LanthaScreen® Terbium Assay Setup Guide on the BMG LABTECH PHERAstar® *FSX* Microplate Readers

The BMG LABTECH PHERAstar® *FSX* Microplate Readers were tested for compatibility with LanthaScreen® Activity Assay, a TR-FRET assay from Thermo Fisher Scientific, using LanthaScreen® Fluorescein-labeled Poly GT (PV3610) and Tb-anti-GST Antibody (PV3550).

The following document is intended to demonstrate setup of this instrument for any Tb-based TR-FRET assay and provide representative data. For more detailed information and technical support of Thermo Fisher Scientific' assays please call 1-800-955-6288 ext. 40266. For more detailed information and technical support of BMG LABTECH instruments or software, please contact BMG LABTECH at 1-877-264-5227 or www.bmglabtech.com.

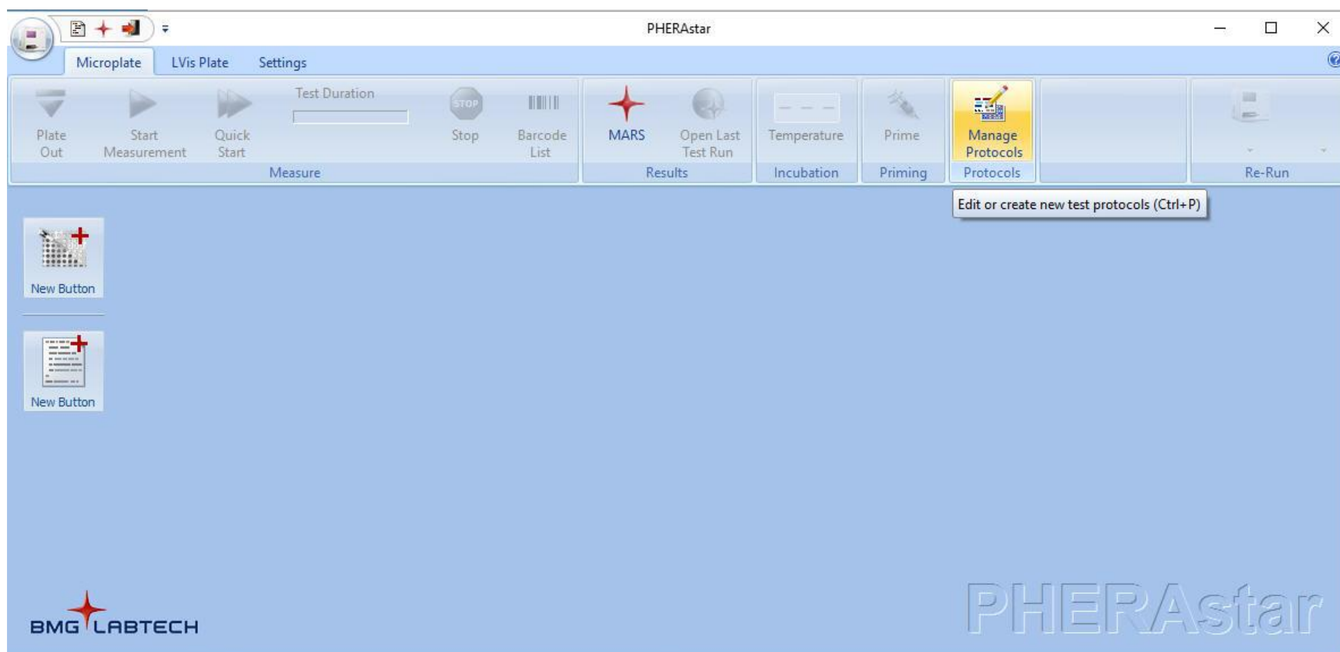
A. Recommended Optics

	Wavelength (nm)	BMG LABTECH Optic module
Excitation	337	LanthaScreen
Emission 1	520	LanthaScreen
Emission 2	490	LanthaScreen
Dichroic Mirror	Included	LanthaScreen

