LanthaScreen™ TR-FRET Coregulator Protocol and Assay Conditions

Revised 31-Jul-2017

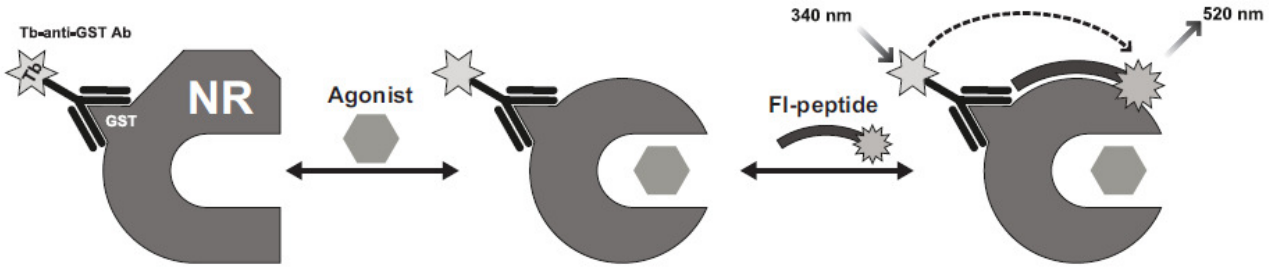
Assay Theory

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.

Binding of agonist to the nuclear receptor (Figure 1) causes a conformational change around helix 12 in the ligand binding domain, resulting in higher affinity for the coactivator peptide. When the terbium label on the anti-GST antibody is excited at 340 nm, energy is transferred to the fluorescein label on the coactivator peptide and detected as emission at 520 nm.

When running the LanthaScreen™ TR-FRET Coregulator Assay, Nuclear Receptor-LBD is added to ligand test compounds followed by addition of a mixture of the fluorescein-coregulator 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 EC50 from a dose response curve of the compound. Based on the biology of the Nuclear receptor-coregulator peptide interaction, this ligand EC50 is a composite value representing the amount of ligand required to bind to receptor, effect a conformational change, and recruit coregulator peptide.



LanthaScreen TR-FRET Nuclear Receptor Coregulator Assay Conditions

Test Compounds
The Test Compounds are screened in 1% DMSO (final) in the well. For 10-point titrations, 3 fold serial dilutions are conducted from the starting concentration of the customer's choosing.

Target/Antibody Mixtures
All Target/Antibody Mixtures are diluted to a 2X working concentration in the appropriate Assay Buffer.

Tracer
The 4X Fluorescein labeled Coregulator peptide is prepared in Assay Buffer.

Agonist Assay Protocol
Bar-coded Corning, low volume, black 384-well plate (Corning Cat. #3677 or #3676)
1. 4.0 µL –160 nL 100X Test Compound in 100% DMSO plus 3.84 µL Assay Buffer
2. 8.0 µL – 2X Target/Antibody Mixture
3. 4.0 µL – 4X Coregulator peptide
4. 2 hour incubation at room temperature
5. Read on fluorescence plate reader and analyze the data

Antagonist Assay Protocol
Bar-coded Corning, low volume, black 384-well plate (Corning Cat. #3677 or #3676)
1. 4.0 µL –160 nL 100X Test Compound in 100% DMSO plus 3.84 µL Assay Buffer or 4X EC80 concentration of agonist
2. 8.0 µL – 2X Target/Antibody Mixture
3. 4.0 µL – 4X Coregulator peptide
4. 2-4 hour incubation at room temperature
5. Read on fluorescence plate reader and analyze the data

LanthaScreen TR-FRET Nuclear Receptor Coregulator Assay Controls

The following controls are made for each individual target and are located on the same plate as the target:

0% Activation Control

The no activation control contains 1% DMSO in the place of agonist. In agonist mode, it is used to determine the lower end of the assay or 0% activation. In antagonist mode, it is used to determine maximal inhibition or 100% inhibition.

100% Activation Control

The full activation control contains 1% DMSO and a maximum concentration of the known agonist. In agonist mode, the full stim control is used to determine the upper end of the assay or 100% activation. In antagonist mode, the full stim control is used to determine the actual EC_{80} used in the assay, with the EC_{80} concentration chosen from previous agonist experiments.

EC_{80} Control (Antagonist Mode Only)

The EC_{80} control is a concentration of the known agonist in assay media that has been determined through an agonist mode experiment. In antagonist mode, the EC_{80} control is used to determine the actual baseline of activation or 0% inhibition.

Known Agonist (Agonist Mode) or Inhibitor (Antagonist Mode) Titration

A known activator or inhibitor control standard curve, 10-point titration, is run for each individual target on the same plate as the target to ensure the coregulator peptide is recruited within an expected EC_{50} range previously determined.

