LanthaScreen® TR-FRET AR Coactivator Assay

Catalog no. A15878 **Shipping Condition:** Dry Ice **Initial Storage:** Varies

Publication no. MAN0009996 **Revision** A.0

TABLE OF CONTENTS

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

Thermo Fisher Scientific Inc. • 5791 Van Allen Way • Carlsbad, CA 92008 • Phone: 760 603 7200 • FAX: 760 602 6500
www.lifetechnologies.com

1. KIT CONTENTS

The LanthaScreen® TR-FRET Androgen Receptor (AR) Coactivator Assay, catalog no. A15878, contains the following:

Component	Composition	Amount	Storage Temperature	Individual Part no.
Fluorescein-D11FxxLF peptide (He *et al.*, 2002)	100 µM in 50 mM HEPES buffer, pH 7.5 Sequence: VESGSSRFMQLFMANDLLT	100 µL	–20°C	PV4382
AR-LBD (GST)	Rat AR ligand-binding domain in a buffer (pH 7.5) containing protein stabilizing reagents and glycerol. See Certificate of Analysis for the recommended molar concentration for this kit.	100 µg	–80°C	A15676
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 A	Proprietary buffer (pH 7.5) including 20% glycerol	2 × 25 mL	4°C	PV4384
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 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®, Cat. no. 4514). Other plate types may give satisfactory results as well
- 96-well polypropylene plate that can accommodate a 400-µL volume per well. We recommend Nalge Nunc, Cat. no. 49944.
- A known AR agonist, such as dihydrotestosterone (DHT), to serve as a positive control in agonist mode. We recommend Sigma, Cat. no. A8380.
- A known AR antagonist, such as cyproterone acetate (CPA), to serve as positive control in antagonist mode. We recommend Sigma, Cat. no. C3412.
- DMSO to perform serial dilutions. We recommend Sigma, Cat. no. 41647.

3. INTRODUCTION

The LanthaScreen® TR-FRET AR Coactivator Assay provides a sensitive and robust method for high-throughput screening of potential AR ligands as agonists or antagonists of ligand-dependent coactivator recruitment. The kit uses a terbium-labeled anti-GST antibody, a fluorescein-labeled coactivator peptide, and a rat AR ligand-binding domain (LBD) that is tagged with hexa-histidine and glutathione-S-transferase (GST) in a homogenous mix-and-read assay format. This kit contains enough reagents for 800 × 20-µL assays.

3.1 Principle of FRET and TR-FRET

For screening libraries of compounds, time-resolved FRET (TR-FRET) is a recognized method for overcoming interference from compound autofluorescence or light scatter from precipitated compounds. The premise of a TR-FRET assay is the same as that of a standard FRET assay: when a suitable pair of fluorophores is brought within close proximity of one another, excitation of the first fluorophore (the donor) can result in energy transfer to the second fluorophore (the acceptor). This energy transfer is detected by an increase in the fluorescence emission of the acceptor and a decrease in the fluorescence emission of the donor. In HTS assays, FRET is often expressed as a ratio of the intensities of the acceptor and donor fluorophores. The ratiometric nature of such a value corrects for differences in assay volumes between wells and corrects for quenching effects due to colored compounds.

In contrast to standard FRET assays, TR-FRET assays use a long-lifetime lanthanide chelate as the donor species. Lanthanide chelates are unique in that their excited-state lifetime (the average time that the molecule spends in the excited state after accepting a photon) can be on the order of a millisecond or longer. This is in sharp contrast to the lifetime of common fluorophores used in standard FRET assays, which are typically in the nanosecond range. Because interference from autofluorescent compounds or scattered light 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.

