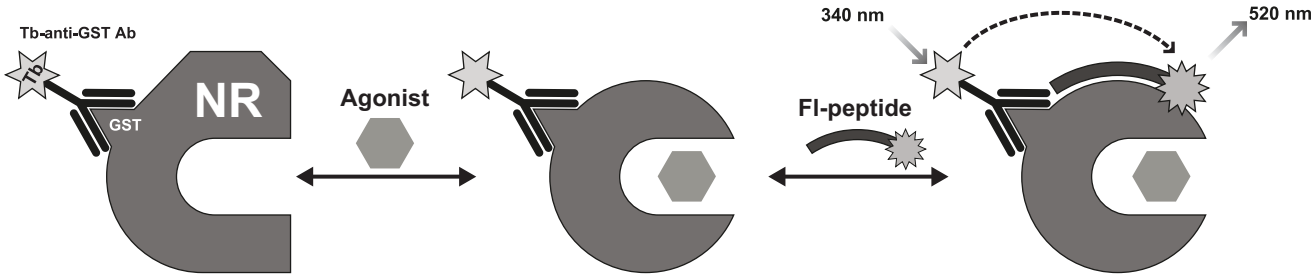


Figure 1. Principle of the nuclear receptor (NR) agonist dependent coactivator peptide recruitment assay: Tb-anti-GST antibody indirectly labels the nuclear receptor by binding to the GST tag. Binding of the agonist to the NR causes a conformational change which results in an increase in the affinity 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.

4. Instrument Settings

The LanthaScreen® Tb Instrument Control Kit, PV5591, has been included to facilitate instrument set-up. We recommend that you use this kit to verify your instrument settings before proceeding with any Nuclear Receptor Biology. For additional help in setting up your instrument to perform LanthaScreen® TR-FRET assays, please contact Invitrogen Drug Discovery technical support at 800-955-6288 (select option 3 and enter 40266), or email drugdiscoverytech@invitrogen.com. Please see www.invitrogen.com/instrumentsetup for instrument specific information, including step-by-step instrument specific guides for optimizing LanthaScreen® Terbium assays on your particular instrument.

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 Instruments Synergy™ 2 and Synergy™ 4; PerkinElmer EnVison® and VICTOR™, and MDS Analytical Technologies Analyst®), CCD-based imagers (PerkinElmer ViewLux™) and monochromator-based instruments (Tecan Safire²™ and Infinite® M1000).

4.1 General Considerations

The excitation and emission spectra of terbium and fluorescein are shown in Figure 2. To read a LanthaScreen® TR-FRET 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 and is achieved with the proper selection of filter bandwidths which cannot be compromised. For this reason a standard fluorescein filter cannot typically be used. After taking the measurements, the signal

from the fluorescein emission is divided (or "ratioed") by the terbium signal to provide a TR-FRET emission ratio and improve the Z' for the assay.

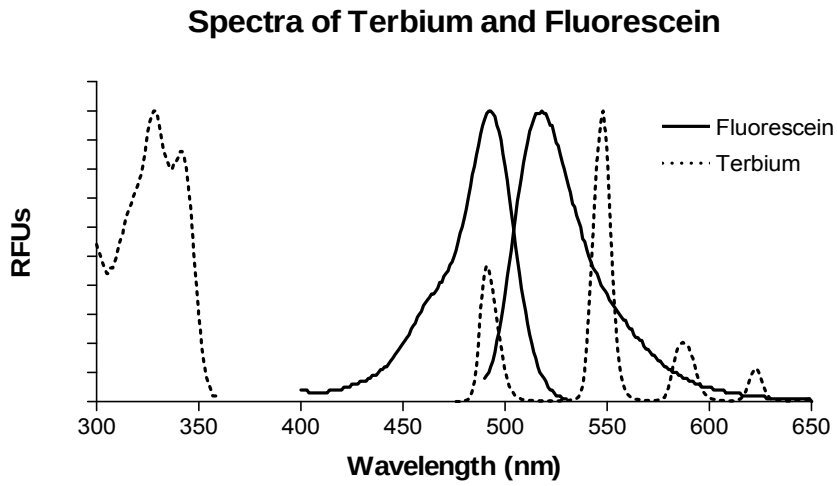


Figure 2. Excitation and emission spectra of fluorescein and terbium.

