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For Research Use Only. Not for use in diagnostic procedures.

1. KIT CONTENTS

The LanthaScreen® TR-FRET Glucocorticoid Receptor (GR) Competitive Binding Assay, catalog no. A15901, contains the following:

Component	Composition	Amount	Storage Temperature	Individual Part no.
Fluormone™ GS1 Green	500 nM in 75% methanol/water	200 µL	–20°C	PV6044
GR-LBD (GST)	Human GR ligand-binding domain (LBD) with a GST tag in buffer (pH 8.0) containing protein stabilizing reagents and 20% glycerol. See Certificate of Analysis for lot specific concentration.	4.5 mg	–80°C	A15668
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
GR Stabilizing Peptide (10X)	1 mM in 10 mM potassium phosphate (pH 7.4)	5 × 1 mL	–20°C to –80°C	P2815
Nuclear Receptor Buffer F	Proprietary buffer (pH 7.4)	2 × 25 mL	Room Temperature	PV4547
DTT, 1 M	In water	1 mL	–20°C	P2325

2. MATERIALS REQUIRED BUT NOT SUPPLIED

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

- A fluorescence plate reader with excitation capabilities at 340 nm and with the appropriate filter sets installed for detecting the fluorescent emission signals of terbium at 495 nm and fluorescein at 520 nm (see **Section 4**).
- Pipetting devices for 1–1000 µL volumes, suitable repeater pipettors, or multi-channel pipettor.
- Black, 384-well assay plates. We recommend black Corning® 384-well, low-volume, round-bottom, black (non-treated surface) assay plates, Cat. no. 4511. 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 Nalge Nunc, Cat. no. 249944.
- A known GR ligand, such as dexamethasone or mometasone furoate, to serve as a positive control. We recommend Sigma, Cat. no. D1756 for dexamethasone, or Sequoia Research Products, Cat. no. SRP066405m for mometasone furoate.
- DMSO to perform serial dilutions. We recommend Sigma, Cat. no. 41647.

3. INTRODUCTION

The LanthaScreen® TR-FRET GR Competitive Binding Assay provides a sensitive and robust method for high-throughput screening of ligands for glucocorticoid receptor. The kit uses a terbium-labeled anti-GST antibody, a fluorescent small molecule GR ligand (Fluormone™ GS1 Green, also referred to as "tracer"), and a human GR 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 micro-seconds 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 fluorescent ligand) 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.

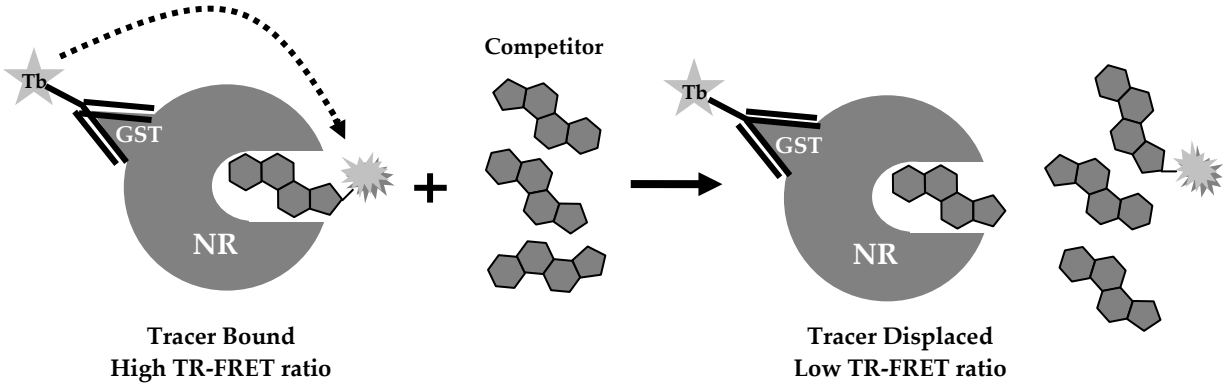
3.2 Assay Overview

A terbium-labeled anti-GST antibody is used to indirectly label a nuclear receptor (NR) by binding to its GST tag. Competitive binding to the NR is detected by a test compound's ability to displace a fluorescent ligand (tracer) from the NR, which results in a loss of FRET signal between the Tb-anti-GST 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.

When running the LanthaScreen® TR-FRET GR Competitive Binding Assay, Fluormone™ GS1 Green tracer is added to ligand test compounds or solvent controls followed by addition of a mixture of the GR-LBD (GST) 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 IC_{50} from a dose response curve of the compound.

