LanthaScreen™ TR-FRET Peroxisome Proliferator Receptor delta Coactivator Assay

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1. KIT CONTENTS

The LanthaScreen™ TR-FRET Peroxisome Proliferator Receptor delta Coactivator Assay, catalog no. PV4685, contains the following:

Component	Composition	Amount	Storage Temp.	Individual Catalog no.
Fluorescein-C33 coactivator peptide (Chang et al, 1999)	100 µM in 25 mM HEPES, pH 7.5, 50% DMSO Sequence: HVEMHPLLMGLLMESQWGA	100 µl	–20 °C	PV4606
PPAR delta LBD, GST	PPAR delta 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	PV4693
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 J	Proprietary buffer (pH 8.0) containing 10% glycerol	25 ml	Room temperature	PV4682
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 capability 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 PPAR delta agonist, such as GW501516, to serve as a positive control. We recommend Alexis Biochemicals #420-043.
- DMSO

3. INTRODUCTION

Invitrogen's LanthaScreen™ TR-FRET Peroxisome Proliferator Receptor alpha Coactivator Assay provides a sensitive and robust method for high-throughput screening of potential PPAR delta ligands as agonists of coactivator recruitment. The kit uses a terbium-labeled anti-GST antibody, a fluorescein-labeled coactivator peptide, and a PPAR delta 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

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 Receptor delta Coactivator Assay, PPAR delta-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 EC_{50} from a dose response curve of the compound. Based on the biology of the PPAR delta-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).

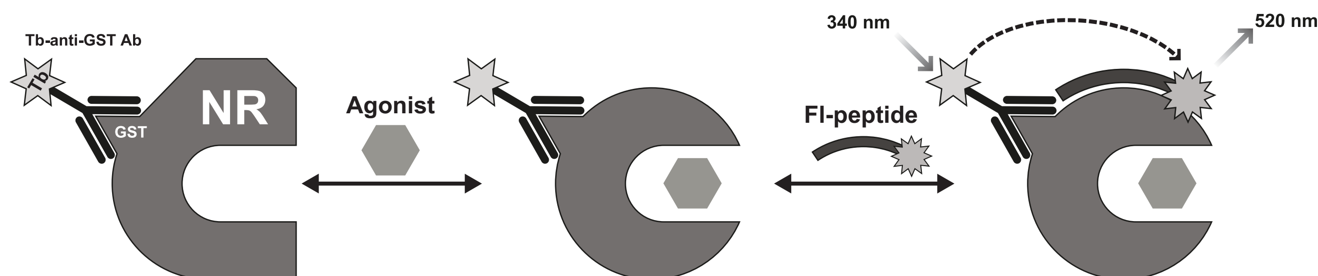


Figure 1. Principle of the nuclear receptor (NR) agonist dependent coactivator peptide recruitment assay: Tb-anti-GST antibody indirectly labels the nuclear receptor by binding to the GST tag. Binding of the agonist to the NR causes a conformational change that results in an increase in the affinity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

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 µs
Integration Time	200 µs

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.

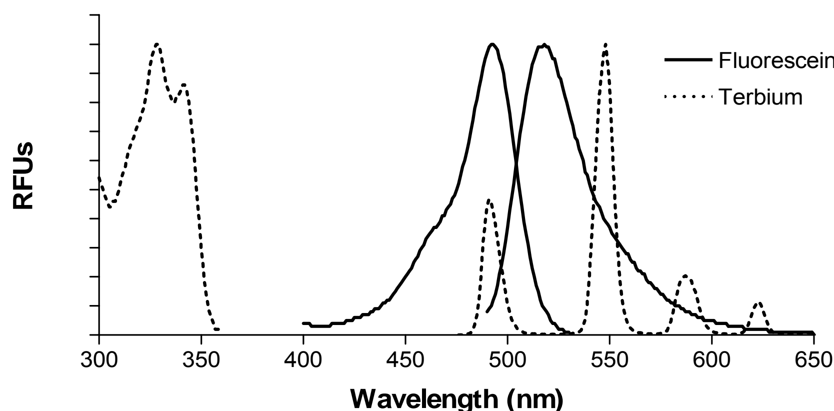


Figure 2. Excitation and emission spectra of fluorescein and terbium.

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 µs followed by a 200-µs 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 Safire^2 and Molecular Devices M5 monochromator-based instrument. Contact Invitrogen Technical Services for instrument-specific setup guidelines.

5. GUIDELINES AND RECOMMENDATIONS

5.1 Reagent Handling

PPAR delta-LBD

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

TR-FRET Coregulator Buffer J

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

LanthaScreen™ Tb anti-GST Antibody

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

Fluorescein-C33 Coactivator Peptide

Store fluorescein-C33 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.