Aside from filter choices, other instrument settings are similar to the settings used with europium-based technologies. A delay time of 100 µs followed by a 200-µs integration time is typical for a LanthaScreen® TR-FRET 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,,GENios Pro, Infinite®) PerkinElmer LANCE®/TRF (EnVision®,, VICTOR™) 380 nm (preferred) or 400 nm (MDS Analytical Technologies Analyst®) Built In (BMG LABTECH 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 most microplate readers are available directly from Invitrogen at www.invitrogen.com/LanthaScreen. A LanthaScreen® TR-FRET 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. 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 (Tecan Safire^{2}™, Tecan Infinite® M1000) give satisfactory performance in LanthaScreen® TR-FRET assays, other monochromator-based instruments (e.g. Tecan Safire™ and MDS Analytical Technologies Gemini series and SpectraMax® M2 and M5) 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. In filter-based instruments the difference is typically negligible.

4.4 General Settings for CCD-based Instruments

Please see www.invitrogen.com/instrumentsetup and click on "ViewLux™" for a step-by-step guide to optimizing LanthaScreen® Terbium assays on the PerkinElmer ViewLux™.

5. Guidelines and Recommendations

5.1 Reagent Handling

PPAR gamma-LBD, GST

Store the GST tagged PPAR gamma-LBD at –80°C. Thaw on ice before use and perform all dilutions while on ice. Mix by gentle pipetting or inversion of the tube. Never vortex the PPAR gamma-LBD stock or dilutions. We recommend aliquotting the PPAR gamma-LBD after the first thaw for subsequent experiments.

Nuclear Receptor Buffer F

Thaw Nuclear Receptor Buffer F 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 20°C–30°C.

LanthaScreen® TR-FRET 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 min) 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.

Fluorescein-TRAP220/DRIP-2 Coactivator Peptide

Store fluorescein-TRAP220/DRIP-2 coactivator peptide at –20°C. Thaw and mix 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 tubes such as those made from polypropylene or HDPE. Dilution in DMSO reduces the occurrence of compound precipitation while performing the dilution series, reduces sticking to plastics, improves EC_{50} reproducibility and maintains constant DMSO in all wells. After addition of all reagents to the assay, the final concentration will be 1X ligand and 1% DMSO. We suggest using this method for compound dilutions.

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.

5.3 Solvent Tolerance

The assay was validated in the presence of 1% DMSO. However, the assay has been performed with up to 4% DMSO, 8% ethanol, and 8% methanol (in addition to the 1% DMSO present from the ligand dilution) with good results. The use of higher concentrations of DMSO may still yield a robust assay, however a decreased assay window was observed at 8% DMSO.

5.4 Note on Reagent Order of Addition

The assay was validated using three additions per well in which PPAR gamma-LBD was added to agonist dilutions, followed by the addition of pre-mixed fluorescein-TRAP220/DRIP-2 coactivator peptide and Tb anti-GST antibody (agonist, receptor, peptide/antibody). A pre-mixture of PPAR gamma-LBD, fluorescein-TRAP220/DRIP-2 coactivator peptide, and Tb anti-GST antibody may also be added to the agonist dilutions for a total of 2 additions per well, although the assay was not fully validated in this manner and equilibration times may differ in this format. The impact of premixing on EC_{50} and IC_{50} values has not been evaluated. It is important to consider the effect of time and temperature on this three-component pre-mixture when developing the assay.

In antagonist mode, we recommend adding PPAR gamma-LBD to antagonist dilutions followed by addition of a mixture of agonist/fluorescein-TRAP220/DRIP-2 peptide/Tb anti-GST antibody, for a total of 3 additions per well (antagonist, receptor, peptide/antibody/agonist).

5.5 Incubation Conditions

Incubation Time

The incubation time can be set by the user. As a guide, results for various time points using GW1929 as the agonist and GW9662 as the antagonist are shown in Table 1 below. The EC_{50} and Z' in agonist mode are stable from 1 to 24 hours (Table 1). Note that the IC_{50} values for the antagonist GW9662 decrease over time due to the irreversible binding of the compound. We suggest maintaining the same incubation time and temperature during testing and screening for consistent results.