The recommended concentrations of GR-LBD (GST), 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.

Figure 1. Principle of a LanthaScreen® TR-FRET NR Competitive Binding assay: Tb-anti-GST antibody indirectly labels the nuclear receptor by binding to the GST tag. Binding of the fluorescently labeled tracer results in close proximity of the tracer to the terbium-labeled antibody, resulting in a high TR-FRET signal. When competitor displaces the fluorescently labeled tracer, there is a decrease in the TR-FRET signal.



4. INSTRUMENT SETTINGS

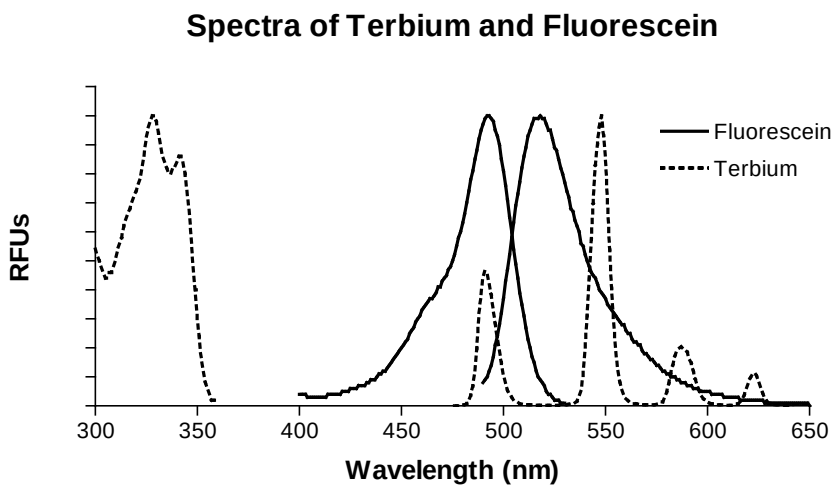
To facilitate instrument set-up we recommend downloading the Terbium Assay Application Note which can be found at **www.lifetechnologies.com/instrumentsetup** and following the instructions. You will need Fluorescein-Poly GT, PV3610 to run this test. For additional help in setting up your instrument to perform LanthaScreen® TR-FRET assays, please contact Technical Support at 800-955-6288 ext. 40266, or email **drugdiscoverytech@lifetech.com**. Please see **www.lifetechnologies.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, GENios™ pro, and Infinite® F500, BMG LABTECH® PHERAstar®; BioTek® Synergy™ 2 and Synergy™ 4; PerkinElmer® EnVision® and VICTOR™, and Molecular Devices® 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, next page. 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 (Molecular Devices® 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 Life Technologies. Visit **www.lifetechnologies.com/instrumentsetup** to purchase filters for your instrument. A LanthaScreen® Tb filter optical module is available directly from BMG LABTECH® for use on the BMG LABTECH® PHERAstar® instrument. PerkinElmer® EnVision® instrument users may require a specific filter holder which is available from PerkinElmer® as part no. 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® Infinite® 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® Safire²™, Tecan® Infinite® M1000) give satisfactory performance in LanthaScreen® TR-FRET assays, other monochromator-based instruments (e.g. Tecan® Safire™ and Molecular Devices® 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.lifetechnologies.com/instrumentsetup** and click on "ViewLux®" for a step-by-step guide to optimizing LanthaScreen® Terbium assays on the PerkinElmer® ViewLux® instrument.

5. GUIDELINES AND RECOMMENDATIONS

5.1 Reagent Handling

GR-LBD(GST)

Store GR-LBD (GST) 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 GR-LBD (GST) stock or dilutions.

Nuclear Receptor Buffer F

Thaw Nuclear Receptor Buffer F at room temperature upon receipt. Mix well before first use, as the buffer may not have thawed evenly. The buffer should be stored at room temperature.

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.

Fluormone™ GS1 Green

Store Fluormone™ GS1 Green at –20 °C. Place on ice for at least 15 minutes and then vortex before use. Keep container closed and on ice when not in use to prevent evaporation.

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 IC_{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 IC_{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, 6% acetonitrile and 8% methanol (in addition to the 1% DMSO present from the ligand dilution) with good results. The use of higher concentrations of DMSO or acetonitrile may still yield a robust assay, however a decreased assay window was observed.

