

LanthaScreen® Europium (Eu) 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® Eu Kinase Binding Assay, a TR-FRET assay from ThermoFisher, using Kinase Tracer 236 (PV5592) and Eu-anti-GST Antibody (PV5594).

The following document is intended to demonstrate setup of this instrument for any Eu-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	337	340x26 (Id. Nr. 54083-01)	Id. Nr. 59542 and 68493
Emission 1	665	665xm7uv (Id. Nr. 60729)	Id. Nr. 59542
Emission 2	620	620xm10uv (Id. Nr. 62793)	Id. Nr. 59542

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*

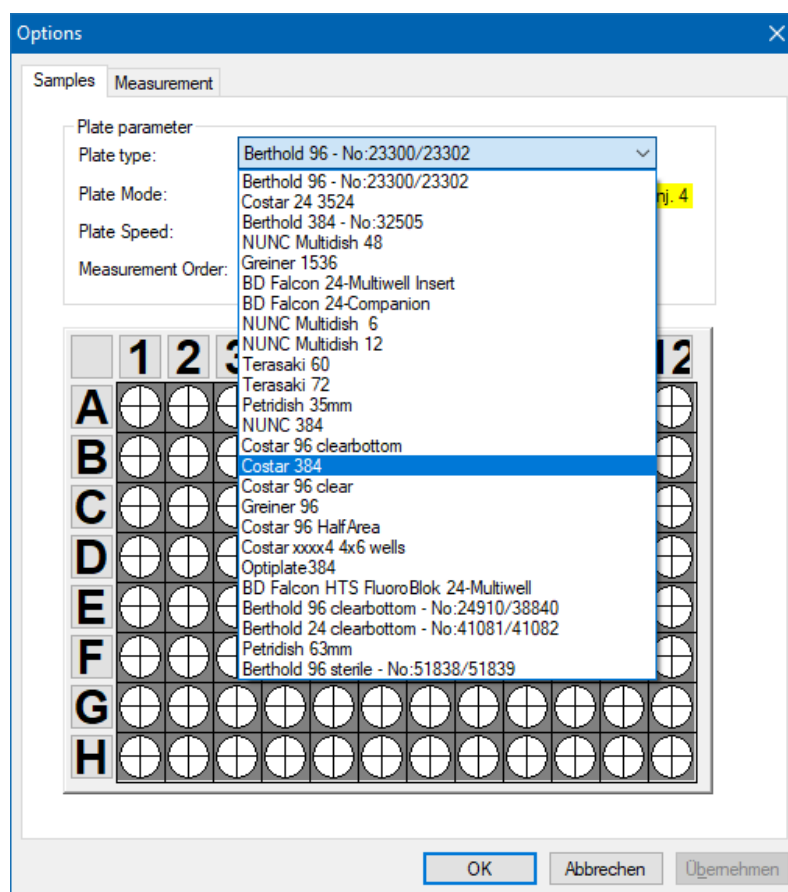
* Not used in this application.

Note: Eu-based TR-FRET and Tb-based TR-FRET use different excitation filters.

Note: Monochromator based detection is not recommended for TR-FRET assays.