	Agonist Mode		Antagonist Mode	
Incubation Time (hours)	EC_{50} GW1929	Z′-Factor	IC_{50} GW9662	Z′-Factor
1	3.2 nM	0.82	240 nM	0.74
2	3.4 nM	0.83	140 nM	0.72
4	4.2 nM	0.82	75 nM	0.71
6	5.3 nM	0.80	51 nM	0.68
24	12 nM	0.70	15 nM	0.40

Table 1. Effect of Incubation Time on Assay Performance. Sample data represents mean values from 3 separate experiments ($n \geq 4$). EC_{50} or IC_{50} 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 agonist and no agonist (agonist mode) or maximum antagonist and no antagonist (antagonist mode). 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. Agonist Assay

The procedure in this section describes a method for determining the EC_{50} of an agonist (n = 4) and the Z′ factor for maximum agonist and no agonist controls (n = 24) using the agonist-induced recruitment of fluorescein TRAP220/DRIP-2 coactivator peptide to PPAR gamma-LBD. The only variable is the agonist concentration. All other assay components (PPAR gamma-LBD, peptide, Tb anti-GST antibody) are fixed at concentrations optimized to produce a satisfactory assay window (Z′-factor >0.5) while using the lowest receptor concentration to achieve the best sensitivity to tight binding ligands. Assays were measured on a BMG LABTECH PHERAstar fluorescent plate reader. Different plate readers may provide very different assay windows because assay window is highly dependent on fluorescent plate reader sensitivity. Although a higher concentration of nuclear receptor may give a larger TR-FRET signal window, it may compromise 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 reoptimized.

Component	Final Assay Concentration
Fluorescein-TRAP220/DRIP-2	125 nM
Tb anti-GST antibody	5 nM
PPAR gamma-LBD GST	See Certificate of Analysis for the recommended molar concentration for this kit

6.1 Agonist Assay—Procedure

Prepare Complete Nuclear Receptor Buffer F and Agonist Controls

Note: The PPAR gamma-LBD should be thawed on ice just prior to use. Equilibrate all other assay components to room temperature.

1. Prepare Complete Nuclear Receptor Buffer F by adding 1 M DTT to Nuclear Receptor Buffer F for a final concentration of 5 mM DTT. Complete Nuclear Receptor Buffer F must be prepared fresh daily.

   For example: Add 30 µL of 1 M DTT to 5.97 mL of Nuclear Receptor Buffer F.

Note: Buffer F 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 F as provided with the kit.

2. For the "no agonist" controls, add DMSO to Complete Nuclear Receptor Buffer F for a final concentration of 2% DMSO (2X solvent). Add 10 µL of this buffer containing DMSO 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 Nuclear Receptor Buffer F.

3. Prepare a solution of control agonist (we recommend GW1929) at 100X of the final desired maximum starting concentration using 100% DMSO.

   For example: If the final desired maximum starting concentration of agonist is 1 µM, prepare a solution of 100 µM agonist in 100% DMSO.

4. For the "maximum agonist" controls, dilute the 100X agonist solution from step 3 to 2X using Complete Nuclear Receptor Buffer F. 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 agonist solution to 490 µL of Complete Nuclear Receptor Buffer F.

Prepare 2X Agonist Dilution Series

Note: Although steps 5 and 6 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 agonist solubility. Dilution with 100% DMSO facilitates compound solubility during serial dilutions, which is important for obtaining consistent EC_{50} values.

5. Prepare a 12-point 100X dilution series of agonist in a 96-well polypropylene plate (DMSO tolerant) by serially diluting the 100X agonist solution from step 3 using 100% DMSO. We recommend a 3-fold dilution series.

   For 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 agonist solution prepared in step 3. Perform a three-fold serial dilution by transferring 10 µL of the 100X 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 agonist serial dilution from step 5 to 2X using Complete Nuclear Receptor Buffer F.

   For example: Transfer 5 µL of each of the 100X 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 Nuclear Receptor Buffer F to each well in row B of the 96-well plate. Mix by pipetting up and down.

7. In order 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**.

   For example: Using the 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.

   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, and B2 all contain the highest concentration of ligand from B1 of the 96-well plate. Wells A3, A4, B3, and B4 all contain the next concentration of ligand from B2 of the 96-well plate, etc for 4 replicates of each concentration with constant percent DMSO.

