LanthaScreen™ TR-FRET Competitive Binding Assay Screening Protocol and Assay Conditions

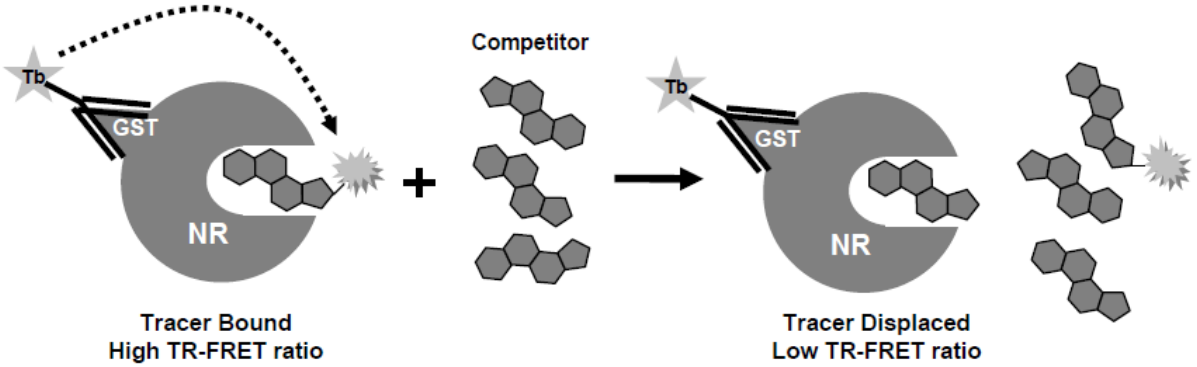
Revised 30-Nov-2016

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.

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 use of radioactivity and enables a homogenous format.



LanthaScreen TR-FRET Competitive Binding 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 Tracer is prepared in Assay Buffer.

Assay Protocol
Bar-coded Corning, low volume, black 384-well plate (Corning Cat. #4511 or #4514)
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 Tracer
4. 60-minute incubation at room temperature
5. Read on fluorescence plate reader and analyze the data

LanthaScreen TR-FRET Competitive Binding Assay Controls

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

0% Displacement Control
The maximum Emission Ratio is established by the 0% Displacement Control wells, which do not contain known inhibitor in the reaction and therefore exhibits no displacement of the tracer.

100% Displacement Control
The minimum Emission Ratio is established by the 100% Displacement Control wells, which contain the highest concentration of the known inhibitor used in that assay.

Known Inhibitor
A known inhibitor control standard curve, 10 point titration, is run for each individual target on the same plate as the target to ensure the inhibitor is displaced within an expected IC_{50} range previously determined.

LanthaScreen TR-FRET Competitive Binding Assay Data Analysis

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

	Equation
Emission Ratio (ER)	$\frac{\text{Fluorescein Emission (520 nm)}}{\text{Terbium Emission (495 nm)}}$
% Displacement	$\left\{ \frac{ER_{0\%\ Disp\ Ctrl} - ER_{Sample}}{ER_{0\%\ Disp\ Ctrl} - ER_{100\%\ Disp\ Ctrl}} \right\} * 100$
Difference Between Data Points (single point only)	$\lvert \%\ Displacement_{Point\ 1} - \%\ Displacement_{Point\ 2} \rvert$
Test Compound Interference	For each emission wavelength, fluorescence interference is flagged for a compound well that is more than 20% outside the range of the controls.
Z′ (using Emission Ratio values)	$1 - \frac{3 * Stdev_{0\%\ Disp\ Ctrl} + 3 * Stdev_{100\%\ Disp\ Ctrl}}{\lvert Mean_{0\%Disp\ Ctrl} - Mean_{100\%\ Disp\ Ctrl} \rvert}$

Graphing Software

SelectScreen Profiling Service uses XL*fit* from IDBS. The dose response curve is curve fit to model number 205. 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)	Antibody	Antibody Conc (nM)	Tracer	Tracer Conc (nM)	Tracer Kd (nM)	Buffer	Known Inhibitor	IC50 (nM)
ER-alpha	2.1	Tb-anti-GST	2	Fluor. ES2 Green	3	3.5	NRK	17-beta-Estradiol	0.600
ER-beta	0.9	Tb-anti-GST	2	Fluor. ES2 Green	3	1.7	NRK	17-beta-Estradiol	0.620
GR	1080	Tb-anti-GST	2	Fluor. GS1 Green	5	2	NRN	Dexamethasone	7.38
PPAR-alpha	2	Tb-anti-GST	5	Pan-PPAR	20	50	NRL	GW7647	3.00
PPAR-delta	2	Tb-anti-GST	5	Pan-PPAR	20	14	NRL	GW0742	0.600
PPAR-gamma	0.5	Tb-anti-GST	5	Pan-PPAR	5	2.8	NRL	GW1929	0.770
PXR (SXR)	5	Tb-anti-GST	10	Fluor. PXR Green	40	400	NRM	SR-12813	250

Kinase Buffer NRK: TR-FRET Coregulator Buffer K, 5 mM DTT
Kinase Buffer NRL: TR-FRET PPAR Assay Buffer, 5 mM DTT
Kinase Buffer NRM: TR-FRET PXR (SXR) Assay Buffer, 5 mM DTT
Kinase Buffer NRN: TR-FRET Coregulator Buffer K, 1X GR stabilizing peptide, 5 mM DTT