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1. Kit Contents

The LanthaScreen™ TR-FRET PPAR α Competitive Binding Assay Kit, PV4892, contains the following:

Component	Composition	Amount	Storage Temp.	Individual Catalog no.
Fluormone™ Pan-PPAR Green	2 μ M in 95% ethanol/water	200 μ l	-80°C	PV4896
PPAR α -LBD(GST)	Human PPAR α ligand-binding domain in buffer (pH 8.0) containing protein stabilizing reagents and glycerol. See Certificate of Analysis for lot specific concentration.	10 μ g	-80°C	PV4691
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 μ g	-20°C	PV3550
TR-FRET PPAR Assay Buffer	Proprietary buffer (pH 7.5)	25 ml	4°C	PV4895
DTT, 1 M	In water.	1 ml	-20°C or -80°C	P2325

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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 filters sets installed for detecting the fluorescent emission signals of terbium at 495 nm and the acceptor fluor at 520 nm (see **Section 4**).
- Pipetting devices for 1–1000 μ l volumes, suitable repeater pipettors, or multi-channel pipettors.
- Black 384-well assay plates. We recommend untreated polypropylene plates (*e.g.*, MatriCal #MP101-1-PP with total assay volume of 20–40 μ l) or untreated polystyrene plates (*ex.* Corning #3677 with total assay volume of 20 μ l).
- A known PPAR α ligand to serve as a positive control for competition. We recommend GW7647 (Tocris #1677).

3. Introduction

Invitrogen's LanthaScreen™ TR-FRET PPAR α Competitive Binding Assay provides a sensitive and robust method for high-throughput screening (HTS) of ligands for peroxisome proliferator-activated receptor-alpha (PPAR α). The kit uses a terbium-labeled anti-GST antibody, a fluorescent small molecule pan-PPAR ligand (Fluormone™ Pan-PPAR Green, also referred to as "tracer"), and human PPAR α 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 400 assays of 40 μ l each.

3.1. Principle of FRET and TR-FRET

For screening libraries of compounds, time-resolved fluorescence resonance energy transfer (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 two suitable fluorophores are 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 emission 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 such as terbium 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 noninstantaneous nature of the flashlamp excitation source.

3.2. Assay Overview

A terbium-labeled anti-GST antibody is used to indirectly label a nuclear receptor (NR) by binding to its GST tag. When a fluorescent ligand (tracer) is bound to the receptor, energy transfer from the antibody to the tracer occurs, and a high TR-FRET ratio is observed. Competitive ligand binding to the NR is detected by a test compound's ability to displace the tracer from the NR, which results in a loss of FRET signal between the antibody and the tracer. This type of binding assay is analogous to radioligand-based assays, except that it eliminates handling of radioactivity and enables a homogenous, "addition-only" format.

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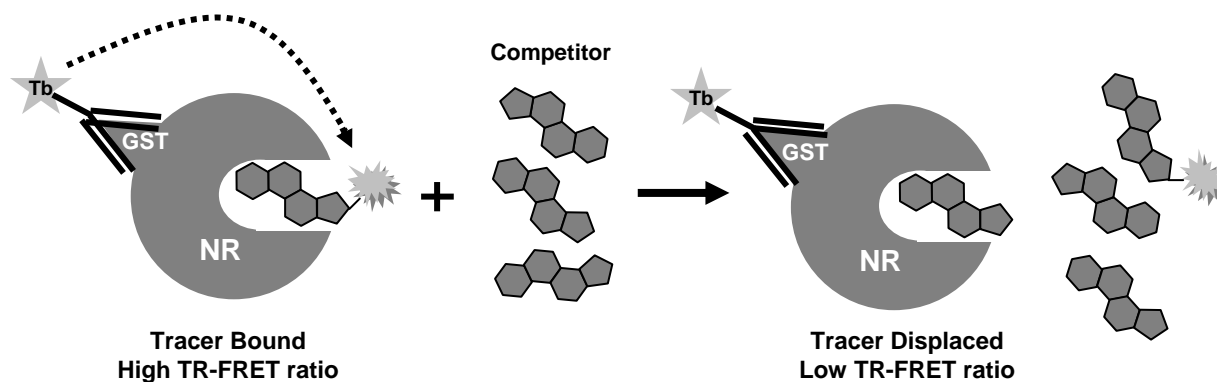


Figure 1. Principle of a LanthaScreen™ nuclear receptor competitive binding assay.