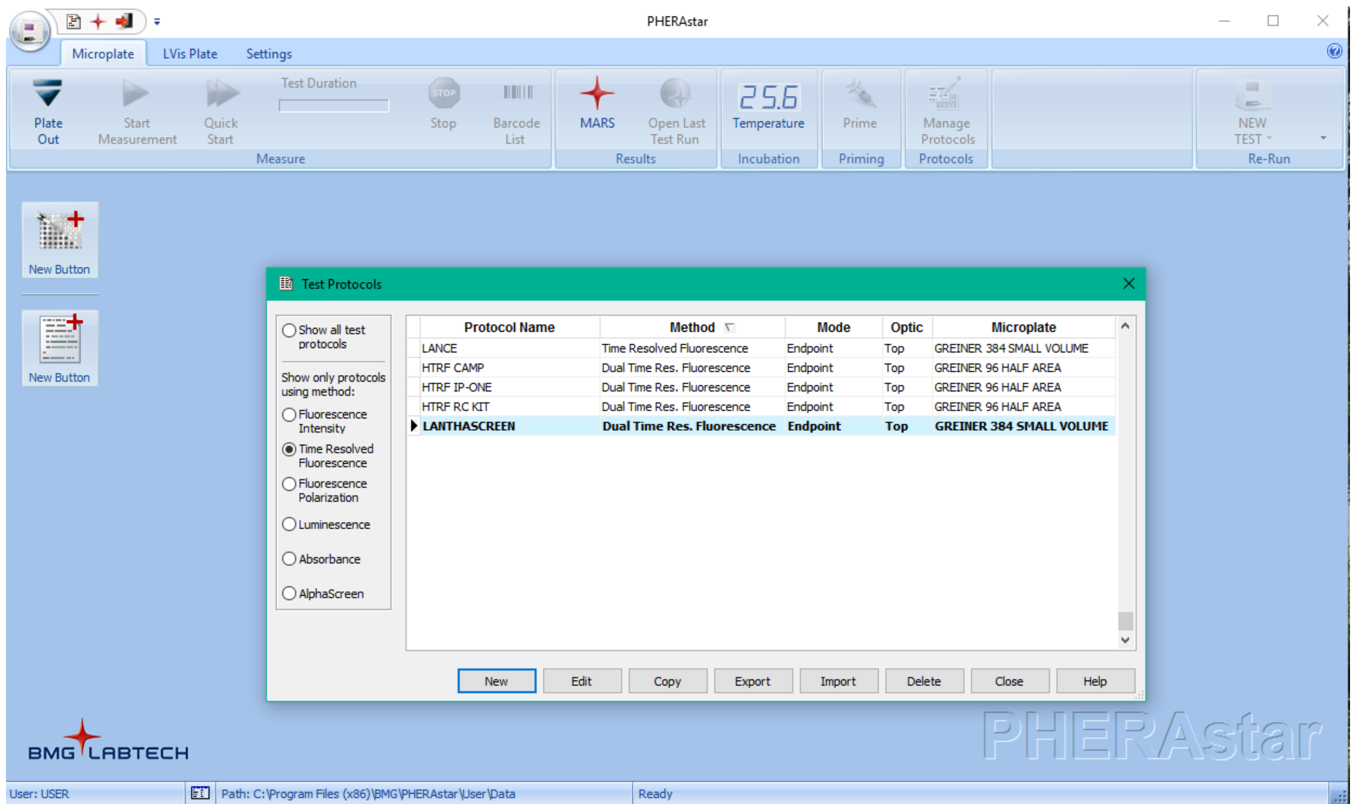
B. Instrument Setup

1. Make certain the plate reader is turned on and then open the PHERAstar® Control software on computer.

2. When PHERAstar® Control software opens, if you do not have a pre-existing protocol for LanthaScreen®, select "Manage Protocols" from the menu bar at the top portion of the window. If you do have a protocol, open the protocol. Use this document to review your settings.



3. At this point, a new screen will open (below). Click on the “Show all test protocols” or "Time Resolved Fluorescence" button on the left side of the screen, then select “New” from the tabs at the bottom.