Prepare 4X PPAR gamma-LBD

8. Prepare 4X PPAR gamma-LBD using Complete Nuclear Receptor Buffer F. The recommended molar concentration of PPAR gamma for this kit is listed on the Certificate of Analysis. ***Never vortex the PPAR 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 PPAR gamma-LBD has a stock concentration of 4000 nM and the recommended concentration for this kit is 5 nM, prepare a 4X solution at 20 nM by adding 4 µL of PPAR gamma-LBD stock to 796 µL of **cold** Complete Nuclear Receptor Buffer F.

9. Add 5 µL of 4X PPAR gamma-LBD to rows A–D, columns 1–24 of the 384-well assay plate. We recommend adding from low to high concentration of ligand (right to left) to prevent ligand carry over.

Prepare 4X Fluorescein-TRAP220/DRIP-2/4X Tb anti-GST Antibody

10. Prepare a solution containing 500 nM fluorescein-TRAP220/DRIP-2 (4X) and 20 nM Tb anti-GST antibody (4X) using Complete Nuclear Receptor Buffer F. The stock concentration of fluorescein-TRAP220/DRIP-2 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).

    For example: Add 5 µL of 100 µM fluorescein-TRAP220/DRIP-2 and 5.7 µL of Tb anti-GST antibody to 989 µL of Complete Nuclear Receptor Buffer F.

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

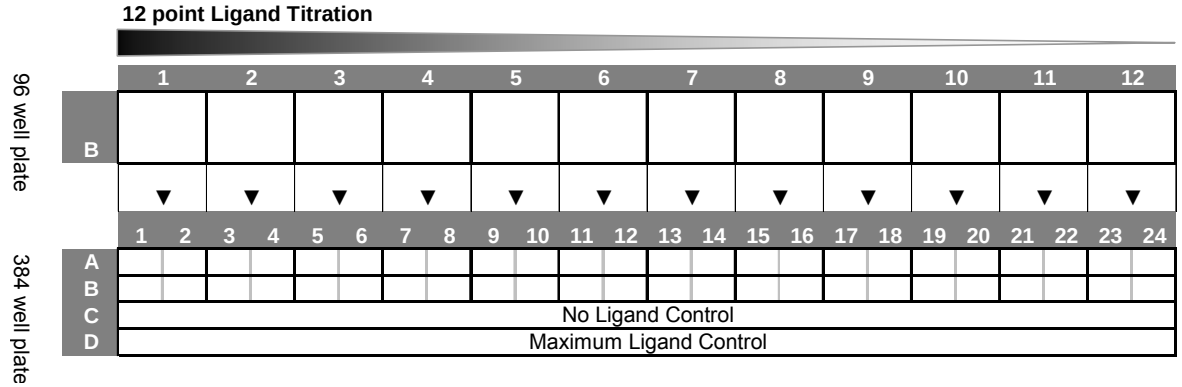
Plate Incubation and Reads

12. Briefly and gently mix the 384-well plate on a plate shaker and incubate at room temperature protected from light for the chosen equilibration time. The plate may be sealed with a cover to minimize evaporation.

Note: If you decide to use 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.

13. Read the plate 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 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.

The "maximum agonist" and "no 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 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 Assay—Reagent Volumes and Controls

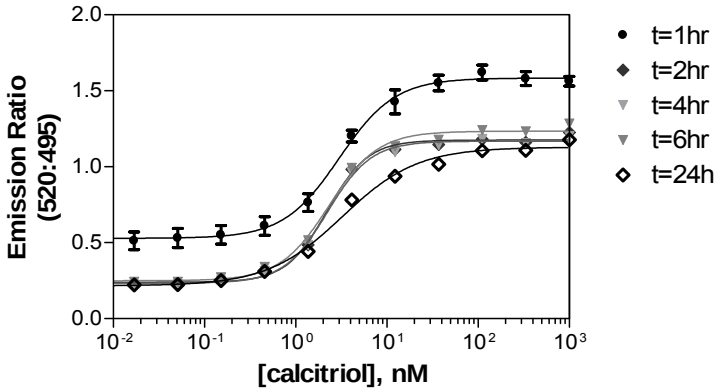
The following table summarizes the reagent volumes, order of addition, and potential controls for developing an agonist mode assay. The protocol in **Section 6.1** may be adapted to screen test compounds using the following summary table as a guide.