5.4 Reagent Order of Addition

The LanthaScreen® GR Competitive Binding Assay has been developed using separate tracer and receptor/antibody additions. Use of a pre-mixture containing all three components is not recommended. If the receptor is first allowed to bind to the tracer in the absence of competitor, more time will be required for binding of the competitor to reach equilibrium, and the assay window will be reduced due to higher a higher background signal.

5.5 Incubation Conditions

Incubation Time

The incubation time can be set by the user. As a guide, results for various time points using mometasone furoate as the ligand are shown in Table 1 below. The IC_{50} and Z' are stable from 1 to 24 hours (Table 1). We suggest maintaining the same incubation time and temperature during testing and screening for consistent results.

Table 1. Effect of Incubation Time on Assay Performance. Sample data represents mean values from 3 separate experiments (n = 24). IC_{50} values were determined by fitting the data to a sigmoidal dose response (variable slope) equation in IDBS XLfit® 4.2 (data not shown). Z'-factors were calculated using the method of Zhang *et al*. (Zhang *et al.*, 1999) on the 24 replicates of maximum ligand and no ligand. 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.

Incubation Time (hours)	IC_{50} Mometasone Furoate (nM)	Z'-Factor
1	3.2	0.62
2	2.8	0.67
4	2.3	0.71
6	2.2	0.68

Temperature

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

6. PROCEDURE

The procedure in this section describes a method for determining the IC_{50} of a ligand and the Z'-factor for maximum ligand and no ligand controls (n = 24). The only variable is the ligand concentration. All other assay components (GR-LBD (GST), tracer, 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. Higher concentrations of nuclear receptor may give 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 reoptimized.

Component	Recommended Final Assay Concentrations
Fluormone™ GS1 Green	5 nM
Tb anti-GST antibody	2 nM
GR-LBD (GST)	See Certificate of Analysis for the recommended molar concentration for this kit

6.1 Assay Set-Up

Prepare Complete Nuclear Receptor Buffer F and Ligand Controls

Note: The GR-LBD (GST) should be thawed on ice just prior to use. During the assay set-up, keep the GR-LBD (GST) and Fluormone™ GS1 Green on ice. Equilibrate all other assay components to room temperature.

1. Prepare Complete Nuclear Receptor Buffer F by adding 10 X stabilizing peptide to a final concentration of 1X and 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 and 600 µL 10X stabilizing peptide to 537 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 ligand" 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 ligand (we recommend dexamethasone or mometasone furoate) at 100X of the final desired maximum starting concentration using 100% DMSO.

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

4. For the "maximum ligand" controls, dilute the 100X ligand 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 ligand solution to 490 µL of Complete Nuclear Receptor Buffer F.

Prepare 2X Ligand Dilution Series

Note: Although steps 5 and 6 below require more pipetting than other methods of preparing a serial dilution of ligand, 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 IC_{50} values.

5. Prepare a 12-point 100X dilution series of ligand in a 96-well polypropylene plate (DMSO tolerant) by serially diluting the 100X ligand 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 ligand solution prepared in step 3. 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.

6. Dilute each 100X agonist serial dilution from step 5 to 2X using Complete 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 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 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.

   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 Fluormone™ GS1 Green

8. Prepare 4X Fluormone™ GS1 Green (20 nM) using Complete Nuclear Receptor Buffer F.

   For example: The Fluormone™ GS1 Green has a stock concentration of 500 nM and the recommended concentration for this kit is 5.0 nM, so prepare a 4X solution at 20 nM by adding 40 µL of Fluormone™ GS1 Green stock to 960 µL of Complete Nuclear Receptor Buffer F.

9. Add 5 µL of 4X Fluormone™ GS1 Green 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 GR-LBD (GST)/4X Tb anti-GST Antibody

10. Prepare a solution containing 4X GR-LBD (GST) and 4X Tb anti-GST antibody (8 nM) using Complete Nuclear Receptor Buffer F. The recommended assay concentration of GR for this kit is listed on the Certificate of Analysis. ***Never vortex the GR-LBD (GST) stock or dilutions***. Mix by pipetting or gentle inversion. Keep this solution on ice until needed for use in the assay. The stock 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: If the GR-LBD (GST) has a stock concentration of 200 nM and the recommended concentration for this kit is 1.0 nM, prepare a 4X solution at 4 nM by adding 20 µL of GR-LBD (GST) stock and 2.4 µL of the Tb anti-GST antibody to 978 µL of Complete Nuclear Receptor Buffer F.