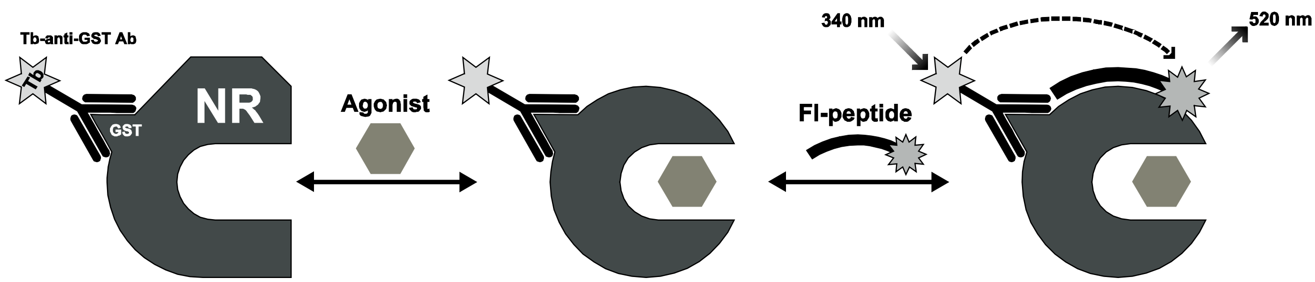
3.2 Assay Overview

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 antagonist is bound to these nuclear receptors, helix 12 adopts a conformation that precludes coactivator peptide binding, and a decrease in TR-FRET is observed.

When running the LanthaScreen® TR-FRET AR Coactivator Assay in agonist mode (to identify agonist compounds), AR-LBD (GST) 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 AR-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, next page).

When the LanthaScreen® TR-FRET AR Coactivator Assay is run in antagonist mode (to identify antagonist compounds), AR-LBD (GST) is added to ligand test compounds followed by addition of a mixture of agonist, fluorescein-coactivator peptide, and terbium anti-GST antibody. The concentration of agonist used in this mode is the EC_{80} concentration as determined by first running the assay in agonist mode.

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.



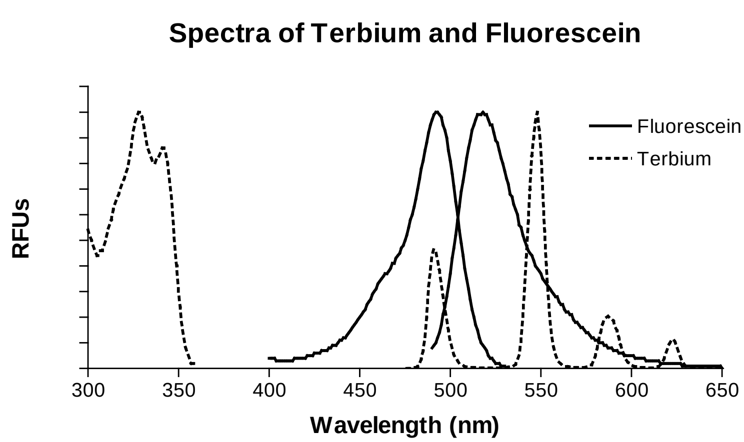
4. INSTRUMENT SETTINGS

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, 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 Figure 2, 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® TR-FRET filter optical module is available directly from BMG LABTECH® for use on the BMG LABTECH® PHERAstar® instrument.

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, BMG LABTECH® PHERAstar®, Molecular Devices® Analyst®, or PerkinElmer® EnVision® instruments. LanthaScreen® assays have also been performed successfully on the Tecan® Safire2™ and Molecular Devices® M5 monochromator-based instrument.

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. 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.

5. GUIDELINES AND RECOMMENDATIONS

5.1 Reagent Handling

AR-LBD (GST)

Store AR-LBD (GST) at –80°C. For best results, thaw on ice for 10 minutes before use. Once thawed, keep on ice and perform all dilutions while on ice. In concentrated stock solutions, AR-LBD is unstable at temperatures >4°C. Never vortex the AR-LBD stock solution or dilutions. This reagent is stable up to at least 8 freeze/thaw cycles.

TR-FRET Coregulator Buffer A

Thaw TR-FRET Coregulator Buffer A 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 room temperature.

LanthaScreen® Tb anti-GST Antibody

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

Fluorescein-D11FxxLF Coactivator peptide

Store fluorescein-D11FxxLF 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 in agonist mode using three additions per well in which AR-LBD (GST) was added to agonist dilutions, followed by the addition of pre-mixed fluorescein-D11FxxLF peptide and Tb anti-GST antibody (agonist, receptor, peptide/antibody). A pre-mixture of AR-LBD (GST), fluorescein-D11FxxLF 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.