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 PPAR delta-LBD was added to agonist dilutions, followed by the addition of pre-mixed fluorescein-C33 coactivator peptide and Tb anti-GST antibody (agonist, receptor, peptide/antibody). A pre-mixture of PPAR delta-LBD, fluorescein-C33 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. 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 GW501516 as the agonist are shown in Table 1 below. The EC_{50} and Z′ are stable from 1 to 24 hours (Table 1).

Incubation Time (hours)	EC_{50} GW501516	Z′-Factor
1	14 nM	0.80
2	12 nM	0.84
4	11 nM	0.85
6	13 nM	0.85
24	14 nM	0.54

Table 1. Effect of Incubation Time on Assay Performance. Sample data represents mean values from 3 separate experiments (n = 24). EC_{50} values were determined by fitting the data to a sigmoidal dose response (variable slope) equation in XLFit4 (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). 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 C33 coactivator peptide to PPAR delta-LBD. The only variable is the agonist concentration. All other assay components (PPAR delta-LBD, peptide, Tb anti-GST antibody) are fixed at concentrations optimized to provide an assay with the lowest EC_{50} with the control 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.

Component	Final Assay Concentration
Fluorescein-C33	100 nM
Tb anti-GST antibody	10 nM
PPAR delta LBD-GST	See Certificate of Analysis for the recommended molar concentration for this kit

6.1 Agonist Assay—Procedure

Prepare Complete TR-FRET Coregulator Buffer J and Agonist Controls

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

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

   For example: Add 6 µl of 1 M DTT to 6 ml of TR-FRET Coregulator Buffer J.

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

2. For the “no agonist” controls, add DMSO to Complete TR-FRET Coregulator Buffer J 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 J.

3. Prepare a solution of control agonist (we recommend GW501516) at 100X of the final desired maximum starting concentration using DMSO.

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

4. For the “maximum agonist” controls, dilute the 100X agonist solution from step 3 to 2X using Complete TR-FRET Coregulator Buffer J. 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 TR-FRET Coregulator Buffer J.

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.

5. Prepare a 12-point 100X dilution series of agonist in a 96-well plate by serially diluting the 100X 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 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 A3–A12.

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

   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 TR-FRET Coregulator Buffer J to each well in row B of the 96-well plate. Mix by pipetting up and down.

7. Transfer 10 µl of each of the 2X 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 PPAR delta-LBD

8. Prepare 4X PPAR delta-LBD using **cold** Complete TR-FRET Coregulator Buffer J from step 1. The recommended molar concentration of PPAR delta for this kit is listed on the Certificate of Analysis. ***Never vortex the PPAR delta-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 delta-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 PPAR delta-LBD stock to 996 µl of **cold** Complete TR-FRET Coregulator Buffer J.

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

Prepare 4X Fluorescein-C33/4X Tb anti-GST Antibody

10. Prepare a solution containing 0.4 µM fluorescein-C33 (4X) and 40 nM Tb anti-GST antibody (4X) using Complete TR-FRET Coregulator Buffer J at room temperature. The stock concentration of fluorescein-C33 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 4 µl of 100 µM fluorescein-C33 and 6 µl of 6.7 µM Tb anti-GST antibody to 990 µl of Complete TR-FRET Coregulator Buffer J.

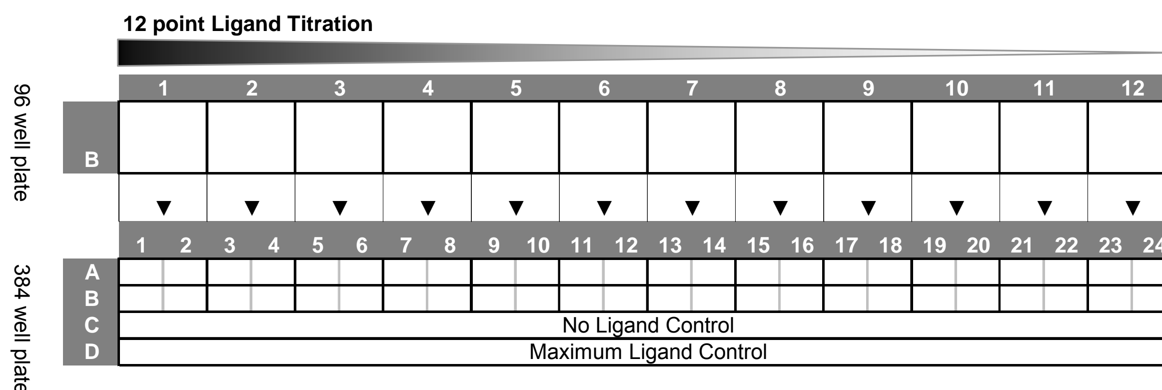
11. Add 5 µl of 4X peptide/4X antibody solution to rows A–D, columns 1–24 of the 384-well assay plate (see plate layout in **Section 6.2**).

