LanthaScreen® Terbium (Tb) Assay Setup Guide on the Berthold Technologies Mithras² LB 943 Microplate Reader

The Berthold Technologies Mithras² LB 943 Microplate Reader was tested for compatibility with LanthaScreen® Activity Assay, a TR-FRET assay from Thermofisher, 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 Thermofisher's assays please call 1-800-955-6288 ext. 40266. For more detailed information and technical support of Berthold Technologies' instruments or software, please contact Berthold Technologies Bioanalytic at +49 7081-177-0 or www.berthold-bio.com.

A. Recommended Optics

	Wavelength (nm)	Berthold Technologies' Filters	Included in Filter Package
Excitation	320	320x40 (Id. Nr. 60361)	Id. Nr. 59542
Emission 1	520	520xm10uv (Id. Nr. 62792)	Id. Nr. 68493 and Id. Nr. 59542
Emission 2	495	495xm10uv (Id. Nr. 68476)	Id. Nr. 68493

The recommended filters are available separately or bundled in TR-FRET-related filter packages.

Filter Package 59542 includes:

Excitation slide:	320x40, Id. Nr. 60361
	340x26, Id. Nr. 54083-01*
Emission slide:	520xm10uv, Id. Nr. 62792
	620xm10uv, Id. Nr. 62793*
	665xm7uv, Id. Nr. 60729*

Filter Package 68493 includes:

Excitation slide:	340x26, Id. Nr. 54083-01*
Emission slide:	495xm10uv, Id. Nr. 68476
	520xm10uv, Id. Nr. 62792

* Not used in this application.

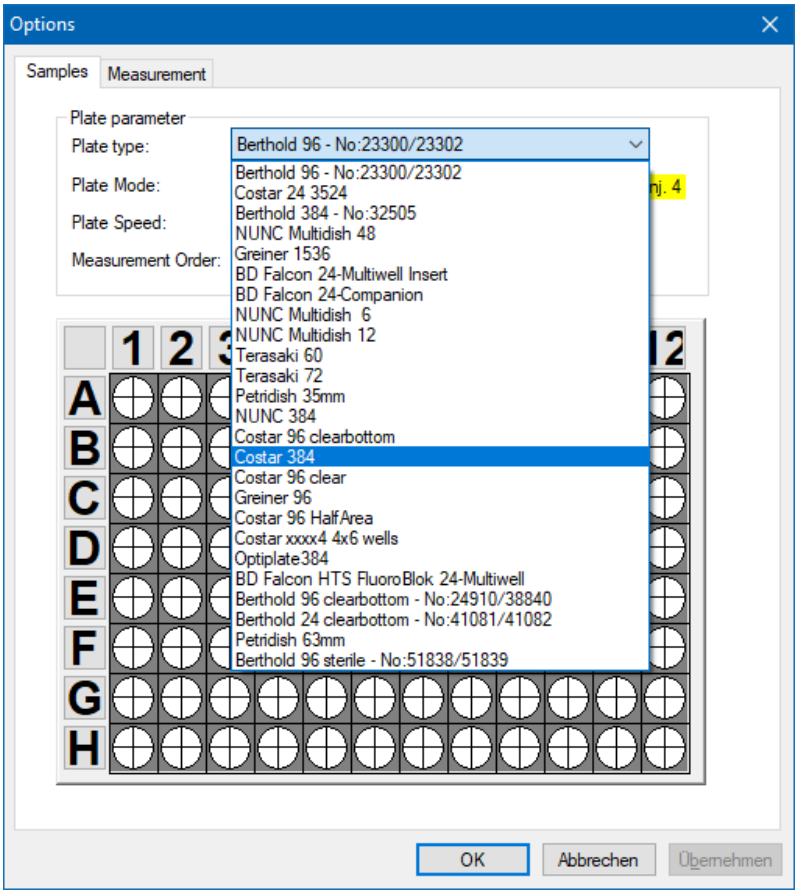
Note: Eu-based TR-FRET and Tb-based TR-FRET use different excitation.
Note: Monochromator based detection is not recommended for TR-FRET assays.

