Test Your Plate Reader Set-up Before Using LanthaScreen® Tb Assays

Purpose

This technical note provides a method for verifying that a fluorescent plate reader is able to detect a change in time-resolved fluorescence energy transfer (TR-FRET) signal, confirming proper instrument set-up and a suitable response. The method is independent of any biological reaction or equilibrium and uses reagents that are on-hand for the LanthaScreen® assay.

At a Glance

Step 1: Go to www.invitrogen.com/instrumentsetup to obtain the specific set-up guide for your instrument.

Step 2: Prepare individual dilutions of the TR-FRET acceptor (fluorescein labeled substrate).
2X = 10,000 nM, 5,000 nM, 2,500 nM, 1,250 nM and 400 nM.

Note: This application is NOT suited for LanthaScreen® GFP-tagged physiological substrates or Nuclear Receptor Fluormores™. Instead, use Fluorescein-poly GT, PV3610 as the acceptor.
Note: To avoid propagating dilution errors, we do NOT recommend using serial dilutions. See page 3.

Step 3: Prepare a dilution of the TR-FRET donor (Tb-Antibody)
2X = 125 nM Tb 3+ chelate.

Note: Concentration is based on the molarity of the Tb chelate (found on the Certificate of Analysis), NOT the molarity of the antibody, to account for normal variation in antibody labeling. See page 3 for calculations and method.

Step 4: Prepare plate and read.

Step 5: Contact Technical Support with your results. E-mail us directly at drugdiscoverytech@lifetech.com or in the US call 1-800-955-6288, select option 3, and enter 40266. We will determine Z'-factors by comparing each concentration of acceptor to the 200 nM acceptor data. Example results and data analysis are available on page 5.

Introduction

This LanthaScreen® Tb plate reader test uses diffusion-enhanced TR-FRET to generate a detectable TR-FRET signal. At high donor or acceptor concentrations, donor and acceptor diffuse to within a suitable distance from one another to allow TR-FRET to take place, resulting in a signal. The response in diffusion-enhanced TR-FRET is easy to control because it is directly proportional to the concentrations of donor and acceptor in solution and is not related to a binding event.

In this method, acceptor concentration varies while the donor concentration remains fixed. As the concentration of acceptor increases, the diffusion-enhanced TR-FRET signal increases. The signal from the acceptor concentrations are compared to the signal from the lowest acceptor concentration to simulate assay windows from high to low to help you assess whether your instrument is properly set-up and capable of detecting TR-FREt signals in LanthaScreen® Assays.

We designed the LanthaScreen® Tb technical note to use components and reagents that are generally used in most LanthaScreen® Assays. Please note that the LanthaScreen® GFP-tagged physiological substrates and Nuclear Receptor Fluormones™ tracers are not suited for this method. Instead, use Fluorescein-poly GT, PV3610 as the acceptor.

Materials Required

Component	Storage	Part Number
LanthaScreen® Tb-labeled antibody (donor)	-20°C	Various
LanthaScreen® fluorescein-labeled substrate (acceptor)	-20°C	Various
TR-FRET Dilution Buffer or any Nuclear Receptor Co-regulator Buffer	Various	PV3574 or Various

96-well plate

384-well plate (typically a white, low-volume Corning 3673 or black, low-volume Corning 3676)

1.5 mL micro-centrifuge tubes

Plate seals

Suitable single and multichannel pipettes

Plate reader capable of reading TR-FRET

Handling

To reread the plate on a different day, seal and store the plate at room temperature for up to 5 days. To reread the plate, centrifuge the plate at 300 x *g* for 1 minute, remove the seal and read.

Important: Prior to use, centrifuge the antibody tube at approximately 10,000 x g for 10 minutes, and carefully pipette the volume needed for the assay from the supernatant. This centrifugation can remove any aggregates present that can interfere with the signal.

Procedure

Step 1: Set up your instrument

Go to www.invitrogen.com/instrumentsetup to obtain the specific set-up guide for your instrument. These guides provide the filter wavelength and dichroic mirror specifications that differ among instruments.

Settings common to all Eu or Tb LanthaScreen® Assays

Note: The settings show here, optimized specifically for LanthaScreen® TR-FRET assays, may differ from those for other commercially available TR-FRET assays. For optimum results, use these settings.

Excitation	340 nm (30 nm bandpass)
Delay Time	100 µs
Integration Time	200 µs

Step 2: Prepare the Acceptor (LanthaScreen® fluorescein substrate or peptide)

Acceptor concentrations (at 2X) are individually prepared from a 30 µM stock to prevent propagation of error that can occur with serial dilutions. We suggest preparing 10 replicates for calculation of a Z'-factor. To accommodate replicates that use 10 µL per well, prepare 120 µL of each concentration. Prepare each concentration in micro-centrifuge tubes or a 96-well polypropylene plate and then transfer it to a 384-well plate.