Plate Incubation and Reads

12. 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 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).

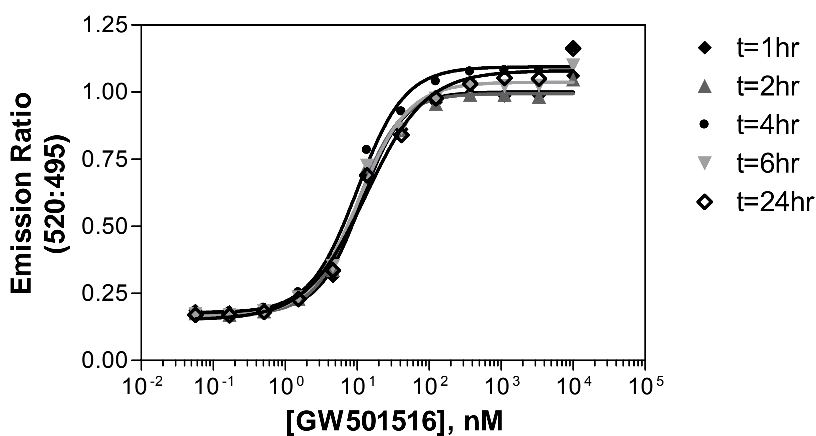
Note: The ligand EC_{50} determined in the assay is a composite of multiple equilibria, including ligand binding to receptor and peptide binding to ligand/receptor complex.

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 delta-LBD 3. 5 µl 4X Fl-C33/Tb anti-GST Ab	Assess coactivator recruitment upon binding of test compound.
Positive Control	1. 10 µl 2X agonist (or dilution series) 2. 5 µl 4X PPAR delta-LBD 3. 5 µl 4X Fl-C33/Tb anti-GST Ab	Assess coactivator recruitment upon binding of a known PPAR delta agonist.
Negative Control	1. 10 µl 2X Compound solvent 2. 5 µl 4X PPAR delta-LBD 3. 5 µl 4X Fl-C33/Tb anti-GST Ab	Provides indication of basal signal or possible ligand-independent coactivator recruitment and accounts for possible interference from a compound's vehicle solvent.
No PPAR delta-LBD Control	1. 10 µl 2X compound solvent 2. 5 µl Complete TR-FRET Coregulator Buffer J 3. 5 µl 4X Fl-C33/Tb anti-GST Ab	Provides absolute bottom baseline for assay, not accounting for ligand-independent coactivator recruitment or diffusion enhanced FRET.

6.5 Agonist Assay—Representative Data



Time Point	EC_{50} (nM)
1 hour	11
2 hour	10
4 hour	10
6 hour	11
24 hour	13

Figure 3. Representative experiment of LanthaScreen™ TR-FRET PPAR delta-Coactivator assay. Assay composition: serial dilution of agonist GW501516, 5 nM PPAR delta-LBD, 100 nM Fluorescein C33, and 10 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. ASSAY PHARMACOLOGY

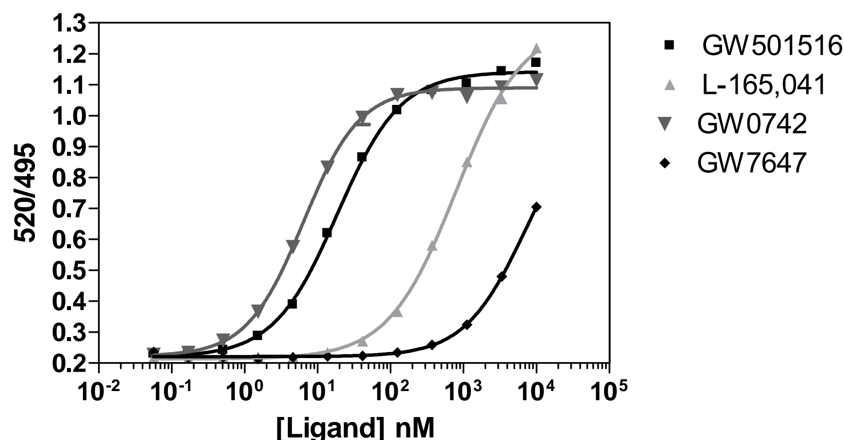


Figure 4: Relative EC_{50} Values of Selected Ligands for PPAR delta-LBD in the LanthaScreen™ TR-FRET PPAR delta Coactivator Assay, Agonist Mode. Serial dilutions of various test compounds (1% final DMSO concentration) were assayed in agonist mode (n = 6). Curves were fit using a sigmoidal dose-response equation (variable slope) in GraphPad™ Prism® 4.0.

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 EC_{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

Chang CY, Norris JD, Grøn H, Paige LA, Hamilton PT, Kenan DJ, Fowlkes D, McDonnell DP (1999) *Mol. Cell. Biol. 19*, 8226-8239.

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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