B. Instrument Setup

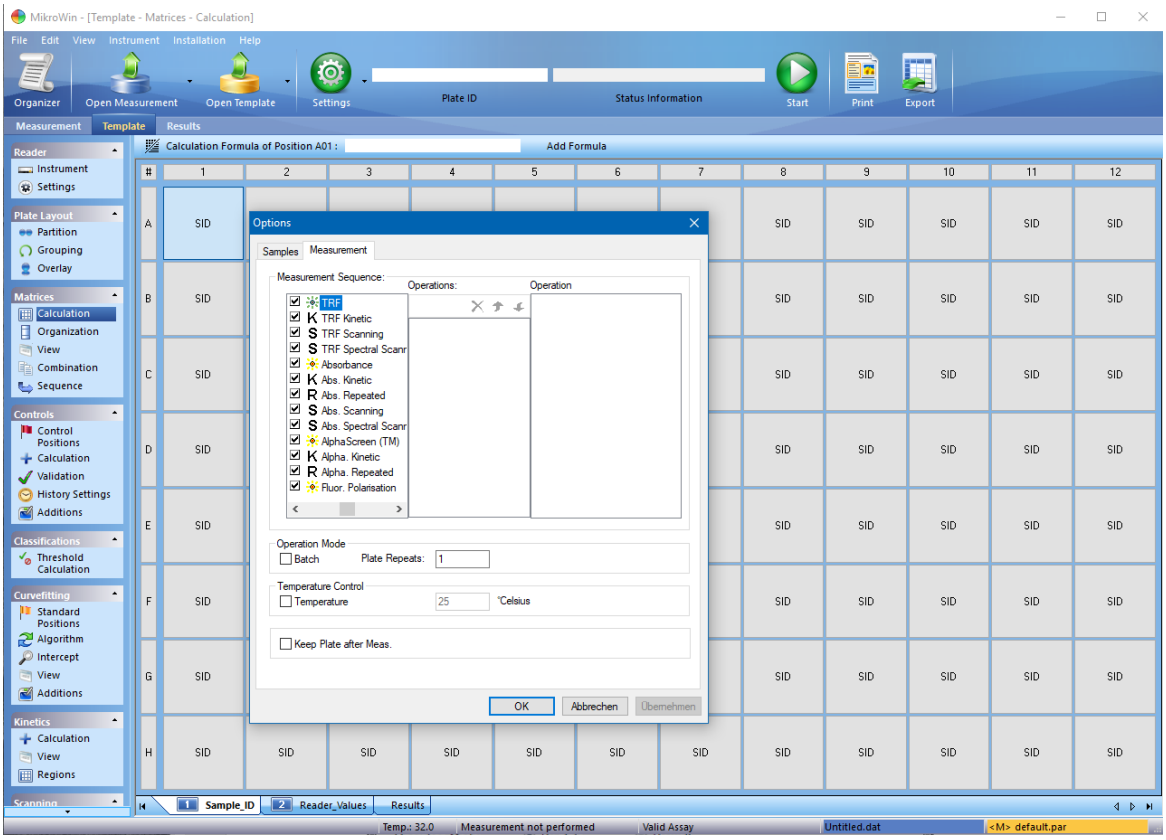
1. Make sure the plate reader is turned on and then open the MikroWin software on the computer.

2. When MikroWin opens, if you already have a pre-existing template for LanthaScreen®, open it and use this document to review your settings; if you don't have yet any suitable template, click on Settings in the menu bar at the top portion of the window to start creating a new template.

3. A new window will open. Select the Plate type corresponding to the plate you are using and highlight the wells you most commonly will measure. If unsure about what plate type to select, contact Berthold Technologies for assistance.