4. Instrument Settings

General Settings:

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

The excitation and emission spectra of terbium and fluorescein are shown in Figure 2 below. 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.

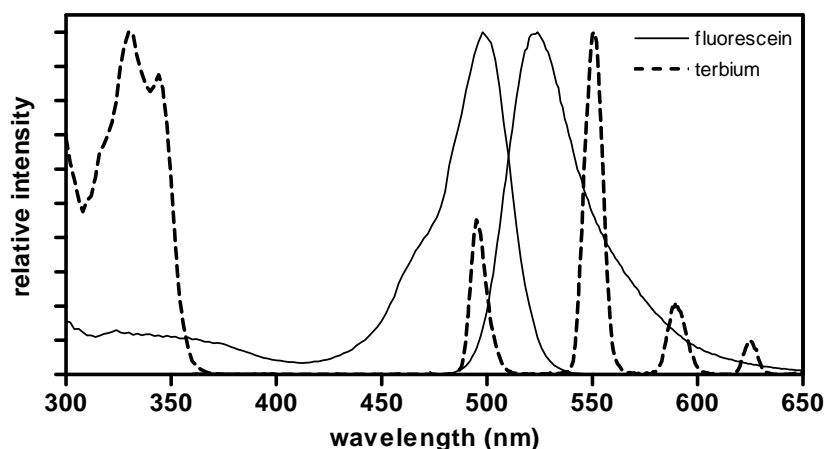


Figure 2. Excitation and emission spectra of fluorescein and terbium.

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 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

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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. Go to www.invitrogen.com/lanthascreen and click on “Instrumentation and Filter Ordering Information” for more instrument specific information. Filters suitable for LanthaScreen™ assays are available directly from Invitrogen at <http://www.invitrogen.com/content.cfm?pageid=10515> or from other vendors. A LanthaScreen™ filter module for the BMG PheraStar is available direct from BMG Instruments.

Aside from filter choices, instrument settings are similar to the settings used with europium-based technologies. In general, the guidelines provided by the instrument manufacturer can be used as a starting point for optimization. A delay time of 100 μ s followed by a 200- μ s integration time is typical for a LanthaScreen™ assay. The number of flashes or measurements per well is highly instrument dependent and should be set as advised by your instrument manufacturer. In general, LanthaScreen™ assays can be run on any filter-based instrument capable of time-resolved FRET, such as the Tecan Ultra, Tecan Infinite F500, BMGLabTech PHERAStar, Molecular Devices Analyst, or PerkinElmer Envision. LanthaScreen™ assays have also been performed successfully on the Tecan Safire2 and Molecular Devices M5 monochromator-based instruments. Contact Invitrogen Technical Services for instrument-specific setup guidelines.

5. Guidelines and Recommendations

5.1. Reagent Handling

PPAR α -LBD

Store PPAR α -LBD at -80°C. Thaw and store on ice before setting up assay. Never vortex the PPAR α -LBD stock or dilutions. This reagent is stable up to 8 freeze-thaw cycles. However, we recommend aliquotting the PPAR α -LBD after the first thaw to maintain optimal activity.

TR-FRET PPAR Assay Buffer

Thaw TR-FRET PPAR Assay Buffer at room temperature before use. Mix by inversion prior to first use, as the buffer may not have thawed evenly. Always allow the buffer to reach ambient room temperature prior to performing assay. The buffer is stable at room temperature for at least 1 week, but for longer term storage 4°C is recommended.