In antagonist mode, we recommend adding AR-LBD (GST) to antagonist dilutions followed by addition of a mixture of agonist/fluorescein-D11FxxLF peptide/Tb anti-GST antibody, for a total of 3 additions per well (antagonist, receptor, peptide/antibody/agonist).

Thermo Fisher Scientific Inc. • 5791 Van Allen Way • Carlsbad, CA 92008 • Phone: 760 603 7200 • FAX: 760 602 6500
www.lifetechnologies.com

5.5 Incubation Conditions

Incubation Time

The incubation time can be set by the user. As a guide, results for various time points using a DHT agonist and CPA antagonist are shown in Table 1 below. In agonist mode, the EC_{50} and Z′ were stable from 2 to 24 hours. However, when the assay was run in antagonist mode, the Z′ was >0.5 only between 2 and 4 hours (Table 1).

Table 1. Effect of Incubation Time on Assay Performance. Sample data represents mean values from 3 separate experiments (n = 24). 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 software (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.

	Agonist Mode		Antagonist Mode	
Incubation Time (hours)	EC_{50} DHT	Z′-Factor	IC_{50} CPA	Z′-Factor
1	2.5 nM	0.27	160 nM	0.20
2	2.2 nM	0.73	200 nM	0.50
4	2.2 nM	0.78	350 nM	0.51
6	2.2 nM	0.79	480 nM	0.47
24	2.5 nM	0.74	1500 nM	0.27

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-D11FxxLF peptide to AR-LBD (GST). The only variable is the agonist concentration. All other assay components (AR-LBD (GST), 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-D11FxxLF	500 nM
Tb anti-GST antibody	5 nM
AR-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 A and Agonist Controls

Note: The AR-LBD (GST) should be thawed on ice just prior to use. Equilibrate all other assay components to room temperature.

1. Prepare Complete TR-FRET Coregulator Buffer A by adding 1 M DTT to TR-FRET Coregulator Buffer A for a final concentration of 5 mM DTT. Complete TR-FRET Coregulator Buffer A must be prepared fresh daily.

   For example: Add 30 µL of 1 M DTT to 5.97 mL of TR-FRET Coregulator Buffer A.

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

2. For the "no agonist" controls, add DMSO to Complete TR-FRET Coregulator Buffer A 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 A.

3. Prepare a solution of control agonist (we recommend DHT) 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 A. 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 A.

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 A2–A12.

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

   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 A 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 AR-LBD (GST)

8. Prepare 4X AR-LBD (GST) using **cold** Complete TR-FRET Coregulator Buffer A from step 1. The recommended molar concentration of AR for this kit is listed on the Certificate of Analysis. ***Never vortex the AR-LBD (GST) 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 AR-LBD (GST) has a stock concentration of 380 nM and the recommended concentration for this kit is 5 nM, prepare a 4X solution at 20 nM by adding 42 µL of AR-LBD (GST) stock to 756 µL of **cold** Complete TR-FRET Coregulator Buffer A.

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

Prepare 4X Fluorescein-D11FxxLF/4X Tb anti-GST Antibody

10. Prepare a solution containing 2 µM fluorescein-D11FxxLF (4X) and 20 nM Tb anti-GST antibody (4X) using Complete TR-FRET Coregulator Buffer A at room temperature. The stock concentration of fluorescein-D11FxxLF 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 16 µL of 100 µM fluorescein-D11FxxLF and 2.4 µL of 6.7 µM Tb anti-GST antibody to 782 µL of Complete TR-FRET Coregulator Buffer A.