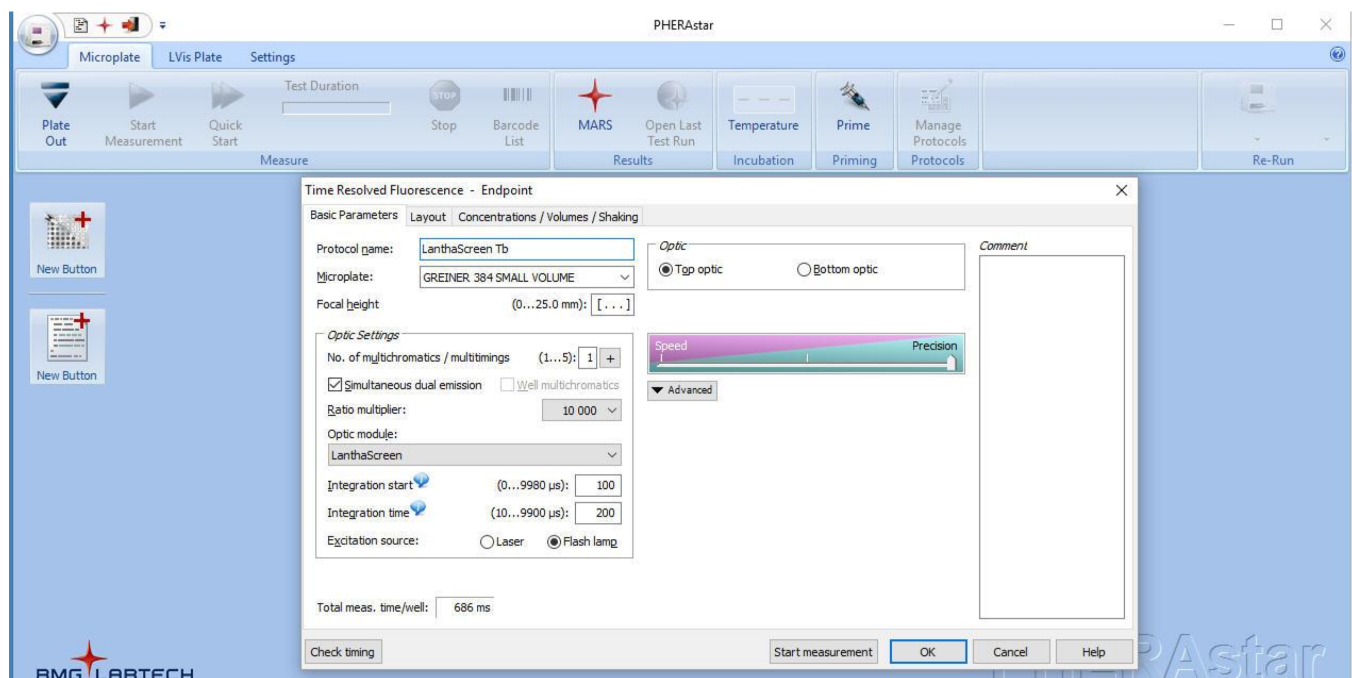
4. A new window will pop up. Select “Time-Resolved Fluorescence” and “Endpoint” and then select “OK.”

5. A new protocol window will open automatically. Enter a test name, select plate typ. We recommend Corning and Greiner 384 well low volume plate for LanthaScreen® Terbium assays. Because LanthaScreen® is a Time-Resolved FRET assay, set the Integration Start and Integration Time to 100 and 200 µseconds, respectively, as shown below. Check the “Simultaneous dual emission” box and select the “LanthaScreen” optic module form the “Optic Module” menu. Finally, select the desired “Excitation Source.