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

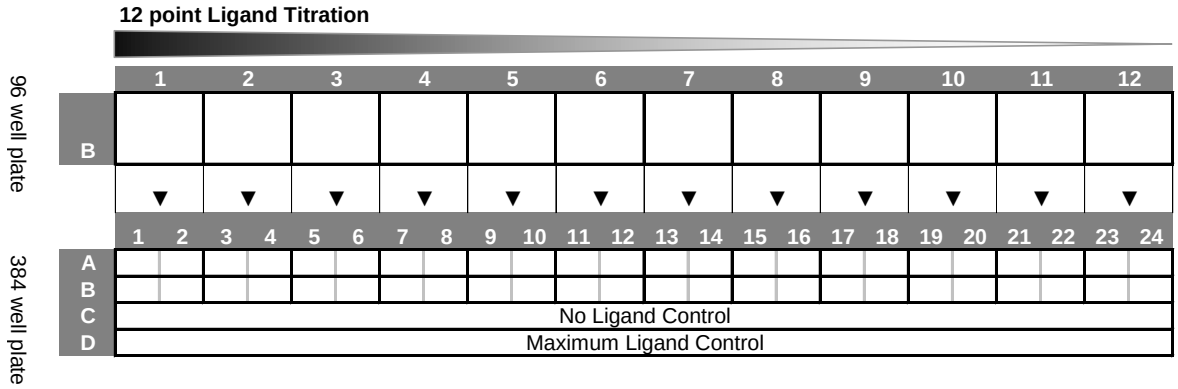
Plate Incubation and Reads

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: When using assay plates that are not coated such as Corning® 3677, 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 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 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 IC_{50} value, fit the data using an equation for a sigmoidal dose response (variable slope), as provided by GraphPad Prism™ 4.0 software or another comparable graphing program.

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

6.4 Reagent Volumes and Controls

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

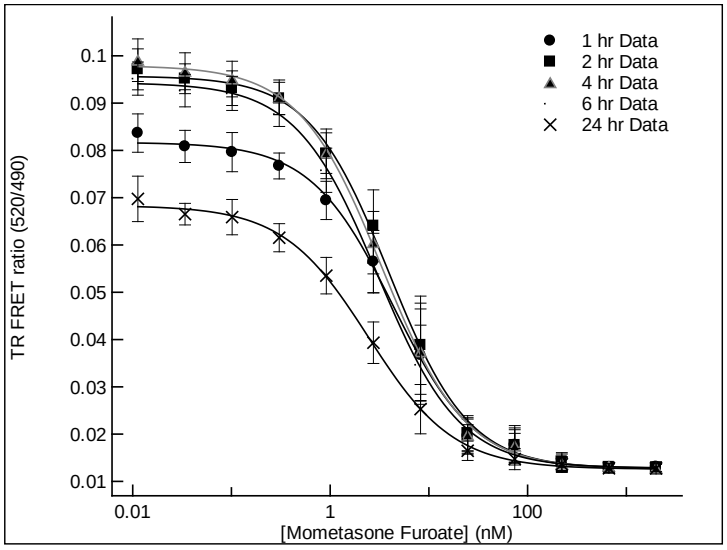
Assay Condition	Reagent Additions	Purpose
Test Compound	1. 10µL 2X Test Compound 2. 5 µL 4X Fluormone™ GS1 Green 3. 5 µL 4X GR-LBD (GST)/Tb-anti-GST Ab	Assess competition by test compound of interest using a single concentration or dilution series.
Positive Control	1. 10 µL 2X Control Competitor 2. 5 µL 4X Fluormone™ GS1 Green 3. 5 µL 4X GR-LBD (GST)/Tb-anti-GST Ab	Represents 100% tracer displacement (minimum TR-FRET ratio) by a known GR ligand. We recommend using 20 µM dexamthasone or mometasone furoate as the Control Competitor.
Negative Control	1. 10 µL 2X Test Compound Solvent 2. 5 µL 4X Fluormone™ GS1 Green 3. 5 µL 4X GR-LBD (GST)/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. 10 µL 2X Test Compound Solvent 2. 5 µL 4X Fluormone™ GS1 Green 3. 5 µL 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.*

***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 (GS1 Green) 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. The concentrations of GS1 Green used in this assay would result in minimal diffusion enhanced TR-FRET.