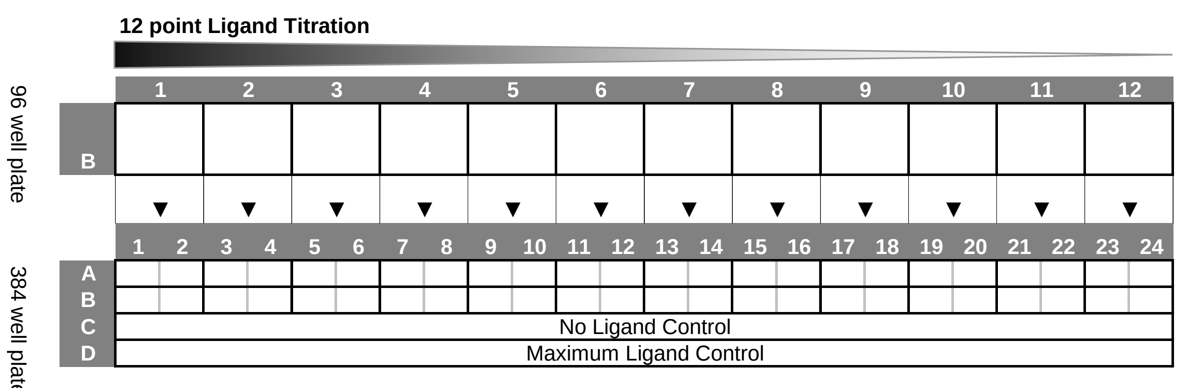
11. Add 5 µL of 4X peptide/4X antibody solution to rows A–D, columns 1–24 of the 384-well assay plate. (see the plate layout in **Section 6.2)**

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 2 and 24 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 software or another comparable graphing program. To calculate the EC_{80} value for performing an antagonist assay, see **Section 7.1**.

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.

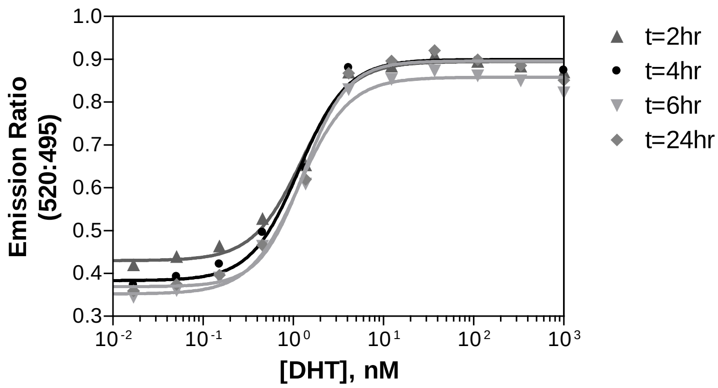
6.4 Agonist Assay—Reagent Volumes and Controls

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 AR-LBD (GST) 3. 5 µL 4X Fl-D11FxxLF/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 AR-LBD (GST) 3. 5 µL 4X Fl-D11FxxLF/Tb anti-GST Ab	Assess coactivator recruitment upon binding of a known AR agonist.
Negative Control	1. 10 µL 2X Compound solvent 2. 5 µL 4X AR-LBD (GST) 3. 5 µL 4X Fl-D11FxxLF/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 AR-LBD (GST) Control	1. 10 µL 2X compound solvent 2. 5 µL Complete TR-FRET Coregulator Buffer A 3. 5 µL 4X Fl-D11FxxLF/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

Figure 3. Representative experiment of LanthaScreen® TR-FRET AR Coactivator assay run in agonist mode. Assay composition: serial dilution of agonist DHT, 5 nM AR-LBD (GST), 500 nM Fluorescein-D11FxxLF, and 5 nM Tb anti-GST antibody. Results for 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 software.



Time Point	EC_{50}
2 hour	1.2 nM
4 hour	1.2 nM
6 hour	1.2 nM
24 hour	1.3 nM

7. ANTAGONIST ASSAY

This procedure describes a method for determining the IC_{50} of an antagonist (n = 4) and the Z'-factor for maximum antagonist and no antagonist controls (n = 24) using the antagonist competition of agonist-induced recruitment of fluorescein-D11FxxLF peptide to AR-LBD (GST). The only variable is the antagonist concentration. All other assay components (AR-LBD (GST), peptide, Tb anti-GST antibody, and EC_{80} agonist concentrations) must be fixed in the assay, as shown in the following table. Figure 4 was generated using a method adapted from this procedure.

