LanthaScreen® Europium (Eu) Assay Setup Guide on the Berthold Technologies Tristar² S LB 942 Microplate Reader

The Berthold Technologies Tristar² LB 942 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	340/26 (Id. Nr. 54083)	Id. Nr. 62772
Emission 1	665	665/7uv (Id. Nr. 52544[1])	Id. Nr. 62772
Emission 2	620	620/10uv (Id. Nr. 47731[1])	Id. Nr. 62772

[1] Although filters 52544 and 47731 are available separately, they need to be mounted in a special way to be suitable for LanthaScreen®. Use only the filters provided in filter package 62772 (Measurement technology "TR-FRET / HTRF"). Do not disassemble, change or move the filters included in the filter slides, as that could render them unsuitable for LanthaScreen®. Do not use filters purchased separately.

Filter Package 62772 includes:

Excitation slide:	320/40, Id. Nr. 52733*
	340/26, Id. Nr. 54083
Emission slide:	620/10uv, Id. Nr. 47731
	665/7uv, Id. Nr. 52544
	520/10uv, Id. Nr. 38836*

* 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.

IMPORTANT: the fluorescence module with **extended spectral range** is required to perform the LanthaScreen® Europium Assay in the Tristar² S.

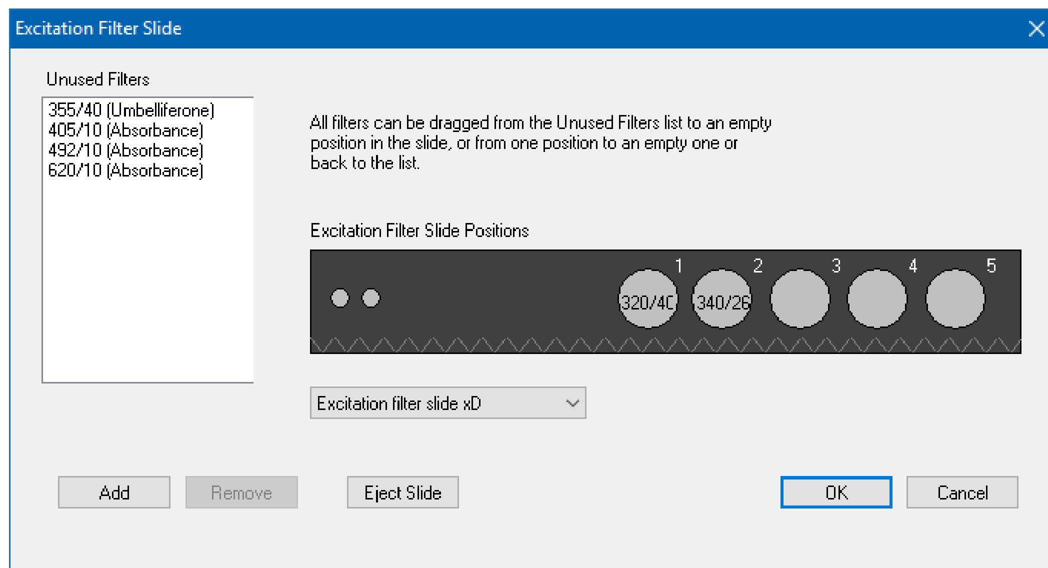
B. Instrument Setup

The following instructions are provided for the MikroWin software. The ICE software is also compatible with LanthaScreen®, and the same instrument settings can be easily programmed in ICE. Contact Berthold Technologies if you need support to program in ICE the instrument settings detailed here.