LanthaScreen™ Tb-anti-GST antibody

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

Fluormone™ Pan-PPAR Green

Store Fluormone™ Pan-PPAR Green at -80°C. Place on ice for at least 15 minutes to bring the temperature up to ~4°C and then vortex before use. Keep container closed when not in use to prevent evaporation. The tracer is stable for at least 8 freeze-thaw cycles.

5.2. Solvent Tolerance

The LanthaScreen™ TR-FRET PPAR α Competitive Binding Assay has been developed and validated in the presence of 1% DMSO. The use of higher amounts of solvents can affect the assay window. However, when using up to 8% DMSO, 8% methanol, or 8% ethanol, the IC₅₀ value of the control competitor GW7647 was unaffected and Z'-factor values >0.7 were obtained (data not shown). Regardless of the solvent concentration used, we always recommend that the test compound vehicle solvent be included in all of the control assay wells.

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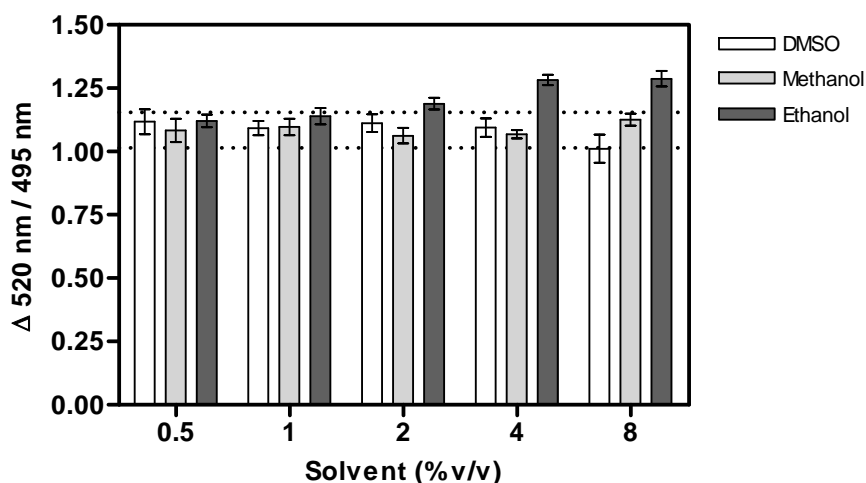


Figure 3. Effect of different solvents on assay performance.

$\Delta 520$ nm/495 nm was determined by the change in TR-FRET ratio between assay wells without competitor to those containing 1 μ M GW7647. Dotted lines represent ± 1 standard deviation from the average $\Delta 520/495$ value of wells containing no solvent.

5.3. Reagent Order of Addition

The LanthaScreen™ TR-FRET PPAR α Competitive Binding Assay has been validated using separate tracer and receptor/antibody additions. Use of a premixture containing all three components is not recommended. If the receptor is first allowed to bind to the tracer in the absence of competitor, it is more difficult to displace the tracer from the receptor, resulting in a longer period of time to reach binding equilibrium for the test compound and a higher background signal. However, the order in which the tracer and receptor/antibody mixtures are added to the assay plate is less critical; the order of addition for these components may be reversed if desired.

5.4. Incubation Conditions

Assay Component	Recommended 1X Concentration
[PPAR α -LBD]	See Certificate of Analysis for recommended concentration.
Fluormone™ Pan-PPAR Green	20 nM
Tb-anti-GST antibody	5 nM

Incubation Time

The incubation time can be set by the user. An incubation time of 2-6 hours is recommended. As a guide, sample results (average and standard deviation values) from various incubation time points using GW7647 as the competitor in three independent experiments are shown in the table below.

Incubation Time (hrs)	GW7647 IC ₅₀ (nM) Avg \pm Std dev	Z'-Factor Avg \pm Std dev
1	1.4 \pm 0.1	0.67 \pm 0.13
2	1.2 \pm 0.2	0.78 \pm 0.05
4	0.9 \pm 0.2	0.78 \pm 0.03
6	0.9 \pm 0.2	0.77 \pm 0.03
24	0.9 \pm 0.2	0.78 \pm 0.01

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Incubation Temperature

We recommend that assays be conducted at room temperature (20-25°C).