Assay	Reagent Additions	Purpose
Test Compound	1. 10 µL 2X Test Compound (or dilution series) 2. 5 µL 4X PPAR gamma-LBD 3. 5 µL 4X Fl-TRAP220/DRIP-2/Tb anti-GST Ab	Assess coactivator recruitment upon binding of test compound.
Positive Control (Max agonist)	1. 10 µL 2X agonist (or dilution series) 2. 5 µL 4X PPAR gamma-LBD 3. 5 µL 4X Fl-TRAP220/DRIP-2/Tb anti-GST Ab	Assess coactivator recruitment upon binding of a known PPAR gamma agonist.
Negative Control (No agonist)	1. 10 µL Assay Buffer with 2X Compound Solvent 2. 5 µL 4X PPAR gamma-LBD 3. 5 µL 4X Fl-TRAP220/DRIP-2/Tb anti-GST Ab	Provides baseline signal for the assay including possible ligand-independent coactivator recruitment.
No PPAR gamma-LBD Control	1. 10 µL Assay Buffer 2X Compound Solvent 2. 5 µL Complete Nuclear Receptor Buffer F 3. 5 µL 4X Fl-TRAP220/DRIP-2/Tb anti-GST Ab	Can be compared to the negative control to determine ligand-independent coactivator recruitment. May also be used to determine diffusion enhanced TR-FRET.*

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

* Diffusion enhanced TR-FRET occurs when the donor (Tb-anti-GST Antibody) passes by the acceptor (Fl-TRAP220/ DRIP-2) in solution during the excited state lifetime of the donor and 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



Time Point	EC_{50}
1 hour	2.1 nM
2 hour	2.2 nM
4 hour	2.8 nM
6 hour	3.5 nM
24 hour	10.6 nM

Figure 3. Representative experiment of LanthaScreen® TR-FRET PPAR gamma-Coactivator assay. Assay composition: serial dilution of agonist GW1929, 5 nM PPAR gamma-LBD, 125 nM Fluorescein TRAP220/DRIP-2, and 5 nM Tb anti-GST antibody. Results for 1-hour, 2-hour, 4-hour, 6-hour, and 24-hour incubations are shown with the corresponding EC_{50} values. The curves were generated using a sigmoidal dose response (variable slope) equation in GraphPad™ Prism® 4.0.

7. Antagonist Assay

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. 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 which can then result in displacement of coregulator peptides relative to the agonist.

Component	Final Assay Concentration
Fluorescein-TRAP220/DRIP-2	125 nM
Tb anti-GST antibody	5 nM
Agonist concentration	EC_{80} calculated from assay performed in agonist mode
PPAR gamma LBD-GST	See Certificate of Analysis for the recommended molar concentration for this kit

Note: We recommend determining the EC_{80} of the agonist as described in **Section 6.0**. Although a concentration of agonist 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

Prepare Complete Nuclear Receptor Buffer F and Controls

1. Prepare Complete Nuclear Receptor Buffer F by adding 1 M DTT to Nuclear Receptor Buffer F for a final concentration of 5 mM DTT. Complete Nuclear Receptor Buffer F must be prepared fresh daily.

   For example: Add 30 µL of 1 M DTT to 5.97 mL of Nuclear Receptor Buffer F.

2. For the "no antagonist" controls (Negative Control) in the presence of EC_{80} agonist (row C) and for the "no agonist, no antagonist" controls (Alternative Positive Control) (row E), prepare a solution containing 2% DMSO in Complete Nuclear Receptor Buffer F. Add 10 µL of this solution to rows C & E, columns 1–24 of a 384-well assay plate (see the plate layout in **Section 7.3**).

   For example: Add 20 µL of 100% DMSO to 980 µL of Complete Nuclear Receptor Buffer F.

3. Prepare a solution of control antagonist (we recommend GW9662) at 100X of the final desired maximum starting concentration using 100% DMSO.

   For example: If the final desired maximum starting concentration of antagonist is 1 µM, prepare a solution of 100 µM antagonist in 100% DMSO.

4. For the "maximum antagonist" controls (Positive Control) dilute the 100X antagonist solution from step 3 to 2X using Complete Nuclear Receptor Buffer F. 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**).

   For example: Add 10 µL of 100X antagonist solution to 490 µL of Complete Nuclear Receptor Buffer F.