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

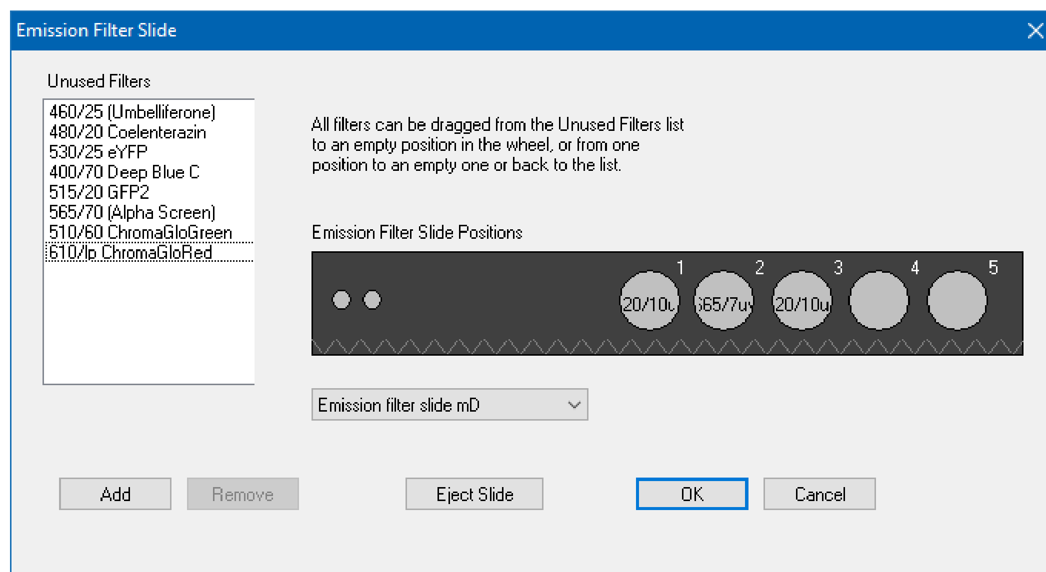
2. Click on Instrument >> Excitation Filter Slide. Check if the right filters are assigned to the right positions of the filter slides; if they are not, assign each filter to the corresponding position in the filter slide. Please follow the example below:

   Excitation slide xD
   Slot 1: 320/40 (HTRF Eu cryptate); usage: TRFluorescence
   Slot 2: 340/26 (HTRF Tb cryptate); usage: TRFluorescence



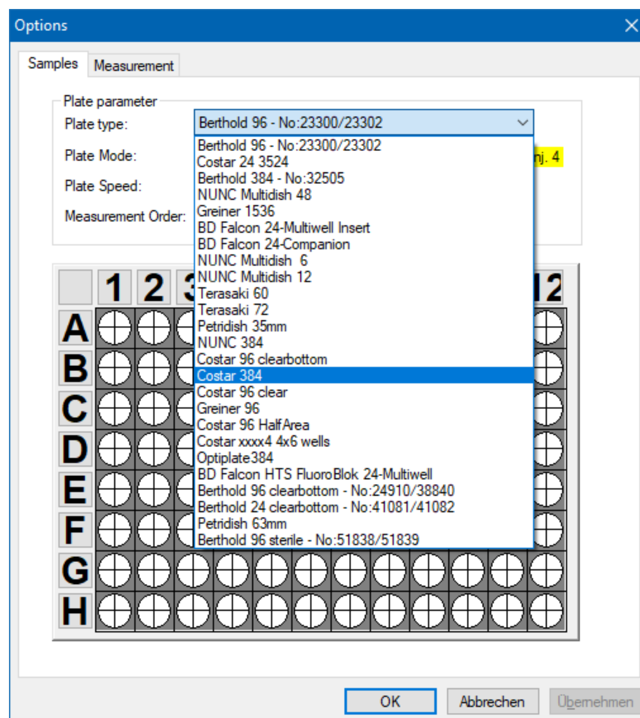
3. Click on Instrument >> Emission Filter Slide. Check if the right filters are assigned to the right positions of the filter slides; if they are not, assign each filter to the corresponding position in the filter slide (add new filters and enter the settings below if needed). Please follow the example below:

   Emission Slide mD
   Slot 1: 620/10uv (HTRF Eu cryptate); usage: TRFluorescence
   Slot 2: 665/7uv (HTRF XL665/APC); usage: TRFluorescence
   Slot 3: 520/10uv; usage: TRFluorescence