4. Click on the Measurement tab and look for the TRF operation.

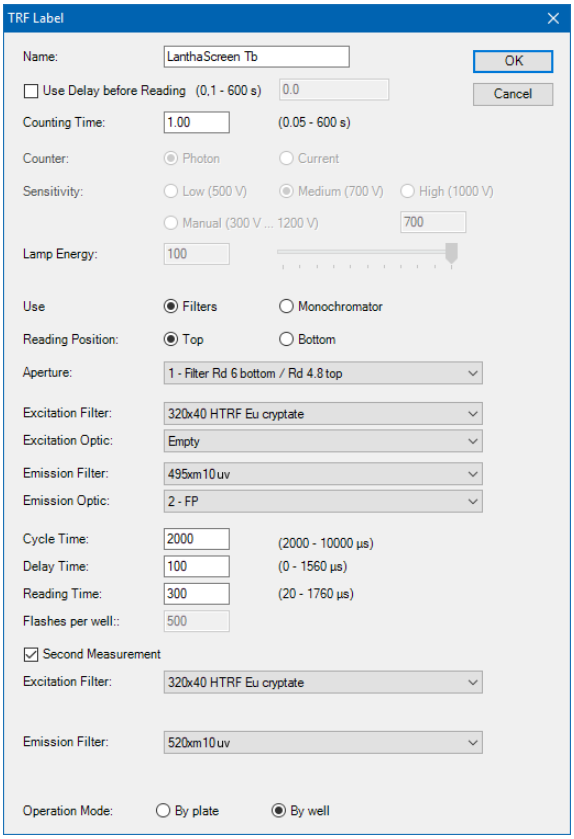


5. Double click on TRF to insert a TRF measurement operation. A new window will appear. If desired, enter a Name for the measurement operation. Configure the settings as shown in the screenshot below:

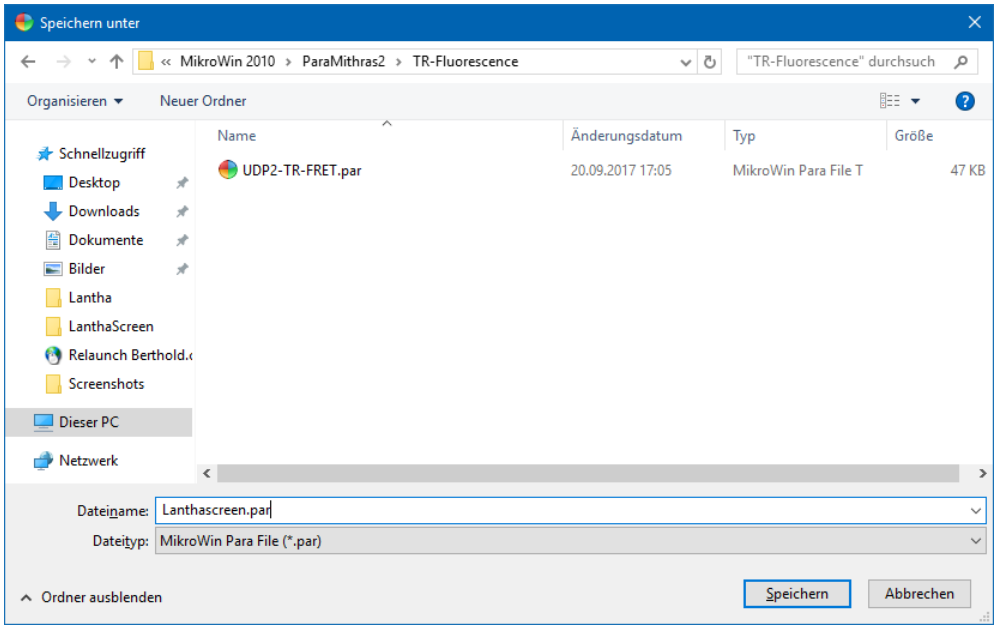
- Enter Counting Time: 1.00
- Select Aperture 1 (Filter Rd 6 bottom / Rd 4.8 top)
- Select Excitation Filter 320x40 HTRF Eu cryptate*
- Select Excitation Optic Empty
- Select Emission Filter 495xm10uv
- Select Emission Optic 2 – FP
- Enter Timing settings: Cycle Time 2000, Delay Time 100, Reading Time 300
- Check Second Measurement
- Select Excitation Filter: 320x40 HTRF Eu cryptate*
- Select Emission Filter: 520xm10uv

When finished, click OK.