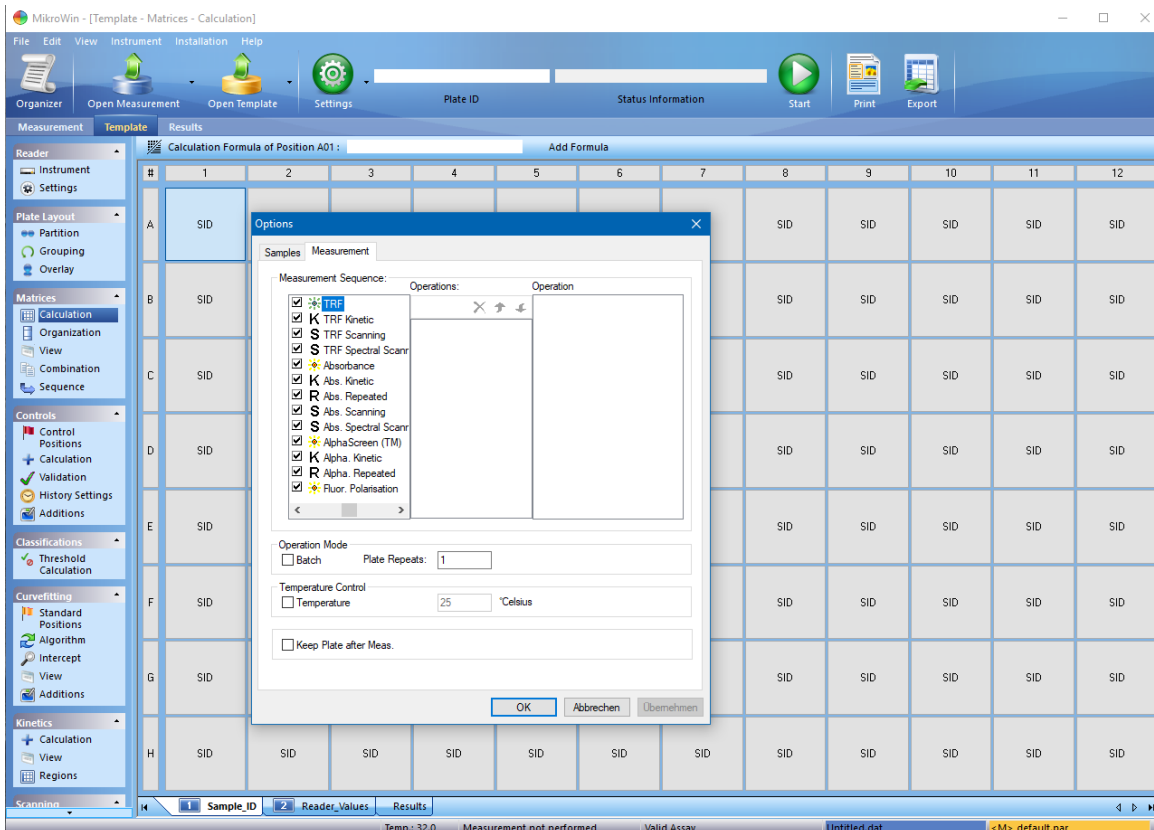
B. Instrument Setup

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.



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



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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: 340x26 HTRF Tb cryptate*
- Select Excitation Optic: Empty
- Select Emission Filter: 620xm10uv Eu cryptate*
- Select Emission Optic: 2 – FP
- Enter Timing settings: Cycle Time 2000, Delay Time 100, Reading Time 300
- Check Second Measurement
- Select Excitation Filter: 340x26 HTRF Tb cryptate*
- Select Emission Filter: 665xm7uv XL665, APC*

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.

The screenshot shows the 'TRF Label' dialog box with the following settings:

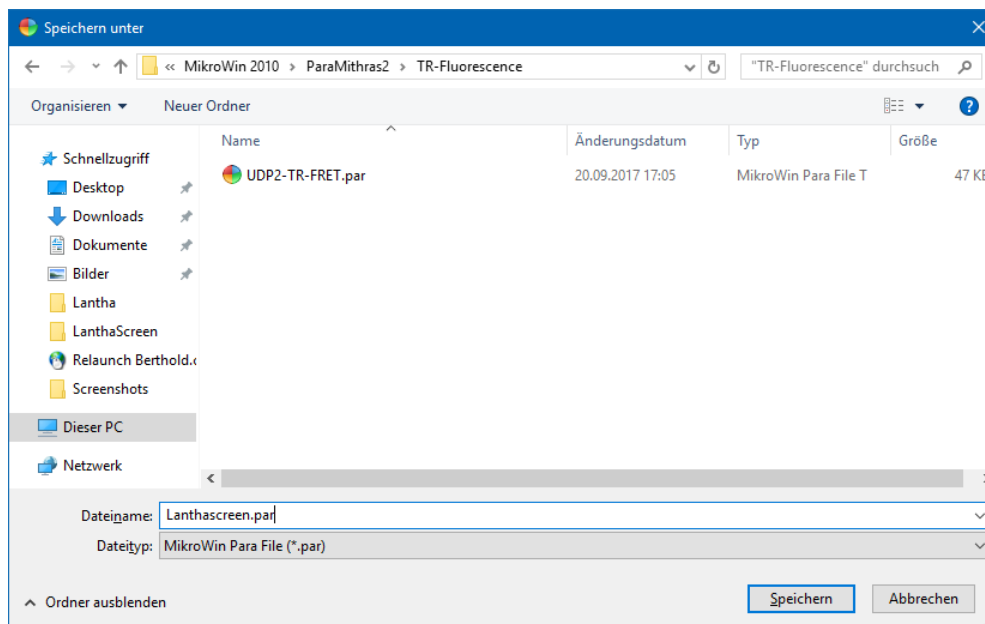
- Name: Lantha Eu
- Use Delay before Reading (0.1 - 600 s): 0.0
- Counting Time: 1.00 (0.05 - 600 s)
- Counter: Photon
- Sensitivity: Medium (700 V)
- Lamp Energy: 100
- Use: Filters
- Reading Position: Top
- Aperture: 1 - Filter Rd 6 bottom / Rd 4.8 top
- Excitation Filter: 340x26 HTRF Tb cryptate - Slot 3
- Excitation Optic: Empty
- Emission Filter: 620xm10uv Eu cryptate
- Emission Optic: 2 - FP
- Cycle Time: 2000 (2000 - 10000 μs)
- Delay Time: 100 (0 - 1560 μs)
- Reading Time: 300 (20 - 1760 μs)
- Flashes per well: 500
- Second Measurement: Second Measurement
- Excitation Filter: 340x26 HTRF Tb cryptate - Slot 3
- Emission Filter: 665xm7uv XL665, APC
- Operation Mode: By well

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- 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.



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

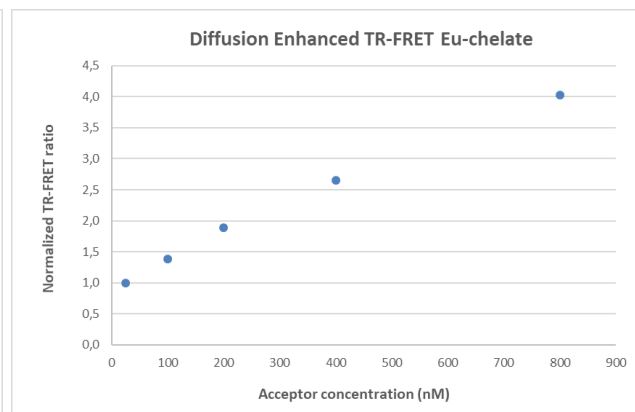
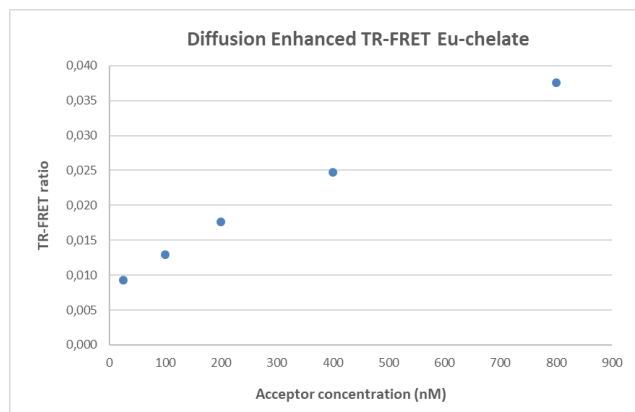
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Donor signal (620 nm)					
[Acceptor]	800 nM	400 nM	200 nM	100 nM	25 nM
A	125230	152733	171404	187660	184181
B	120648	151811	206664	185483	180673
C	122730	158532	176642	184181	183392
D	122797	157505	178534	190161	187137
E	123519	152800	164854	177441	192385
F	125306	152039	160006	187147	180968
G	133187	156098	166461	181167	192176
H	128672	147523	169854	181928	205124
I	126771	153579	164882	181671	204525
J	126362	154872	167934	185711	209373
Acceptor signal (665 nm)					
[Acceptor]	800 nM	400 nM	200 nM	100 nM	25 nM
A	4744	3869	2995	2386	1797
B	4525	3860	3755	2282	1702
C	4867	3793	3137	2348	1749
D	4639	3945	3014	2538	1778
E	4544	3850	2890	2263	1797
F	4706	3574	2880	2443	1778
G	4772	3860	2966	2282	1816
H	4696	3708	2928	2367	1806
I	4725	3793	2928	2386	1844
J	4896	3708	2976	2491	1797