6.5 Ligand Assay—Representative Data

Figure 3. Representative experiment of LanthaScreen® TR-FRET GRCompetitive Binding assay. Assay composition: serial dilution of mometasone furoate (final 1% DMSO concentration), 1 nM GR-LBD (GST), 5 nM Fluormone™ GS1 Green, and 2 nM Tb anti-GST antibody. Results for 1-hour, 2-hour, 4-hour, and 6-hour incubations are shown with the corresponding IC_{50} values. The curves were generated using a sigmoidal dose response (variable slope) equation in IDBS XLfit® 4.2.

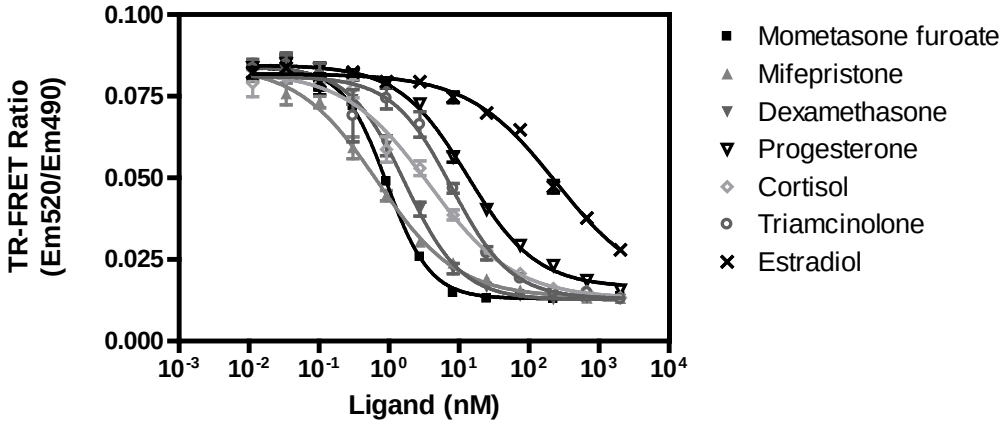


Time Point	IC_{50} (nM)
1 hour	4.34
2 hour	3.92
4 hour	3.34
6 hour	3.04

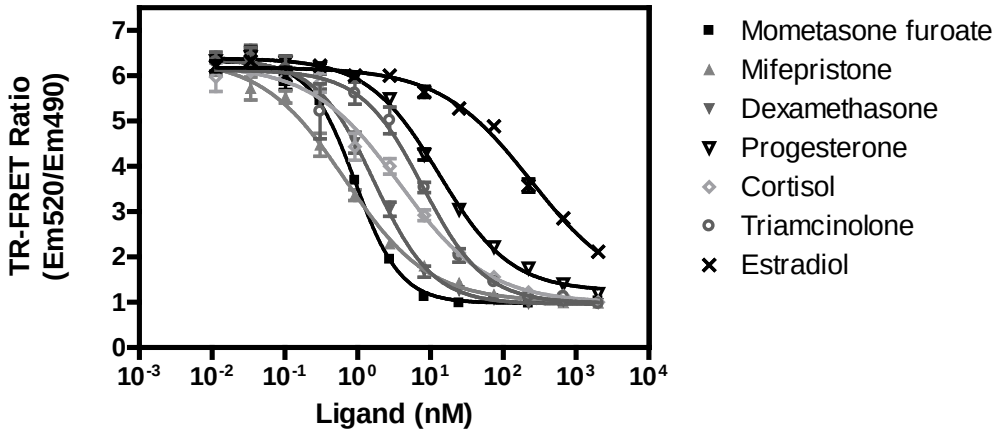
7. ASSAY PHARMACOLOGY

Figure 4: Relative IC_{50} Values of Selected Ligands for GR-LBD (GST) in the LanthaScreen® TR-FRET GR Competitive Binding Assay. Serial dilutions of various test compounds (1% final DMSO concentration) were assayed (n = 4). Curves were fit using a sigmoidal dose-response equation (variable slope) in GraphPad Prism™ 4.0 software. Figures 4A and 4B are the same data plotted differently. The values plotted in 4B have been normalized by the minimum TR-FRET ratio obtained for mometasone furoate so that the emission ratio for mometasone furoate starts at 1.0. There is no effect on the Z′ or calculated IC_{50}'s.

A.



B.



8. REFERENCES

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

9. LIMITED PRODUCT WARRANTY

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at www.lifetechnologies.com/termsandconditions. If you have any questions, please contact Life Technologies at www.lifetechnologies.com/support.

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