Prepare 2X Antagonist Titration

5. Prepare the serial dilution of antagonist as described for the agonist in **Section 6.1**, steps 5 through 7.

Prepare 4X PPAR gamma-LBD

6. Prepare 4X PPAR gamma-LBD as described in **Section 6.1**, step 8.
7. Add 5 µL of 4X PPAR gamma-LBD to rows A–E, columns 1–24 of the 384-well assay plate. We recommend adding from low to high concentration of ligand (right to left) to prevent ligand carry over

Prepare 4X Fluorescein-TRAP220/DRIP-2/4X Tb anti-GST Antibody

8. Prepare 4X Fluorescein-TRAP220/DRIP-2/4X Tb anti-GST Antibody as described in **Section 6.1**, step 10.
9. Add 5 µL of 4X peptide/4X antibody solution to row E ONLY, columns 1–24 of the 384-well assay plate (see plate layout in **Section 7.3**).

Prepare 4X Fluorescein-TRAP220/DRIP-2/4X Tb anti-GST Antibody/4X EC_{80} Agonist

10. Prepare a 1000X solution of the EC_{80} of the agonist in Complete Buffer F. This will add only 0.1% DMSO to the assay in addition to the 1% DMSO from the antagonist.

    For example: If the EC_{80} of the agonist is 8 nM, and the 100X agonist solution from Step 6.1.3 is 100 µM, prepare a 1000X solution at 8 µM in Complete Buffer F by adding 8 µL of the 100 µM from step 6.1.3 to 92 µL of Complete Nuclear Receptor Buffer F.

11. Prepare a solution containing 0.5 µM fluorescein-TRAP220/DRIP-2 (4X), 20 nM Tb anti-GST antibody (4X), and 4X of the agonist EC_{80} using Complete Nuclear Receptor Buffer F. The concentration of fluorescein-TRAP220/DRIP-2 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).

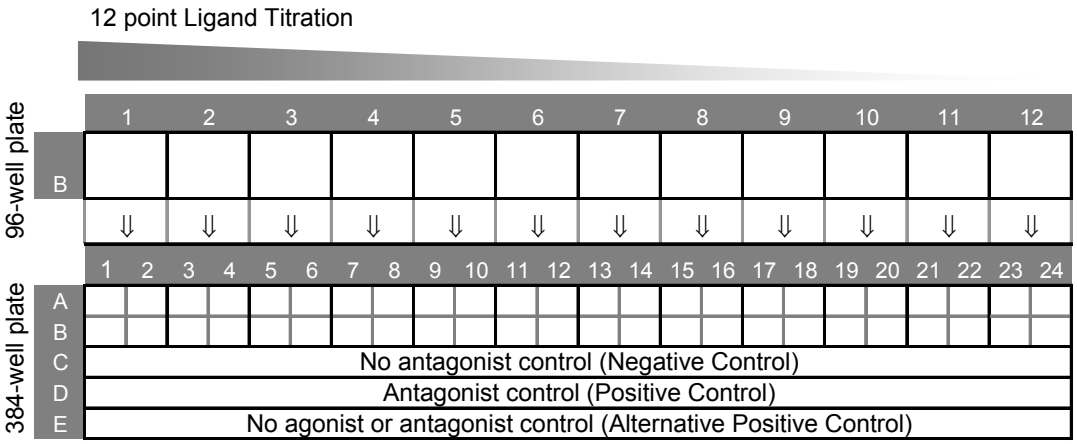
For example: Add 4 µL of the 1000X solution of the EC_{80} agonist, 5 µL of 100 µM fluorescein-TRAP220/DRIP-2, and 5.8 µL of Tb anti-GST antibody to 985 µL of Complete Nuclear Receptor Buffer F.

12. 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)**

Plate Incubation and Reads

13. For plate incubations, follow Steps 12 and 13 in **Section 6.1**.

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). The "no antagonist" and "alternative positive control" (rows C and E) can also be used to calculate Z´-factor.