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® Eu Assays

Purpose

This LanthaScreen® Eu 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 (tracer, e.g. PV5592).
2X = 1,600 nM, 800 nM, 400 nM, 200 nM and 50 nM.

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 (Eu-Antibody, e.g. PV5594).
2X = 125 nM Eu-chelate.

Note: Concentration is based on the molarity of the Eu 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.

Introduction

This LanthaScreen® Eu 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 a suitable distance from one another to allow TR-FRET to occur, 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, a acceptor concentration varies while the donor concentration remains fixed. As the concentration of a acceptor increases, the diffusion-enhanced TR-FRET signal increases. The signal from the a acceptor concentrations are compared to the signal from the lowest a acceptor concentration to simulate assay windows from high to low allowing you to assess if your instrument is properly set-up and capable of detecting TR-FRET signals in the LanthaScreen® Assays.

We designed the LanthaScreen® Eu technical note to use components and reagents that are generally used in the LanthaScreen® Eu Kinase Binding Assays. If you are using a Eu-based LanthaScreen® Activity or Adapta™ assay, call Technical Support for additional information.

Materials Required

Component	Storage	Part Number	Example Reagents
LanthaScreen® Eu-labeled antibody (donor)	-20°C	Various	PV5594
LanthaScreen® Tracer (acceptor)	-20°C	Various	PV5592
5X Kinase Buffer	Room Temperature	PV3189	PV3189

*If you are using a Eu-based LanthaScreen® Activity or Adapta™ assay, call Technical Support for additional information.

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 xg for 1 minute, remove seal and read.

Important: Prior to use, centrifuge the antibody at approximately 10,000 xg 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® Kinase Tracer 236)

Acceptor concentrations (2X) are individually prepared from the Kinase Tracer stock (either 25 µM or 50 µM) 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.

First prepare **1X Kinase Buffer A** by adding 4 mL of 5X Kinase Buffer A to 16 mL of highly purified water. Diluted 1X Kinase Buffer A can be stored at room temperature.

1. Prepare 2,500 nM acceptor stock solution:

LanthaScreen® Kinase Tracer	Cat #	Concentration as Sold	Dilution to prepare a 30 µM solution
Tracer 178	PV5593	25 µM	Add 17 µL of tracer to 153 µL of 1X Kinase Buffer A
Tracer 199	PV5830	25 µM	Add 17 µL of tracer to 153 µL of 1X Kinase Buffer A
Tracer 236	PV5592	50 µM	Add 8.5 µL of tracer to 161.5 µL of 1X Kinase Buffer A
Tracer 314	PV6087	25 µM	Add 17 µL of tracer to 153 µL of 1X Kinase Buffer A
Tracer 1710	PV6088	25 µM	Add 17 µL of tracer to 153 µL of 1X Kinase Buffer A

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	1,600 nM	800 nM	400 nM	200 nM	50 nM
Final 1X Acceptor Concentration	800 nM	400 nM	200 nM	100 nM	25 nM
Volume 1X Kinase Buffer A	43 µL	81.6 µL	100.8 µL	110.4 µL	117.6 µL
Volume 2,500 nM Acceptor (prepared above)	77 µL	38.4 µL	19.2 µL	9.6 µL	2.4 µL

Step 3: Prepare the Donor (Eu-Chelate Labeled Antibody)

Prepare a 2X stock of Eu-chelate at 125 nM that will result in a final assay concentration of 62.5 nM. This method relies on the concentration of Eu-chelate, NOT the concentration of antibody. The lot-to-lot variation in the number of Eu-chelates covalently bound to antibody can be accounted for by referring to the Eu-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 Eu-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 Eu-chelate:

Eu-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
			[Stock]				[2X]
Eu-Chelate	V ₁	x	36,300 nM	=	1,000 µL	x	125 nM

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$V_1 = 3.4 \mu\text{L}$

Add 3.4 μL of 36,300 nM stock to 996.6 μL 1X Kinase Buffer A.