* The name of the filters in the software sometimes does not match the LanthaScreen® naming conventions, and sometimes filters named as “Tb cryptate” are mentioned in a Eu assay, or the other way around. This is not an error; filter naming was designed for HTRF® assays, but for LanthaScreen® different filter combinations are sometimes chosen for the best performance.



6. To save the template, click on File in the main menu, then Template and Save as. Browse to the desired folder, enter the desired filename and click OK.



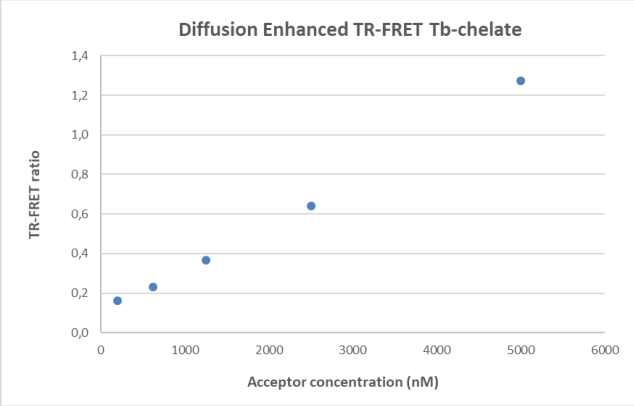
7. To start the measurement, enter the desired Plate ID to identify the measurement. If you want to edit the wells to be measured, click on Settings and select the desired wells (see point 3). When you are ready, click Start. The plate tray will open; insert the plate and click OK to start the measurement.

8. When the measurement has finished, click Export to export the data for further calculation, if necessary. Example raw data values are displayed below.

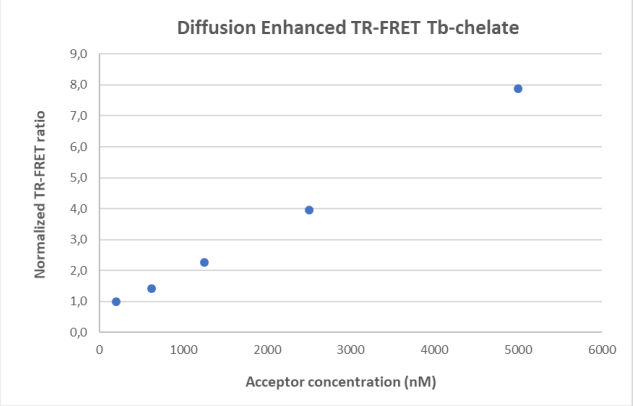
Donor signal (495 nm)					
[Acceptor]	5000 nM	2500 nM	1250 nM	625 nM	200 nM
A	44301	63913	76357	80597	89638
B	43863	54330	85075	93022	93554
C	42418	60643	79751	77412	87042
D	41268	63523	70758	86681	90341
E	41468	57182	79817	90617	91320
F	46050	60804	76937	93136	89457
G	47257	62867	81186	97595	95579
H	40213	61251	77441	91263	79152
I	44405	58409	77308	77270	93032
J	40726	60006	78030	89951	89581
Acceptor signal (520 nm)					
[Acceptor]	5000 nM	2500 nM	1250 nM	625 nM	200 nM
A	52876	44548	27094	20648	15030
B	50765	38597	28729	20563	14032
C	52524	37199	28834	18205	15648
D	54511	35279	28653	20087	14963
E	56916	38454	28872	20401	14469
F	51706	36116	28377	21029	13927
G	57924	36391	29841	20544	15277
H	59331	41915	27835	20059	13709
I	53836	35374	28567	18918	14498
J	58133	41620	29081	21276	13442

9. Plots of ratios corresponding to these raw data are displayed below.

A. Ratio Data



B. Normalized Data



10. These values were obtained using the procedure detailed in the next section. Additional representative data from the Berthold Technologies Mithras² are available at the end of the section.