Note: This method is NOT for use with the LanthaScreen® GFP-tagged physiological substrates or Nuclear Receptor FluormoneTM tracers. Instead, use Fluorescein-poly GT, PV3610, as the acceptor.

1. Prepare 30 µM acceptor stock solution:

Fluorescein Substrate/Peptide	Cat #	Stock Concentration	Dilution to prepare a 30 nM solution
Fluorescein-Poly GT	PV3610	30 µM	No dilution needed
Fluorescein-Poly GAT	PV3611	30 µM	No dilution needed
Fluorescein peptides for kinases	Various	Various	Add 6 µL of 1 mg/mL peptide stock to 94 µL of TR-FRET Dilution Buffer (1 mg/mL with a MW ~ 2kDa = ~500 µM)
Fluorescein co-regulator peptides for NRs	Various	100 µM	Add 30 µL of 100 µM peptide stock to 70 µL of TR-FRET Co-regulator Buffer. Do not add DTT.

2. Prepare 120 µL of each 2X acceptor concentration from the 30 µM stock:

96-well plate or tubes	A1	B1	C1	D1	EI
2X Acceptor Concentration	10,000 nM	5,000 nM	2,500 nM	1,250 nM	400 nM
Final 1X Acceptor Concentration	5,000 nM	2,500 nM	1,250 nM	625 nM	200 nM
Volume TR-FRET Dilution Buffer or NR Coregulator Buffer	80.0 µL	100.0 µL	110.0 µL	115 µL	117.5 µL
Volume 30 µM Acceptor (prepared above)	40.0 µL	20.0 µL	10.0 µL	5.0 µL	2.5 µL

Step 3: Prepare the Donor (Tb-chelate labeled antibody)

Prepare a 2X stock of Tb-chelate at 125 nM that will result in a final assay concentration of 62.5 nM. This method relies on the concentration of Tb-chelate, NOT the concentration of antibody. The lot-to-lot variation in the number of Tb-chelates covalently bound to antibody can be accounted for by referring to the Tb-chelate-to-antibody ratio listed on the lot-specific Certificate of Analysis for your antibody. Multiply this ratio by the antibody concentration to calculate the Tb-chelate concentration.

Example chelate concentrations

Antibody Concentration	Antibody Molarity	Chelate: Antibody Ratio	Chelate Concentration
0.5 mg/mL	3.3 µM	11	36.3 µM = 36,300 nM
0.25 mg/mL	1.7 µM	8	13.6 µM = 13,600 nM

Example Calculation: Prepare 1,000 µL of Tb-chelate:

Tb-antibody = 0.5 mg/mL (3.3 µM) with a chelate:antibody ratio of 11

Chelate: Stock = 3.3 µM x 11 = 36.3 µM = 36,300 nM.

1X = 62.5 nM; 2X = 125 nM

	V_1	x	C_1	=	V_2	x	C_2
			[Stock]				**[2X]**
Tb-Chelate	V_1	x	36,300 nM	=	1,000 µL	x	125 nM
	V_1 = **3.4** µL						

Add 3.4 µL of 36,300 nM stock to 996.6 µL TR-FRET dilution buffer or NR coregulator buffer.

Step 4: Add Reagents to the 384-well plate and read

1. Donor

Transfer 10 µL of 2X Tb-chelate to rows A through J and columns 1 through 5 of the 384-well assay plate. Since you need only a single concentration, you can transfer this solution with a multichannel pipette from a basin to all 50 wells. We recommend preparing the 1 mL solution in a 1.5 mL micro-centrifuge tube before transferring into the basin.

2. Acceptor

Note: To eliminate carryover, we recommend changing pipette tips for each concentration of acceptor.
Note: After adding 2X acceptor, mix the reagents by pipetting up and down.

Transfer 10 µL of the indicated concentration of 2X acceptor to the rows A-J of the corresponding column of the 384- well plate.

2X Acceptor	Column
10,000 nM	1
5,000 nM	2
2,500 nM	3
1,250 nM	4
400 nM	5

3. Read plate

This step does not require any equilibration time.

Step 5: Contact Technical Support

Send us your results by e-mailing us directly at drugdiscoverytech@lifetech.com or in the US call 1-800-955-6288, select option 3, and enter 40266.