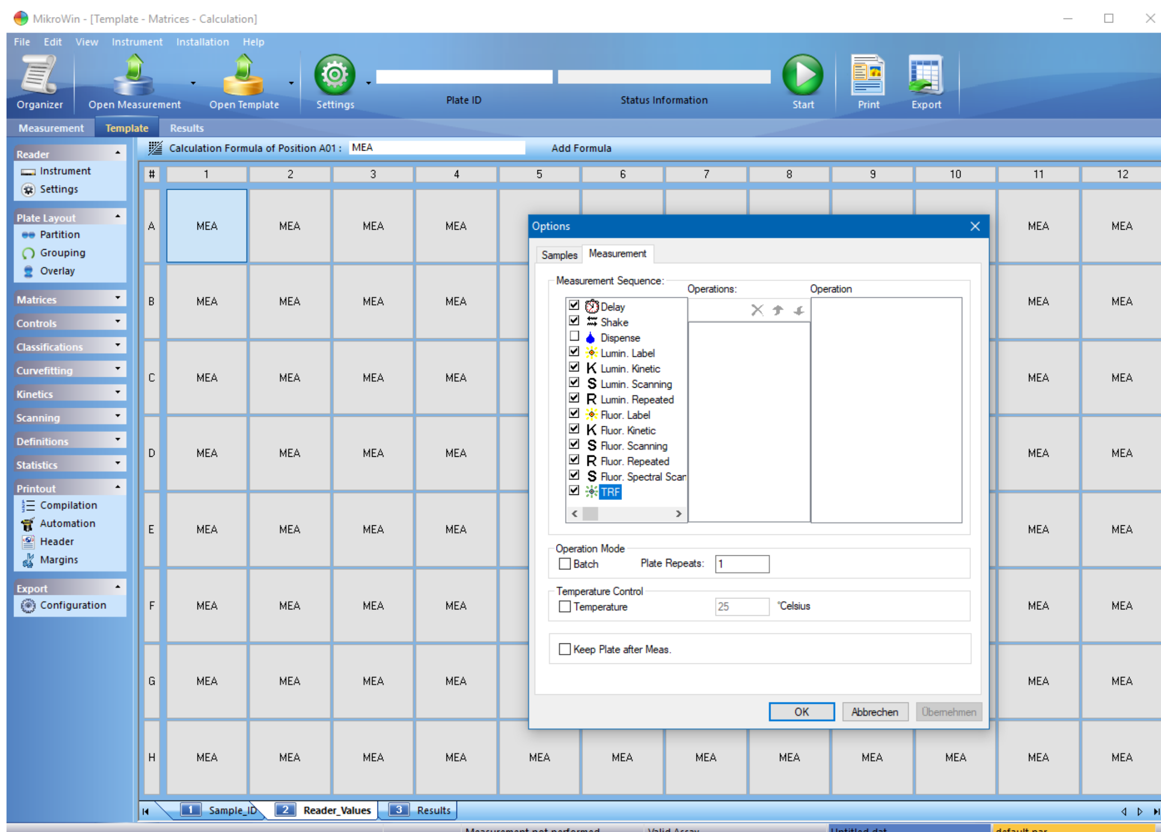


4. If you already have a pre-existing template for LanthaScreen®, open it and use this document to review your settings; if you don't have yet any suitable template, click on Settings in the menu bar at the top portion of the window to start creating a new template.

5. 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.



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



7. 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:

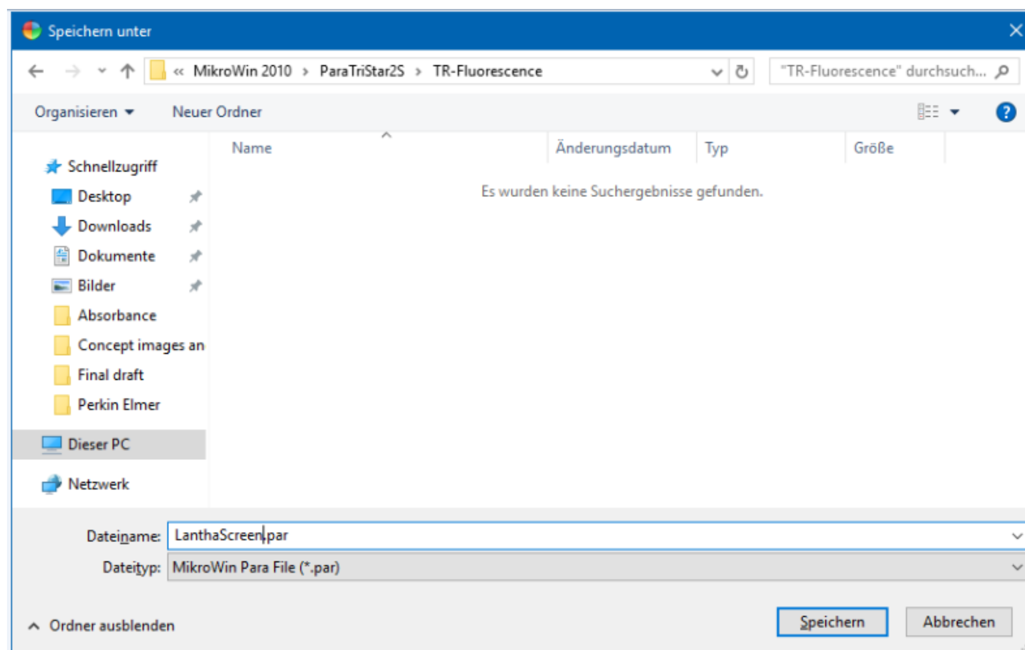
- Enter Counting Time: 1.00
- Select Aperture: 3 - Rd 2
- Select Excitation Filter: 340/26 (HTRF Tb cryptate)*
- Select Excitation Optic: 3 – Wide Filter 0.45mm
- Select Emission Filter: 620/10uv (HTRF Eu cryptate)*
- Enter Timing settings: Cycle Time 5000, Delay Time 100, Reading Time 300
- Check Second Measurement
- Select Excitation Filter: 340/26 HTRF Tb cryptate*
- Select Emission Filter: 665/7uv (HTRF 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.