Component	Final Assay Concentration
Fluorescein-D11FxxLF	500 nM
Tb anti-GST antibody	5 nM
Agonist concentration	EC_{80} calculated from assay performed in agonist mode
AR-LBD (GST)	See Certificate of Analysis for the recommended molar concentration for this kit

Note: We recommend determining the EC_{80} using your assay conditions in agonist mode. Although an agonist concentration greater than the EC_{80} will give a larger assay window, the sensitivity of the assay as defined by the ability to identify antagonists will be compromised.

7.1 Calculating the EC_{80} from the Agonist Assay

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

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

7.2 Antagonist Assay—Procedure

Note: The AR-LBD (GST) should be thawed on ice just prior to use. Equilibrate all other assay components to room temperature.

Prepare Complete TR-FRET Coregulator Buffer A and Antagonist Controls

1. Prepare Complete TR-FRET Coregulator Buffer A by adding 1 M DTT to TR-FRET Coregulator Buffer A for a final concentration of 5 mM DTT. Complete TR-FRET Coregulator Buffer A must be prepared fresh daily.

   For example: Add 30 µL of 1 M DTT to 5.97 mL of TR-FRET Coregulator Buffer A.

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

2. For the "no antagonist" controls (in the presence of EC_{80} agonist), add DMSO to Complete TR-FRET Coregulator Buffer A for a final concentration of 2% DMSO. Add 10 µL of this solution to row C, columns 1–24 of a 384-well assay plate (see the plate layout in **Section 7.3**)

   For example: Add 10 µL of DMSO to 490 µL of Complete TR-FRET Coregulator Buffer A.

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

   For example: If the final desired maximum starting concentration of antagonist is 50 µM, prepare a solution of 5 mM antagonist in DMSO.

4. For the "maximum antagonist" controls, dilute the 100X antagonist solution from step 3 to 2X using Complete TR-FRET Coregulator Buffer A. 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 TR-FRET Coregulator Buffer A.

Prepare 2X Antagonist Titration

5. Prepare a 12-point 100X dilution series of antagonist by serially diluting the 100X antagonist solution from step 3 five-fold using DMSO.

   For example: Add 20 µL of DMSO to wells A2–A12 in a 96-well polypropylene plate. To well A1, add 25 µL of the 100X antagonist solution prepared in step 3. Perform a five-fold serial dilution by transferring 5 µL of the 100X antagonist 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 antagonist serial dilution from step 5 to 2X using Complete TR-FRET Coregulator Buffer A.

   For example: Transfer 5 µL of each of the 100X antagonist 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 A 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 antagonist serial dilutions to duplicate columns of rows A and B of a 384-well assay plate as shown in the plate layout in **Section 7.3** (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 AR-LBD (GST)

8. Prepare 4X AR LBD (GST) using **cold** Complete TR-FRET Coregulator Buffer A. The recommended molar concentration of AR for this kit is listed on the Certificate of Analysis. ***Never vortex the AR-LBD (GST) 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 AR-LBD (GST) has a stock concentration of 380 nM and the recommended concentration for this kit is 5 nM, prepare a 4X solution at 20 nM by adding 42 µL of AR-LBD (GST) stock to 756 µL of **cold** Complete TR-FRET Coregulator Buffer A.

9. Add 5 µL of 4X AR-LBD (GST) to rows A–D, columns 1–24 of the 384-well assay plate. (see the plate layout in **Section 7.3)**.

Prepare 4X Fluorescein-D11FxxLF/4X Tb anti-GST Antibody/4X EC_{80} Agonist

10. Prepare a 100X solution of agonist in DMSO.

    For example: If the EC_{80} of the agonist is 5 nM, prepare a 100X solution at 500 nM in DMSO.

Note: A 100X solution of agonist results in an overall final assay concentration of 2% DMSO for this antagonist mode protocol.

11. Prepare a solution containing 2 µM fluorescein-D11FxxLF (4X), 20 nM Tb anti-GST antibody (4X), and 4X of the agonist EC_{80} using Complete TR-FRET Coregulator Buffer A. The concentration of fluorescein-D11FxxLF 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 40 µL of the 100X solution, 20 µL of 100 µM fluorescein-D11FxxLF, and 3.0 µL of 6.7 µM Tb anti-GST antibody to 937 µL of Complete TR-FRET Coregulator Buffer A.