6. Procedure

6.1. Preparing the Reagents

Before proceeding with the assay, prepare the reagents as described in this section. All steps should be carried out at room temperature (20-25°C).

Complete TR-FRET PPAR Assay Buffer

Allow the buffer to reach ambient room temperature before preparing complete TR-FRET PPAR Assay Buffer. Add 1 M DTT to TR-FRET PPAR Assay Buffer for a final concentration of 5 mM DTT and mix thoroughly. Complete TR-FRET PPAR Assay Buffer must be prepared fresh daily; prepare only the amount required for your experiment. Store at room temperature and use for the preparation of all assay reagents as described below.

Example: Add 5 μ l of 1 M DTT to each 1 ml of TR-FRET PPAR Assay Buffer required.

2X Test Compound, Solvent Control, and Control Competitor

Dilute test compound to a 2X concentration in complete TR-FRET PPAR Assay Buffer and mix well. Also prepare a solvent control containing an equivalent amount of the test compound's vehicle solvent in complete TR-FRET PPAR Assay Buffer. Include this solvent control as part of the Negative Control. For positive control of competition, prepare a 2X solution of a known PPAR δ ligand (we recommend 1 μ M GW7647 final 1X concentration) in complete TR-FRET PPAR Assay Buffer.

Note that many test compounds are hydrophobic and have low solubility in aqueous solutions. When performing serial dilutions of these compounds in an aqueous buffer, precipitation at high concentrations or carry-over between wells can occur. As an alternative, we recommend performing serial dilutions at 100X the final concentration of test compounds in 100% DMSO in a multiwell polypropylene plate (such as Corning #3657 or #3363), followed by dilution of each compound sample to its 2X concentration using complete TR-FRET PPAR Assay Buffer.

4X Fluormone™ Pan-PPAR Green

Prepare a 4X Fluormone™ Pan-PPAR Green solution (80 nM) at room temperature by diluting the supplied stock solution 25-fold using complete TR-FRET PPAR Assay Buffer and vortex well. This reagent is stable for at least two hours prior to performing the assay when stored at room temperature. Note: for best results, prepare this reagent in a glass container in order to minimize non-specific binding to plastics.

Example: To prepare 1 ml of 4X Fluormone™ Pan-PPAR Green, add 40 μ l of 2 μ M Fluormone™ Pan-PPAR Green to 960 μ l complete TR-FRET PPAR Assay Buffer and vortex well.

4X PPAR α -LBD/Tb-anti-GST Ab

Prepare a solution containing 20 nM Tb anti-GST antibody (4X) and 4X PPAR α protein (based on the concentration recommended in the Certificate of Analysis) using complete TR-FRET PPAR Assay Buffer at room temperature. The stock concentrations of Tb anti-GST antibody and PPAR α protein are indicated on both the vial label and the Certificate of Analysis (1 mg/ml = ~6.7 μ M antibody). Mix gently several times by inversion. Do not vortex. This reagent is stable for at least two hours prior to performing the assay when stored at room temperature.

Example: To prepare 1 ml of 4X PPAR α -LBD/Tb-anti-GST Ab, add 3 μ l of Tb-anti-GST Ab (if the stock solution is 6.6 μ M, for example), and 4 μ l of PPAR α -LBD (if the stock solution is 2 μ M and the recommended 1X concentration is 2 nM, for example) to 993 μ l Complete TR-FRET PPAR Assay Buffer and mix gently by inversion.

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4X Tb-anti-GST Ab (Optional, see “No Receptor Control” in Section 6.2)

Add Tb-anti-GST antibody to complete TR-FRET PPAR Assay Buffer at a concentration of 20 nM (4X). Mix gently several times by inversion. Do not vortex. This reagent is stable for at least two hours prior to performing the assay when stored at room temperature.