NOTE:

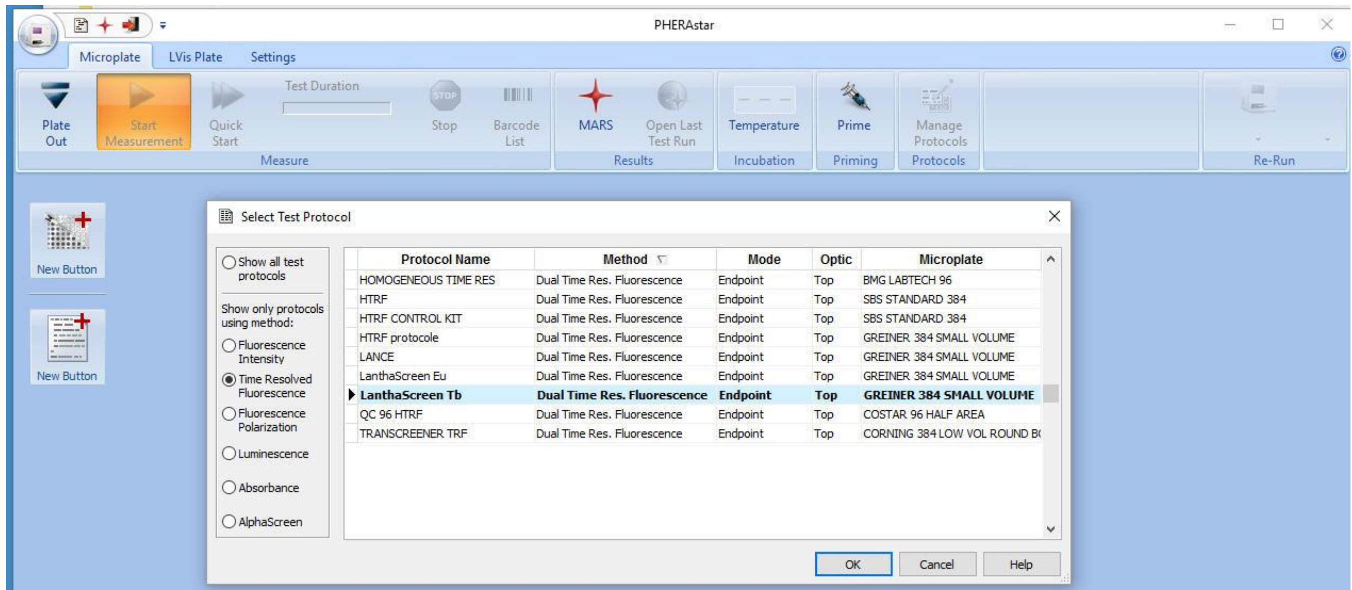
i) Under “Excitation Source” you may select either “Laser” or “Flash Lamp”, we have obtained similar results between the flash lamp and laser (data not shown).

i) The LanthaScreen optic module must be installed on the instrument. If it is not installed, there will not be the option to select it from the “Optic Module” menu.



6. Check the “Layout” tab at the top of the protocol window. Select the wells you wish to read. Note: in this step you can choose to designate blanks, positive controls, etc. For this case, we marked all wells "Sample" and calculations were performed manually. When finished, select “OK”.

7. You will return to the main window. From the menu bar at the top, select "Start Measurement." A new window will appear allowing you to select which test protocol(s) you wish to run. Select the protocol you created for LanthaScreen®, and then press “OK.”



8. A new window will appear. Before beginning the measurement of your samples, the focal height should be set to ensure the most optimal signal measurement of the samples. Ensure the plate is in the reader. The terbium signal is read through channel B. Select “Focus Adjustment” and “Channel B” then select a well containing a sample with terbium labeled antibody to use for adjusting focal height. Calibrate the height by clicking the “Start Adjustment” button. In a moment, the instrument will have calculated the optimal focal height. When finished, click on the "Start Measurement" tab to read.

9. When PHERAstar® has finished reading, you can view your data from the completed test by clicking “Open Last Test Run” on the toolbar at the top of the window. This will automatically redirect you to a MARS file which collects run data. Alternatively, you can open MARS and select your run of interest from the list to open and view data in a plate layout format.

10. Depending on the test protocol and plate layout selections, some calculations may have been performed. Raw data values are displayed below.