TRF Label
Name: TRF
OK
Cancel
Use Delay before Reading (0,1 - 600 s) 0.0
Counting Time: 1.00 (0.05 - 600 s)
Counter: Photon Current
Sensitivity: Low (500 V) Medium (700 V) High (1000 V)
Manual (500 V ... 1000 V) 700
Lamp Energy: 100
Use Filters Monochromator
Aperture: 3 - Rd 2
Excitation Filter: 340/26 (HTRF Tb cryptate) - Slot xD2
Excitation Optic: 3 - Wide Filter 0.45mm
Emission Filter: 620/10uv (HTRF Eu cryptate) - Slot mD1
Cycle Time: 5000 (2000 - 10000 µs)
Delay Time: 100 (0 - 4560 µs)
Reading Time: 300 (20 - 4760 µs)
Flashes per well:: 200
Second Measurement
Excitation Filter: 340/26 (HTRF Tb cryptate) - Slot xD2
Emission Filter: 665/7uv (HTRF XL665/APC) - Slot mD2
Operation Mode: By plate By well

8. 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.



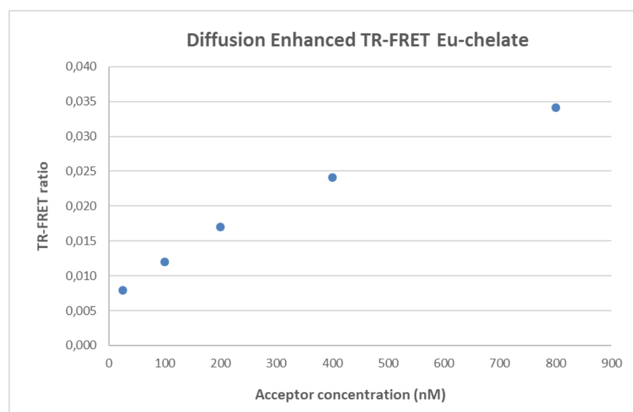
9. To start the measurement, enter the desired Plate ID to identify the measurement. If you want to edit the wells to be measured, click on Settings and select the desired wells (see point 3). When you are ready, click Start. The plate tray will open; insert the plate and click OK to start the measurement.

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

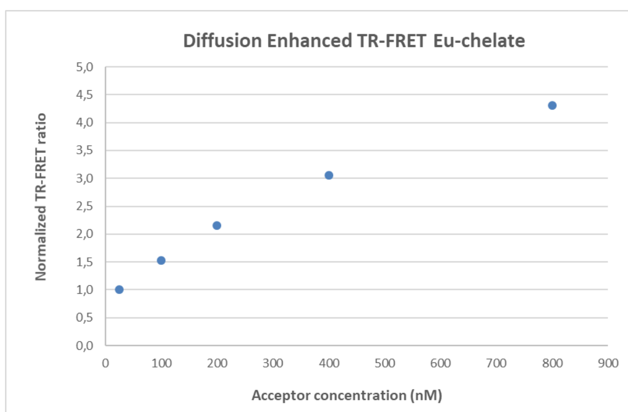
Donor signal (620 nm)					
[Acceptor]	800 nM	400 nM	200 nM	100 nM	25 nM
A	110528	129634	148313	156848	149439
B	113437	137878	249280	155079	152560
C	116080	139495	151717	156899	154052
D	111089	128652	145243	154200	158500
E	105329	126392	145103	152604	151368
F	113156	127965	148588	153275	154803
G	110845	126580	134606	150311	168533
H	120640	129266	134158	156390	160466
I	112618	141038	144094	151048	158793
J	115701	133246	146835	156954	164534
Acceptor signal (665 nm)					
[Acceptor]	800 nM	400 nM	200 nM	100 nM	25 nM
A	4112	3180	2467	1936	1176
B	4089	3528	4399	1904	1295
C	3907	3455	2649	1854	1246
D	3660	3057	2450	1833	1261
E	3788	3170	2425	1805	1131
F	4054	2965	2567	1793	1194
G	3590	3240	2348	1827	1331
H	4016	2772	2096	1897	1288
I	3579	3442	2456	1868	1237
J	3686	3082	2578	1862	1274