7.5 Antagonist Assay—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 PPAR gamma-LBD 3. 5 µL 4X Fl-TRAP220/DRIP-2/Tb anti-GST Ab/ EC_{80} agonist	Assess disruption of coactivator recruitment by competition of test compound and a known PPAR gamma agonist at the EC_{80} for binding to PPAR gamma.
No antagonist (Negative control)	1. 10 µL 2X Compound Solvent 2. 5 µL 4X PPAR gamma-LBD 3. 5 µL 4X Fl-TRAP220/DRIP-2/Tb anti-GST Ab/ EC_{80} agonist	Provides maximum FRET ratio for the antagonist assay.
Max Antagonist (Positive Control)	1. 10 µL 2X known antagonist (or dilution series) 2. 5 µL 4X PPAR gamma-LBD 3. 5 µL 4X Fl-TRAP220/DRIP-2/Tb anti-GST Ab/EC_{80} agonist	Assess disruption of coactivator recruitment by competition of a known PPAR gamma antagonist at the EC_{80} of a known agonist for binding to PPAR gamma.
No Agonist or Antagonist (Alternate Positive Control)	1. 10 µL Assay Buffer with 2X Compound Solvent 2. 5 µL Complete Nuclear Receptor Buffer F 3. 5 µL 4X Fl-TRAP220/DRIP-2/Tb anti-GST Ab	May stand in for the positive control. Provides bottom baseline for assay and can be used to assess diffusion enhanced TR-FRET.*

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

* Diffusion enhanced TR-FRET occurs when the donor (Tb-anti-GST Antibody) passes by the acceptor (Fl-TRAP220/DRIP-2) in solution during the excited state lifetime of the donor and 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.

7.6 Antagonist Assay—Representative Data

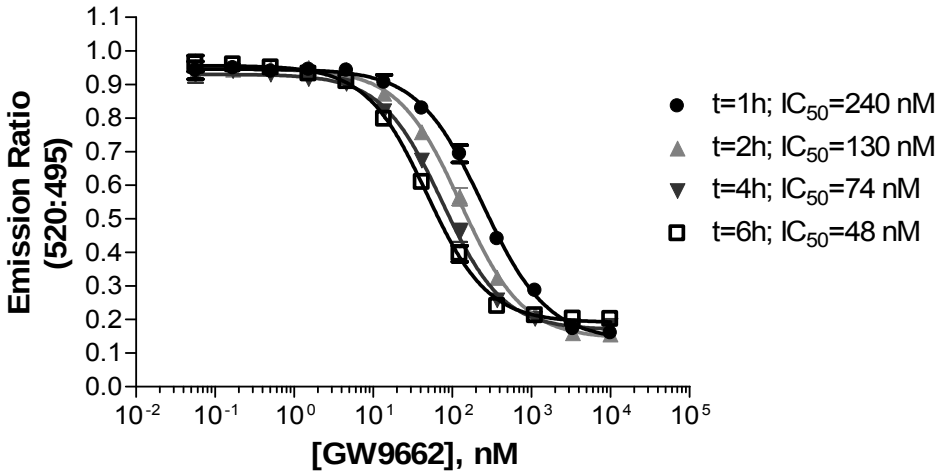


Figure 4. Representative experiment of LanthaScreen™ TR-FRET PPAR gamma Coactivator assay run in antagonist mode. Assay Composition: dilutions of antagonist GW9662, 5 nM PPAR gamma-LBD, 125 nM Fluorescein TRAP220/DRIP-2, 5 nM Tb anti-GST antibody, and 10 nM GW1929. Results for 1-hour, 2-hour, 4-hour, and 6-hour incubations are shown with the corresponding IC_{50} values. Curves were generated using a sigmoidal dose response equation (variable slope) in GraphPad™ Prism® 4.0. Note that the IC_{50} values for the antagonist GW9662 decrease over time due to the irreversible binding of the compound.