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

1. Donor

Transfer 10 μL of 2X Eu-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
1,600 nM	1
800 nM	2
400 nM	3
200 nM	4
50 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:

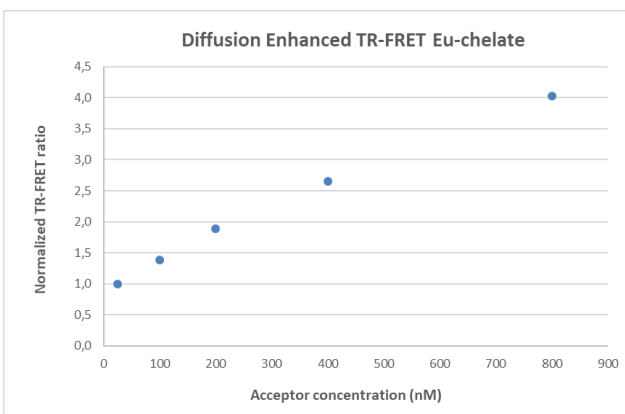
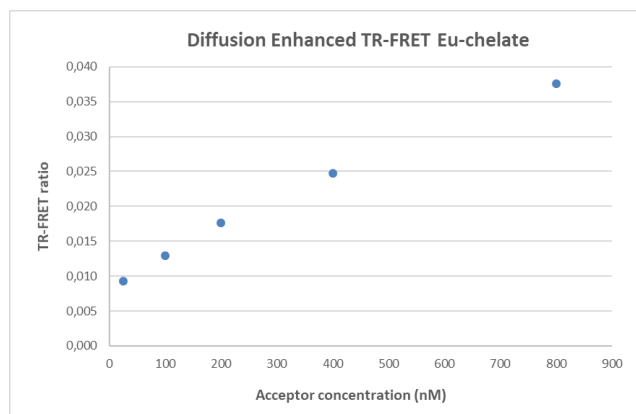
- Obtain the emission ratios by dividing the acceptor signal (665 nm) by the donor signal (615 nm, exact wavelength varies with instrument) for each well.
- Calculate the average ratio for each column (1 through 5). These values can be plotted against the final 1X concentrations (800 nM, 400 nM, 200 nM, 100 nM, and 25 nM) of acceptor (see graph A). Dilution curves from diffusion-enhanced TR-FRET do not plateau and, therefore, do not fit the normal sigmoidal shape produced by binding curves.
- Using the data from column 5 (25 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.

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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 (200 nM to 25 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.

[Eu-chelate]	800 nM	400 nM	200 nM	100 nM	25 nM
Row A	0.038	0.025	0.017	0.013	0.010
Row B	0.038	0.025	0.018	0.012	0.009
Row C	0.040	0.024	0.018	0.013	0.010
Row D	0.038	0.025	0.017	0.013	0.010
Row E	0.037	0.025	0.018	0.013	0.009
Row F	0.038	0.024	0.018	0.013	0.010
Row G	0.036	0.025	0.018	0.013	0.009
Row H	0.036	0.025	0.017	0.013	0.009
Row I	0.037	0.025	0.018	0.013	0.009
Row J	0.039	0.024	0.018	0.013	0.009

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Data Analysis:

[Acceptor]	800 nM	400 nM	200 nM	100 nM	25 nM
Average Ratio	0.038	0.025	0.018	0.013	0.009
St dev	0.0011	0.0007	0.0004	0.0003	0.0004
% CV	2.91	2.73	2.11	2.68	4.31
Assay Window	4.03	2.65	1.89	1.38	Reference
Z'-factor	0.84	0.79	0.72	0.37	

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