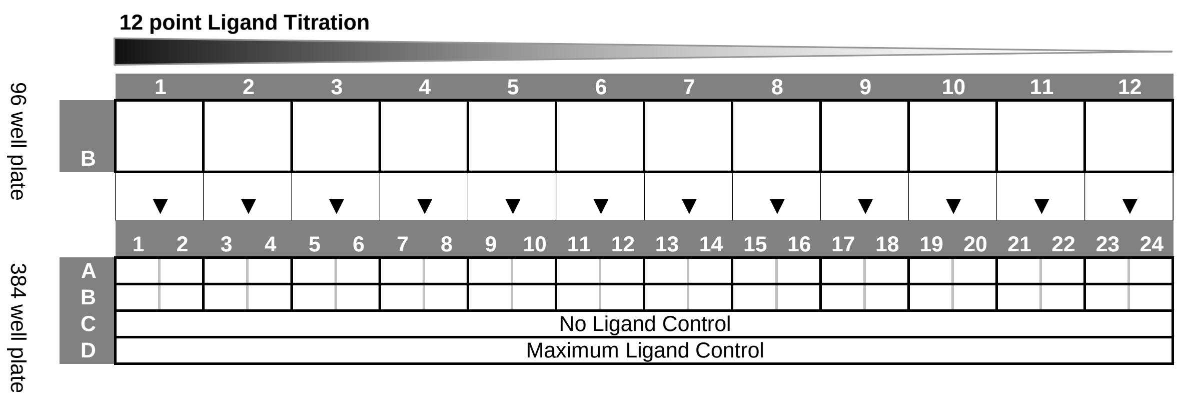
12. Add 5 µL of 4X peptide/4X antibody/4X EC_{80} agonist solution to rows A–D, columns 1–24 of the 384-well assay plate. (see the plate layout in **Section 7.3)**

Plate Incubation and Reads

13. 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.

14. Read the plate between 2 and 4 hours at wavelengths 520 nm and 495 nm, using the instrument settings 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 plate gently, and read. Then proceed to data analysis as described in the next section.

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 [ligand]. 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 software or other comparable graphing program.

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

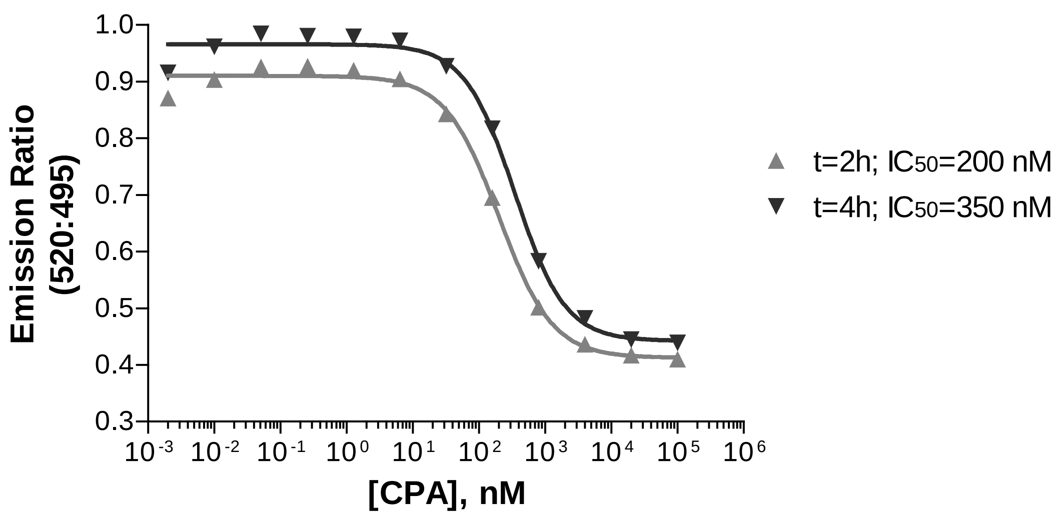
7.5 Antagonist Assay—Reagent Volumes and Controls