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.thermofisher.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	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

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 (495 nm, exact wavelength varies with instrument) for each well.

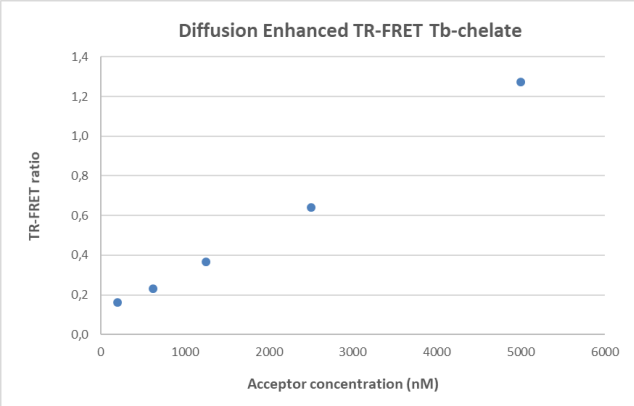
2. Calculate the average ratio for each column (1 through 5). These values can be plotted against the final 1X concentrations (5,000 nM, 2,500 nM, 1,250 nM, 625 nM, and 200 nM) of acceptor (see graph A). Dilution curves form diffusion-enhanced TR-FRET do not plateau and, therefore, do not fit the normal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

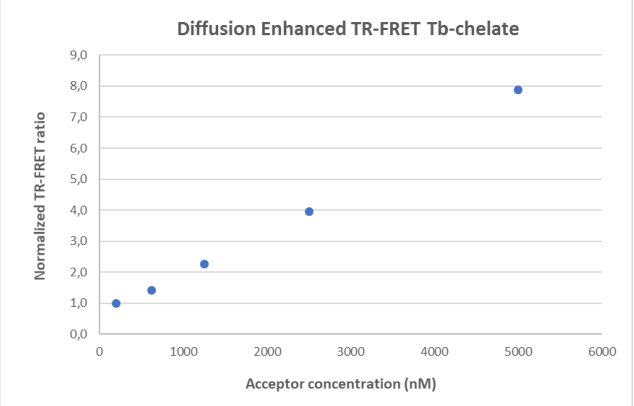
4. Calculate the Z'-factor for each "assay window." Very general guidance is that you should observe a satisfactory Z'-factor (>0.5) for at least the "small window" that compares columns 3 to 5 (1,250 nM to 200 nM). In our hands and on

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

Ratiometric data obtained on a Berthold Technologies Mithras² LB 943 microplate reader.

[acceptor]	5,000 nM	2,500 nM	1,250 nM	625 nM	200 nM
Row A	1.194	0.697	0.355	0.256	0.168
Row B	1.157	0.710	0.338	0.221	0.150
Row C	1.238	0.613	0.362	0.235	0.180
Row D	1.321	0.555	0.405	0.232	0.166
Row E	1.373	0.672	0.362	0.225	0.158
Row F	1.123	0.594	0.369	0.226	0.156
Row G	1.226	0.579	0.368	0.211	0.160
Row H	1.475	0.684	0.359	0.220	0.173
Row I	1.212	0.606	0.370	0.245	0.156
Row J	1.427	0.694	0.373	0.237	0.150

Data Analysis:

[Acceptor]	5,000 nM	2,500 nM	1,250 nM	625 nM	200 nM
Average Ratio	1.275	0.641	0.366	0.231	0.162
St dev	0.118	0.057	0.017	0.013	0.010
% CV	9.3	8.9	4.6	5.7	6.1
Assay Window	**7.89**	**3.96**	**2.26**	**1.43**	**Reference**
Z'-factor	0.65	0.58	0.61	0.01	

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