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

A. Ratio Data



B. Normalized Data



12. These values were obtained using the procedure detailed in the next section. Additional representative data from the Berthold Technologies Tristar² S 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, 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 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 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® 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

	V_1 = **3.4** µL

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:

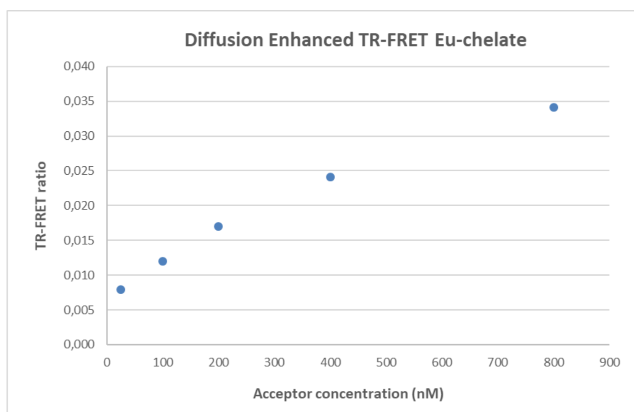
1. Obtain the emission ratios by dividing the acceptor signal (665 nm) by the donor signal (615 nm, exact wavelength varies with instrument) for each well.

2. 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 form diffusion-enhanced TR-FRET do not plateau and, therefore, do not fit the normal sigmoidal shape produced by binding curves.

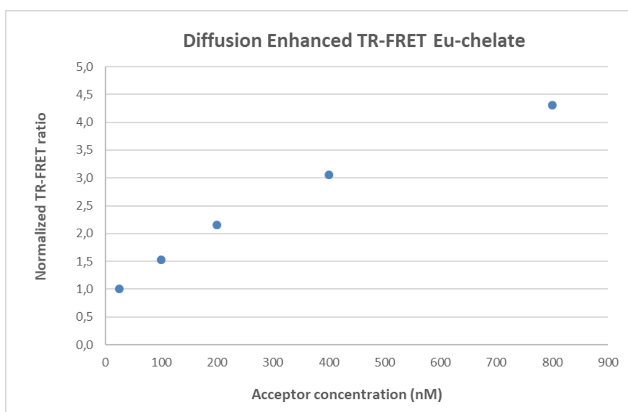
3. Using the data from column 5 (25 nM acceptor) as the bottom of the “assay window,” divide the average ratios from the other columns by the average ratio from column 5 to obtain a range of simulated “assay window” sizes. See the example data below. This “normalized” data can be plotted against the acceptor concentration as shown below in graph B.

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 Tristar² S LB 942 microplate reader.

[Eu-chelate]	800 nM	400 nM	200 nM	100 nM	25 nM
Row A	0.037	0.025	0.017	0.012	0.008
Row B	0.036	0.026	0.018	0.012	0.008
Row C	0.034	0.025	0.017	0.012	0.008
Row D	0.033	0.024	0.017	0.012	0.008
Row E	0.036	0.025	0.017	0.012	0.007
Row F	0.036	0.023	0.017	0.012	0.008
Row G	0.032	0.026	0.017	0.012	0.008
Row H	0.033	0.021	0.016	0.012	0.008
Row I	0.032	0.024	0.017	0.012	0.008
Row J	0.032	0.023	0.018	0.012	0.008

Data Analysis:

[Acceptor]	800 nM	400 nM	200 nM	100 nM	25 nM
Average Ratio	0.034	0.024	0.017	0.012	0.008
St dev	0.0020	0.0013	0.0006	0.0002	0.0003
% CV	5.82	5.36	3.58	2.04	3.42
Assay Window	**4.31**	**3.05**	**2.15**	**1.52**	**Reference**
Z'-factor	0.74	0.71	0.71	0.63	

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