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

Purpose

This LanthaScreen® Eu Microplate Reader Test provides a method to verify the ability of your fluorescent plate reader 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 instrument specific set-up guide.

Step 2: Prepare individual dilutions of the TR-FRET acceptor (tracer), 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 3.

Step 3: Prepare a dilution of the TR-FRET donor (Eu-Antibody), 2X = 125 nM Eu-chelate.
Note: Concentration is based on the molarity of Eu chelate (found on the Certificate of Analysis), NOT the molarity of 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. Example results and data analysis are available beginning on page 5.

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 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
LanthaScreen® Eu-Labeled Antibody (donor)	-20°C	Various
LanthaScreen® Tracer (acceptor)	-20°C	Various
5X Kinase Buffer	Room temperature	PV3179

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

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 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

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 shown here, optimized specifically for LanthaScreen® TR-FRET assays, may differ from 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 Acceptor (such as LanthaScreen® Kinase Tracer 236)

Acceptor concentrations (at 2X) are individually prepared from a 2,500 nM 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.

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 #	Stock Concentration	Dilution to prepare a 2,500 nM 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 2,500 nM solution:

96-well plate or tubes	A1	B1	C1	D1	EI
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 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 on 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

	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 antibody 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 pipette from a basin to all 50 wells. We recommend preparing the 1 mL solution in a 1.5 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. Refer to the chart below:

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.

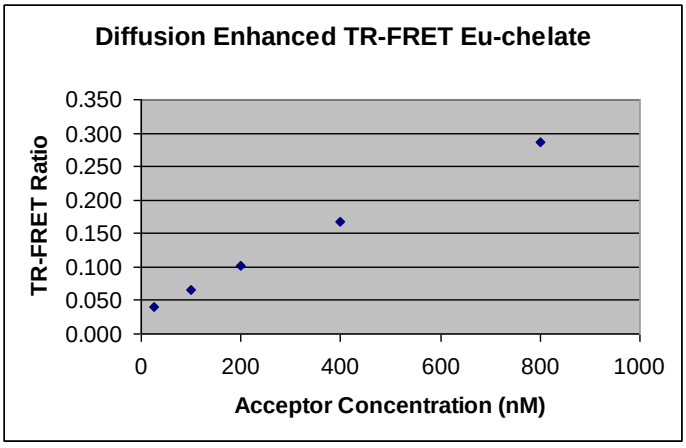
Step 5: Contact Technical Support

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

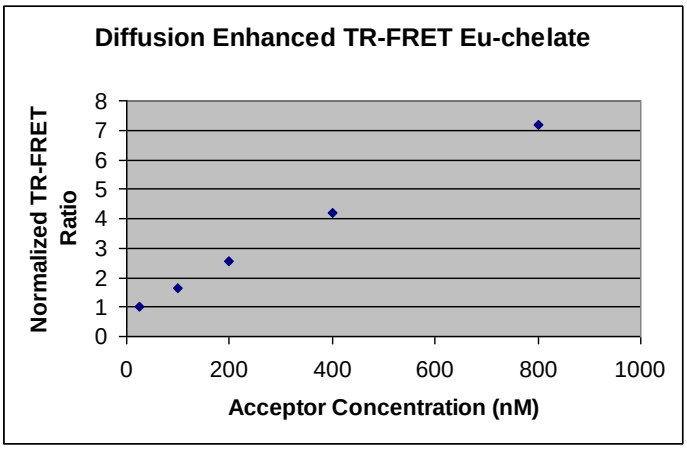
We will help you evaluate your results by performing the following data analysis:

1. Obtain the emission ratios by dividing the acceptor signal (665 nm) by the donor signal (615 nm) for each well.
2. Calculate the average ratio for each column (1 through 5). 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.
3. Using the data from column 5 (25 nM acceptor) as the bottom of the "assay window", divide the average rations from the other columns by the average ration 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 show 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

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

[Eu-chelate]	800 nM	400 nM	200 nM	100 nM	25 nM
Row A	0.297	0.164	0.101	0.069	0.039
Row B	0.289	0.167	0.096	0.066	0.041
Row C	0.282	0.162	0.099	0.067	0.038
Row D	0.282	0.172	0.103	0.066	0.039
Row E	0.285	0.164	0.108	0.064	0.047
Row F	0.275	0.170	0.110	0.067	0.040
Row G	0.294	0.171	0.103	0.069	0.039
Row H	0.293	0.169	0.101	0.064	0.040
Row I	0.286	0.166	0.103	0.067	0.039
Row J	0.291	0.176	0.099	0.067	0.039

Data Analysis:

[Acceptor]	**800 nM**	**400 nM**	**200 nM**	**100 nM**	**25 nM**
Average Ratio	0.287	0.168	0.102	0.067	0.040
St dev	0.0066	0.0042	0.0042	0.0016	0.0026
% CV	2.31	2.52	4.14	2.45	6.54
Assay Window	**7.17**	**4.20**	**2.56**	**1.66**	**Reference**
Z'-factor	0.89	0.84	0.67	0.52	

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