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 AR-LBD (GST) 3. 5 µL 4X Fl-D11FxxLF/Tb anti-GST Ab/agonist	Assess disruption of coactivator recruitment by competition of test compound and a known AR agonist for binding to AR.
Positive Control	1. 10 µL 2X CPA (or dilution series) 2. 5 µL 4X AR-LBD (GST) 3. 5 µL 4X Fl-D11FxxLF/Tb anti-GST Ab/agonist	Assess disruption of coactivator recruitment by competition of a known AR antagonist and a known AR agonist for binding to AR.
Negative control	1. 10 µL 2X Compound Solvent 2. 5 µL 4X AR-LBD (GST) 3. 5 µL 4X Fl-D11FxxLF/Tb anti-GST Ab/agonist	Provides maximum FRET ratio for the antagonist assay. Accounts for possible interference from a compound's vehicle solvent
No AR-LBD (GST)	1. 10 µL 2X CPA at maximum concentration 2. 5 µL Complete TR-FRET Coregulator Buffer A 3. 5 µL 4X Fl-D11FxxLF/Tb anti-GST Ab/agonist	Provides absolute bottom baseline for assay, not accounting for ligand-independent coactivator recruitment or diffusion enhanced FRET

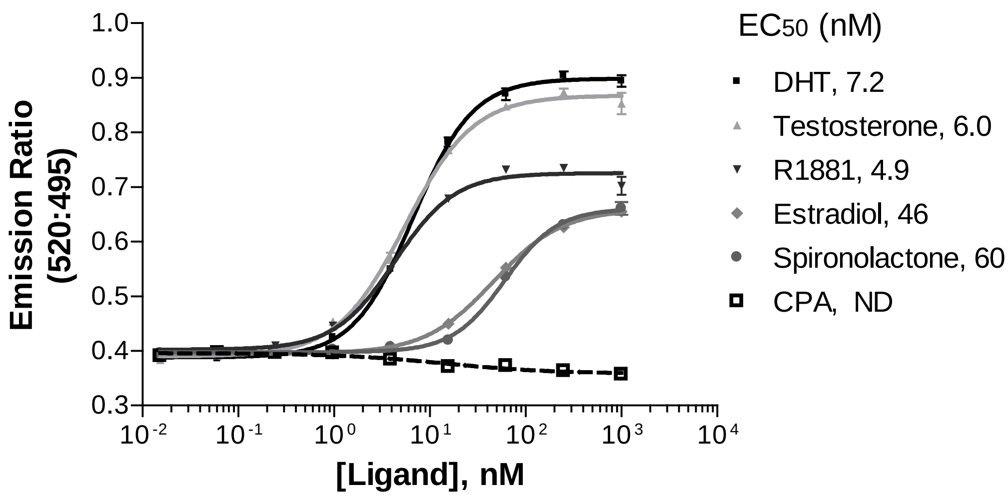
7.6 Antagonist Assay—Representative Data

Figure 4. Representative experiment of LanthaScreen® TR-FRET AR Coactivator assay run in antagonist mode. Assay Composition: dilutions of antagonist CPA, 5 nM AR-LBD (GST), 500 nM Fluorescein-D11FxxLF, 5 nM Tb anti-GST antibody, and 5 nM DHT. Results for 2-hour and 4-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 software.



8. ASSAY PHARMACOLOGY

Figure 5. Relative EC_{50} Values of Selected Ligands for AR-LBD (GST) in the LanthaScreen® TR-FRET AR 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 software. ND, not determined.



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

He, B., Minges, J. T., Lee, L. W., and Wilson, E. M. (2002) The FXXLF motif mediates androgen receptor-specific interactions with coregulators. *J. Biol. Chem.*, *277*, 10226-10235

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

10. 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.

11. PURCHASER NOTIFICATION

Important Licensing Information

This product may be covered by one or more Limited Use Label Licenses. By use of this product, you accept the terms and conditions of all applicable Limited Use Label Licenses.

DISCLAIMER

TRADEMARKS

Thermo Fisher Scientific Inc. • 5791 Van Allen Way • Carlsbad, CA 92008 • Phone: 760 603 7200 • FAX: 760 602 6500

www.lifetechnologies.com