LanthaScreen TR-FRET Nuclear Receptor Coregulator Assay Data Analysis

The following equations are used for each set of data points:

	Equation
Emission Ratio (ER)	$\frac{\text{Fluorescein Emission (520 nm)}}{\text{Terbium Emission (495 nm)}}$
% Activation – Agonist Assays	$\left\{ \frac{ER_{\text{Sample\% Act Ctrl}} - ER_{\text{0\% Act Ctrl}}}{ER_{\text{100\% Act Ctrl}} - ER_{\text{0\% Act Ctrl}}} \right\} * 100$
% Inhibition – Antagonist Assays	$\left\{ 1 - \frac{ER_{\text{Compound}} - ER_{\text{0\% Act Ctrl}}}{ER_{\text{EC80Ctrl}} - ER_{\text{0\% Act Ctrl}}} \right\} * 100$
Z´ - Agonist Assays (using Emission Ratio values)	$1 - \frac{3*\text{Std Dev}_{\text{100\% Act Ctrl}} + 3*\text{Std Dev}_{\text{0\% Act Ctrl}}}{\text{Mean}_{\text{100\% Act Ctrl}} - \text{Mean}_{\text{0\% Act Ctrl}}}$
Z´ - Antagonist Assays (using Emission Ratio values)	$1 - \frac{3*\text{Std Dev}_{\text{EC80 Ctrl}} + 3*\text{Std Dev}_{\text{0\% Act Ctrl}}}{\text{Mean}_{\text{EC80 Ctrl}} - \text{Mean}_{\text{0\% Act Ctrl}}}$

Graphing Software

SelectScreen Biochemical Nuclear Receptor Profiling Service uses XL*fit* from IDBS. The dose response curve is curve fit to model number 205 (sigmoidal dose-response model). For antagonist mode only, if the bottom of the curve does not fit between -20% & 20% inhibition, it is set to 0% inhibition. If the top of the curve does not fit between 70% and 130% inhibition, it is set to 100% inhibition.

Specific Assay Conditions

Target	Target Conc (nM)	Buffer	Antibody	Antibody Conc (nM)	Coregulator Peptide	Coregulator Conc (nM)	Known Activator	EC50 (nM)	Known Inhibitor	IC50 (nM)
AR	56	NRA	Tb-anti-GST	5	D11-FXXLF	500	R1881	2.5	Cyproterone acetate	696.87
CAR	10	NRG	Tb-anti-GST	5	PGC1a	125	CITCO	35.4	Clotrimazole	259.39
ER-alpha	7.3	NRE	Tb-anti-GST	5	PGC1a	250	17-beta-Estradiol	1.8	4-hydroxytamoxifen	8
ER-beta	7	NRE	Tb-anti-GST	5	PGC1a	250	17-beta-Estradiol	4	4-hydroxytamoxifen	12
ERR-alpha	5	NRG	Tb-anti-GST	5	PGC1a	500	None	---	XCT790	11.9
ERR-beta	10	NRG	Tb-anti-GST	5	PGC1a	250	None	---	4-hydroxytamoxifen	247
ERR-gamma	5	NRB	Tb-anti-GST	5	PGC1a	500	None	---	4-hydroxytamoxifen	130
FXR	10	NRG	Tb-anti-GST	5	SRC2-2	500	GW4064	103	None	---
GR	540	NRF	Tb-anti-GST	5	SRC1-4	300	Mometasone Furoate	2.707	Mifepristone	112.4
LXR-alpha	2.5	NRH	Tb-anti-GST	10	TRAP220/DRIP2	250	T0901317	4.5	None	---
LXR-beta	5	NRI	Tb-anti-GST	10	D22	100	T0901317	7.5	None	---
PPAR-alpha	5	NRJ	Tb-anti-GST	5	PGC1a	250	GW7647	2.6	GW9662	726.77
PPAR-delta	5	NRJ	Tb-anti-GST	10	C33	100	GW501516	18	None	---
PPAR-gamma	5	NRF	Tb-anti-GST	5	TRAP220/DRIP2	125	GW1929	9.1401	GW9662	240
PR	30	NRF	Tb-anti-GST	5	SRC1-4	250	Progesterone	7.7	Mifepristone	4.6
RAR-alpha	3.5	NRD	Tb-anti-GST	5	D22	50	ATRA	1.7191	None	---
RAR-beta	2.5	NRE	Tb-anti-GST	5	SRC2-2	125	ATRA	2.1	AGN193109	44.687
RAR-gamma	3	NRC	Tb-anti-GST	5	PGC1a	250	ATRA	2.5	None	---
RXR-alpha	10	NRG	Tb-anti-GST	5	PGC1a	500	9-cis-RA	34.1	None	---
RXR-beta	6	NRA	Tb-anti-GST	2	D22	350	9-cis-RA	52.848	None	---
TR-alpha	0.25	NRC	Tb-anti-GST	2	SRC2-2	200	T3	0.2	None	---

TR-beta	0.5	NRC	Tb-anti-GST	2	SRC2-2	200	T3	0.25	None	---
VDR	0.8	NRG	Tb-anti-GST	2	TRAP220/DRIP2	100	Calcitrol	0.4	None	---

Buffer NRA: TR-FRET Coregulator Buffer A (PV4384) + 5 mM DTT (P2325)
Buffer NRB: TR-FRET Coregulator Buffer B (PV4418) + 5 mM DTT (P2325)
Buffer NRC: TR-FRET Coregulator Buffer C (PV4419) + 5 mM DTT (P2325)
Buffer NRD: TR-FRET Coregulator Buffer D (PV4420) + 5 mM DTT (P2325)
Buffer NRE: TR-FRET Coregulator Buffer E (PV4540) + 5 mM DTT (P2325)
Buffer NRF: TR-FRET Coregulator Buffer F (PV4547) + 5 mM DTT (P2325)
Buffer NRG: TR-FRET Coregulator Buffer G (PV4553) + 5 mM DTT (P2325)
Buffer NRH: TR-FRET Coregulator Buffer H (PV4661) + 5 mM DTT (P2325)
Buffer NRI: TR-FRET Coregulator Buffer I (PV4662) + 5 mM DTT (P2325)
Buffer NRJ: TR-FRET Coregulator Buffer J (PV4682) + 5 mM DTT (P2325)