8. Assay Pharmacology

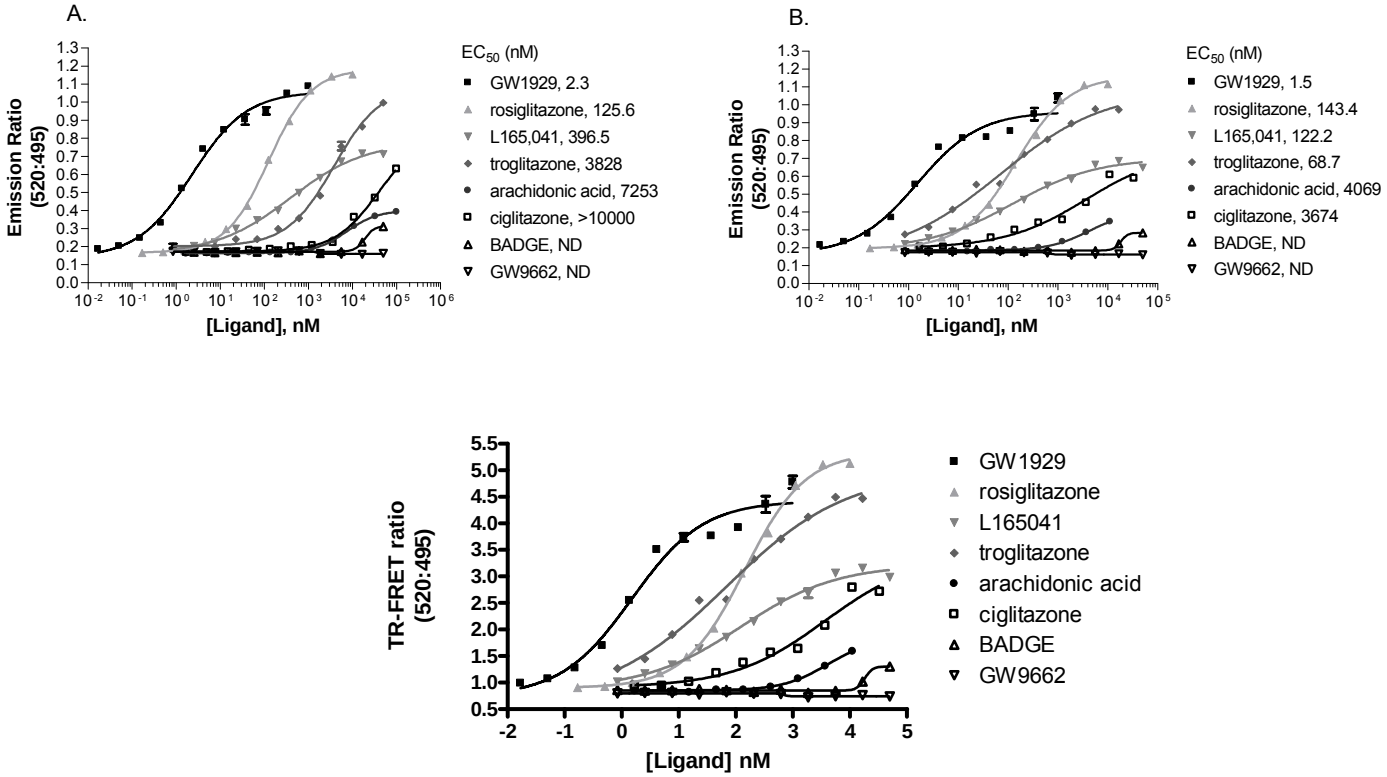


Figure 5. Relative EC_{50} Values of Selected Ligands for PPAR gamma-LBD in the LanthaScreen™ TR-FRET PPAR gamma Coactivator Assay, Agonist Mode. Serial dilutions of various test compounds (1% final DMSO concentration) were assayed in agonist mode (n = 4). Curves were fit using a sigmoidal dose-response equation (variable slope) in GraphPad™ Prism® 4.0. ND, not determined. **A.** Black Corning® 384-well, low-volume, round-bottom (non-binding surface) assay plates; Corning #3676. **B.** Black Corning® 384-well, low-volume, round-bottom (non-coated) assay plates; Corning #3677. Note that the different plate types result in significantly different EC_{50} values for a couple of the compounds, i.e. troglitazone and ciglitazone. These results suggest that these compounds may be sticking to the 3676 plates. Figures 5B and 5C are the same data plotted differently. The values plotted in 5C have been normalized by the minimum TR-FRET ratio obtained for GW1929 so that the emission ratio for GW1929 starts at 1.0. There is no effect on the Z′ or calculated IC_{50}'s.

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

9. References

Rachez, C., Gamble, M., Chang, C-PB., Atkins, G.B., Lazar, M.A., and Freedman, F.P. (2000) The DRIP complex and SRC-1/p160 coactivators share similar nuclear receptor binding determinants but constitute functionally distinct complexes. *Mol. Cell. Biol., 20*, 2718-2726

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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