Example: To prepare 1 ml of 4X Tb-anti-GST Ab, add 3 μ l of Tb-anti-GST Ab (if the stock solution is 6.6 μ M, for example) to 997 μ l complete TR-FRET PPAR Assay Buffer and mix gently by inversion.

6.2. Reagent Volumes

The following table summarizes the reagent amounts and order of addition for each assay condition. Volumes indicate a total assay volume of 40 μ l/well. The indicated volumes can be scaled up or down accordingly.

Assay Condition	Reagent Additions	Purpose
Test Compound	1. 20 μ l 2X Test Compound 2. 10 μ l 4X Fluormone™ Pan-PPAR Green 3. 10 μ l 4X PPAR α -LBD/Tb-anti-GST Ab	Assess competition by test compound of interest using a single concentration or dilution series.
Positive Control	1. 20 μ l 2X Control Competitor 2. 10 μ l 4X Fluormone™ Pan-PPAR Green 3. 10 μ l 4X PPAR α -LBD/Tb-anti-GST Ab	Represents 100% tracer displacement (minimum TR-FRET ratio) by a known PPAR α ligand. We recommend using 1 μ M GW7647 as the Control Competitor.
Negative Control	1. 20 μ l 2X Test Compound Solvent 2. 10 μ l 4X Fluormone™ Pan-PPAR Green 3. 10 μ l 4X PPAR α -LBD/Tb-anti-GST Ab	Represents 0% tracer displacement (maximum TR-FRET ratio) and accounts for possible interference from a compound's vehicle solvent
No Receptor Control (Optional)	1. 20 μ l 2X Test Compound Solvent 2. 10 μ l 4X Fluormone™ Pan-PPAR Green 3. 10 μ l 4X Tb-anti-GST Ab	Provides absolute minimum TR-FRET ratio possible, where only non-specific, diffusion-enhanced FRET between the antibody and Fluormone™ is detected. This has been found to be equivalent to the TR-FRET ratio of the Positive Control assay wells when using 1 μ M GW7647 as the Control Competitor.

6.3. Performing the Assay

Note: Fluormone™ Pan-PPAR Green has shown non-specific binding to certain plastics. We recommend changing pipette tips between every addition of the Fluormone™ Pan-PPAR Green to the assay plate to ensure consistent results. If you are using a repeat pipettor and want to use the same tips across a titration of compound (from low to high concentration), we recommend preconditioning your tips by pipetting the Fluormone™ Pan-PPAR Green solution up and down several times before addition to your assay plate.

1. In a microtiter plate, pipet the reagents into each well in the order listed in the table above and gently mix the plate on an orbital plate shaker for 30 seconds.
2. Cover the assay plate to protect the reagents from light and evaporation, and incubate at room temperature (20–25°C) for 2 to 6 hours. You may wish to take multiple reads during this time period to ensure that binding of test compound has reached equilibrium.

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3. Measure the fluorescent emission signal of each well at 495 nm and 520 nm, using the instrument settings described in **Section 4**.

6.4. Data Analysis

Calculate the TR-FRET ratio by dividing the emission signal at 520 nm by the emission signal at 495 nm. Generate a competition curve by plotting the TR-FRET ratio vs. the log [test compound]. To determine the IC₅₀ value, fit the data using an equation for a sigmoidal dose-response, as provided by GraphPad™ Prism® 4.0 or another graphing program. See **Section 7** for sample competition data.

The inhibition constant (K_i) for a competitor can be calculated by applying the Cheng-Prusoff equation (Cheng & Prusoff, 1973):

$$K_i = \frac{IC_{50}}{1 + \frac{[tracer]}{K_D}}$$

where IC₅₀ is the concentration of competitor that produces 50% displacement of the tracer, [tracer] is the concentration of Fluormone™ Pan-PPAR Green used in the assay (20 nM), and K_D is the binding constant of Fluormone™ Pan-PPAR Green to PPAR α -LBD. This K_D value has been determined to be 12 ± 2 nM (average ± standard deviation calculated from three separate experiments).