We will help you evaluate your results by performing the following data analysis:

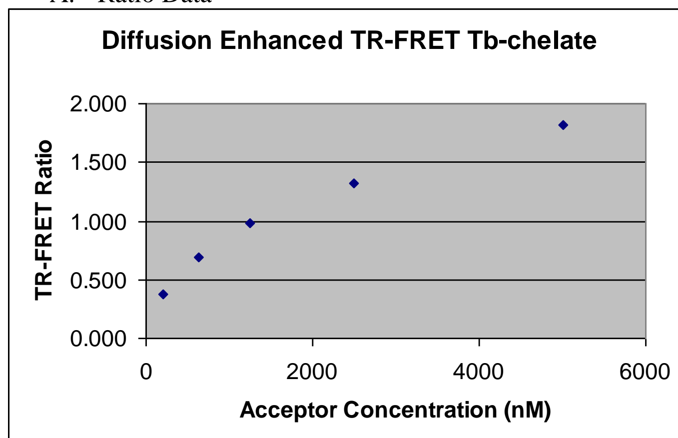
1. Obtain the emission ratios by dividing the acceptor signal (520 nm) by the donor signal (495 nm) for each well.

2. Calculate the average ratio for each column (1 through 5). These values can be plotted against the final 1X concentrations (5,000 nM, 2,500 nM, 1,250 nM, 625 nM, and 200 nM) of acceptor (see graph A). Dilution curves form diffusion-enhanced TR-FRET do not plateau and, therefore, do not fit the normal sigmoidal shape produced by binding curves.

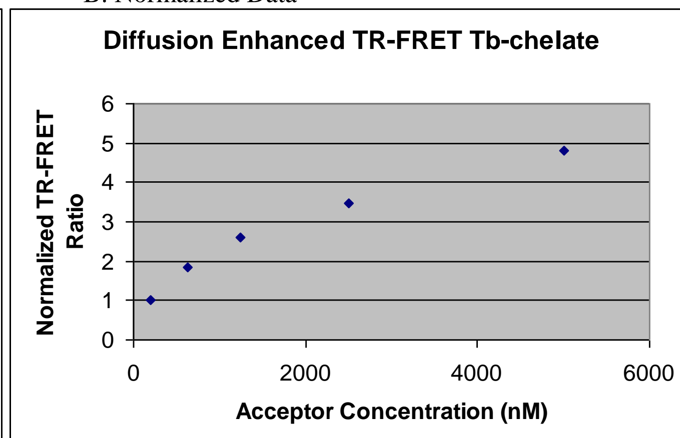
3. Using the data from column 5 (200 nM acceptor) as the bottom of the "assay window", divide the average ratios from the other columns b the average ratio from column 5 to obtain a range of simulated "assay window" sizes. See the example data below. This "normalized" data can be plotted against the acceptor concentration as shown below in graph B.

4. Calculate the Z'-factor for each "assay window". Very general guidance is that you should observe a satisfactory Z'-factor (>0.5) for at least the "small window" that compares columns 3 to 5 (1,250 nM to 200 nM). In our hands and on certain instruments, the data in columns 4 and 5 produces suitable Z'-factors (>0.5) with a simulated assay window of less than 2.

A. Ratio Data



B. Normalized Data



Columns Compared	Description
1 to 5	Largest window
2 to 5	Intermediate window
3 to 5	Small window
4 to 5	Smallest window, less than 2-fold

Example Data: Ratiometric data obtained on a BMG LABTECH PheraStar microplate reader.

[acceptor]	5,000 nM	2,500 nM	1,250 nM	625 nM	200 nM
Row A	1.89	1.40	1.01	0.720	0.378
Row B	1.78	1.31	0.970	0.704	0.378
Row C	1.80	1.32	0.983	0.698	0.382
Row D	1.83	1.27	0.978	0.699	0.378
Row E	1.81	1.31	0.968	0.684	0.377
Row F	1.80	1.34	0.975	0.694	0.378
Row G	1.78	1.36	0.974	0.700	0.377
Row H	1.82	1.36	0.956	0.691	0.371
Row I	1.80	1.17	0.966	0.05	0.379
Row J	1.87	1.35	1.01	0.671	0.390

Data Analysis:

[Acceptor]	5,000 nM	2,500 nM	1,250 nM	625 nM	200 nM
Average Ratio	1.82	1.32	0.979	0.696	0.379
St dev	0.04	0.06	0.02	0.01	0.00
% CV	2.1	4.7	1.9	1.9	1.2
Assay Window	**4.80**	**3.49**	**2.59**	**1.84**	**Reference**
Z'-factor	0.91	0.79	0.88	0.83	

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