LANTHASCREEN - PHERAstar FSX MARS

Test Name: LANTHASCREEN
ID1: 472-0012; ID2: FL, 11.6
Time resolved fluorescence (dual emission)

Sum of range 1

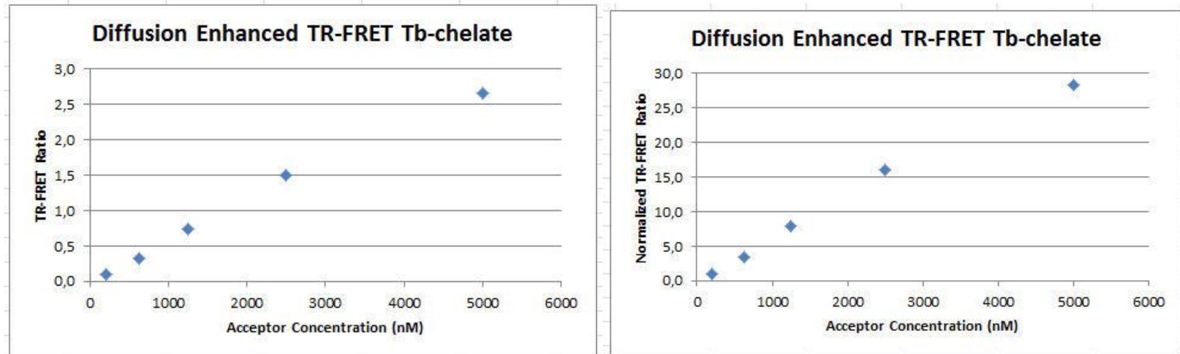
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
A	143635 50886	88586 60936	42334 57384	18524 57054	4955 54862	39 32									
B	149953 56820	86829 59259	40871 57986	17435 55142	4789 51510	40 28									
C	151847 57801	88966 66524	41195 55332	17548 54961	4798 51626	42 30									
D	147214 54683	84897 56351	40131 54661	17273 54165	4823 50809	45 35									
E		83172 54874	40148 53534	17112 53170	4736 50487	46 32									
F	150879 57872	87253 58803	41959 56153	17526 54877	5032 51725										
G	150648 56967	85960 56649	41346 55271	17654 54567	4942 51294										
H	150607 57675	85294 56544	42417 60043	17621 55237	4938 52574										
I	149529 56423	86521 56843	41594 54965	17936 54734	4951 51858										
J	149669 55798	86846 55275	42367 54554	18067 53873	4752 50469										
K	150110 57016														
L	388 45242	417 44214	472 45448	524 48826	435 47300										
M	726 68	443 64	241 39	122 36	81 37										
N															
O															
P															

Legend
1. Raw Data (337/520 A)
2. Raw Data (337/490 B)

Kinetic calculations: 337/490 (B)
Sum of range 1
Based on: Raw Data
Range (1): Startinterval: 11 (100 µs) Stopinterval: 30 (290 µs)

11. Plots of these values are shown here.

A. Ratio Data B. Normalized Data



15. These test values were obtained using the following procedure.

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

Purpose

This LanthaScreen® Tb Microplate Reader Test 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: This document can be found at www.thermofishernologies.com/instrumentsetup.

Step 2: Prepare individual dilutions of the TR-FRET acceptor (fluorescein-labeled substrate, e.g. PV3610 or other).
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 (e.g. PV3610) as the acceptor.

Note: To avoid propagating dilution errors, we do NOT recommend using serial dilutions. See page 17.

Step 3: Prepare a dilution of the TR-FRET donor (Tb-Antibody, e.g. PV3550 or other)
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 17 for calculations and method.

Step 4: Prepare plate and read.

Step 5: Contact Technical Support with your results. E-mail us directly at drugdiscoverytech@thermofisher.com or in the US call 1-800-955-6288 ext 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 19.

Introduction

This LanthaScreen® Tb Microplate 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 the 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	Example Reagents
LanthaScreen® Tb-labeled antibody (donor)	-20°C	Various	PV3550
LanthaScreen® fluorescein-labeled substrate (acceptor)	-20°C	Various	PV3610
TR-FRET Dilution Buffer or any Nuclear Receptor Co-regulator Buffer	Various	PV3574 or Various	PV3574

96-well polypropylene microplate or 1.5 mL microcentrifuge tubes

384-well plate (typically a white, low-volume Corning 4513 or black, low-volume Corning 4514)

Plate seals

Suitable single and multichannel pipettors

Plate reader capable of reading TR-FRET

Handling

To reread the plate on another 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 seal and read.