The positive control and negative control data can be used to calculate a Z'-factor value based on the equation of Zhang *et al.* (Zhang, *et al.*, 1999) in order to assess the robustness of experimental results:

$$Z'\text{-factor} = 1 - \left(\frac{3 \times (\text{standard deviation}_{100\% \text{ Inhibition}} + \text{standard deviation}_{0\% \text{ Inhibition}})}{\text{mean}_{100\% \text{ Inhibition}} - \text{mean}_{0\% \text{ Inhibition}}} \right)$$

where SD_{neg} and SD_{pos} are the standard deviation of the TR-FRET ratios of the negative and positive control wells, respectively, and AVG_{neg} and AVG_{pos} are the average TR-FRET ratios of the negative and positive control wells, respectively. Z'-factor values > 0.5 are generally considered an indication of a robust assay, while a value of 1 indicates a theoretical assay with no variability.

Note: The emission values for the terbium chelate (495 nm) commonly have a much smaller change in intensity than the FRET values (520 nm). This is because there are multiple terbium chelates on the antibody, and not all of them participate in FRET either because the terbium labeled antibody is not fully saturated with receptor, or because not all of the terbium chelates are positioned for efficient FRET. However, the Tb emission serves an important role as in internal reference and improves the noise characteristics of the assay.

7. Sample Competition Data

A serial dilution of test compounds (1% final DMSO concentration, serial dilutions performed in 100% DMSO) were prepared in a 384-well polypropylene assay plate. Fluormone™ Pan-PPAR Green, PPAR α -LBD, and Tb-anti-GST Ab were then added to each sample well as described in the protocol. The assay was incubated for two hours at room temperature prior to measuring the 520 nm / 495 nm emission ratio of each well. The results are shown in Figure 4. Error bars represent the SEM of duplicate wells (n=2). The curve was fit using a sigmoidal dose-response equation with varying slope using Prism® software from GraphPad™ Software, Inc.

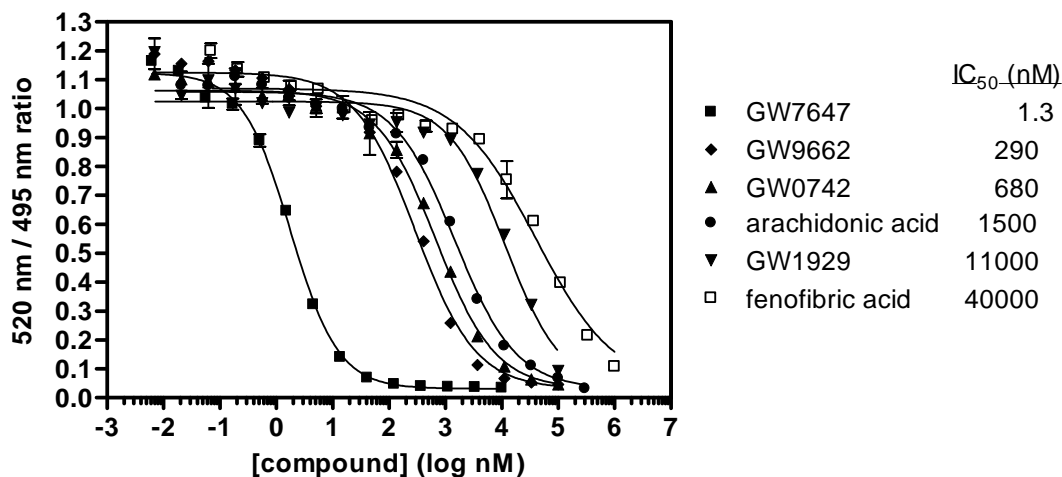


Figure 4. Sample competition data generated using the LanthaScreen™ TR-FRET PPAR α Competitive Binding Assay.

8. References

Cheng, Y., Prusoff, W. H., (1973) Relationship between the inhibition constant (K₁) and the concentration of inhibitor which causes 50 per cent inhibition (I₅₀) of an enzymatic reaction. *Biochem Pharmacol*, 22, (23), 3099-3108.

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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