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

Procedure

Step 1: Set up your instrument using the information in this document.

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

Acceptor concentrations (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.

1. **Prepare 30 µM acceptor stock solution:**

Fluorescein Substrate/Peptide	Cat #	Concentration as Sold	Dilution to prepare a 30 µM 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 (peptide at 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	E1
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	118.4 µL
Volume 30 µM Acceptor (prepared above)	40.0 µL	20.0 µL	10.0 µL	5.0 µL	1.6 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

Formula	V_1	x	C_1	=	V_2	x	C_2
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 pipettor 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@thermofisher.com or in the US call 1-800-955-6288 ext. 40266.

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

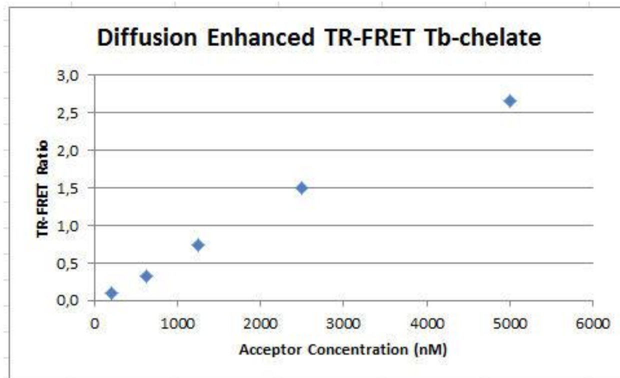
1. Obtain the emission ratios by dividing the acceptor signal (520 nm) by the donor signal (490 nm) for each well.

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.

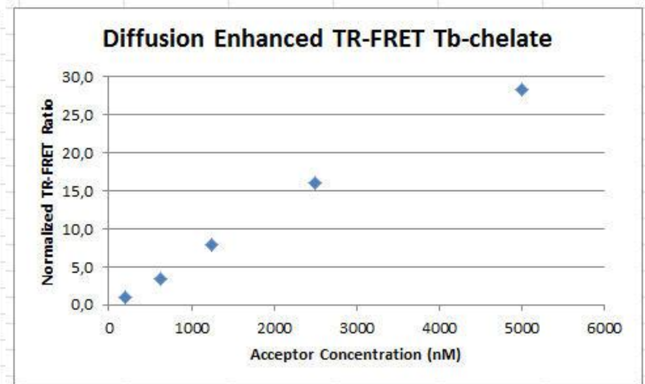
3. Using the data from column 5 (200 nM acceptor) as the bottom of the “assay window,” divide the average ratios from the other columns b the average ratio from column 5 to obtain a range of simulated “assay window” sizes. See the example data below. This “normalized” data can be plotted against the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

Example Data:
Ratiometric data obtained on a BMG LABTECH PHERAstar® *FSX* microplate reader.

[acceptor]	5,000 nM	2,500 nM	1,250 nM	625 nM	200 nM
Row A	2.823	1.454	0.738	0.325	0.090
Row B	2.639	1.465	0.705	0.316	0.093
Row C	2.627	1.337	0.745	0.319	0.093
Row D	2.692	1.507	0.734	0.319	0.095
Row E	2.607	1.516	0.750	0.322	0.094
Row F	2.645	1.484	0.747	0.319	0.097
Row G	2.611	1.517	0.748	0.324	0.096
Row H	2.650	1.509	0.706	0.319	0.094
Row I	2.682	1.522	0.757	0.328	0.096
Row J	2.633	1.571	0.777	0.335	0.094

Data Analysis:

[Acceptor]	5,000 nM	2,500 nM	1,250 nM	625 nM	200 nM
Average Ratio	2.661	1.505	0.741	0.323	0.094
St dev	0.063	0.035	0.022	0.006	0.002
% CV	2.4	2.3	2.9	1.7	2.1
Assay Window	**28.3**	**16.0**	**7.9**	**3.4**	**Reference**
Z’-factor	0.92	0.92	0.89	0.90	

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