ThermoFisher SCIENTIFIC	LanthaScreen® Eu Compatible Microplate Reader Documentation	Version No.: May 2016	Page 1 of 18

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

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

The BMG LABTECH PHERAstar® *FSX* Microplate Readers were tested for compatibility with LanthaScreen® Eu Kinase Binding Assay, a TR-FRET assay from Thermo Fisher Scientific, using the 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 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	HTRF
Emission 1	665	HTRF
Emission 2	620	HTRF
Dichroic Mirror	Included	HTRF

B.   Instrument Setup

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

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NA: 800-955-6288 ext. 40266   Email: drugdiscoverytech@thermofisher.com

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LanthaScreen® Eu Compatible
Microplate Reader Documentation
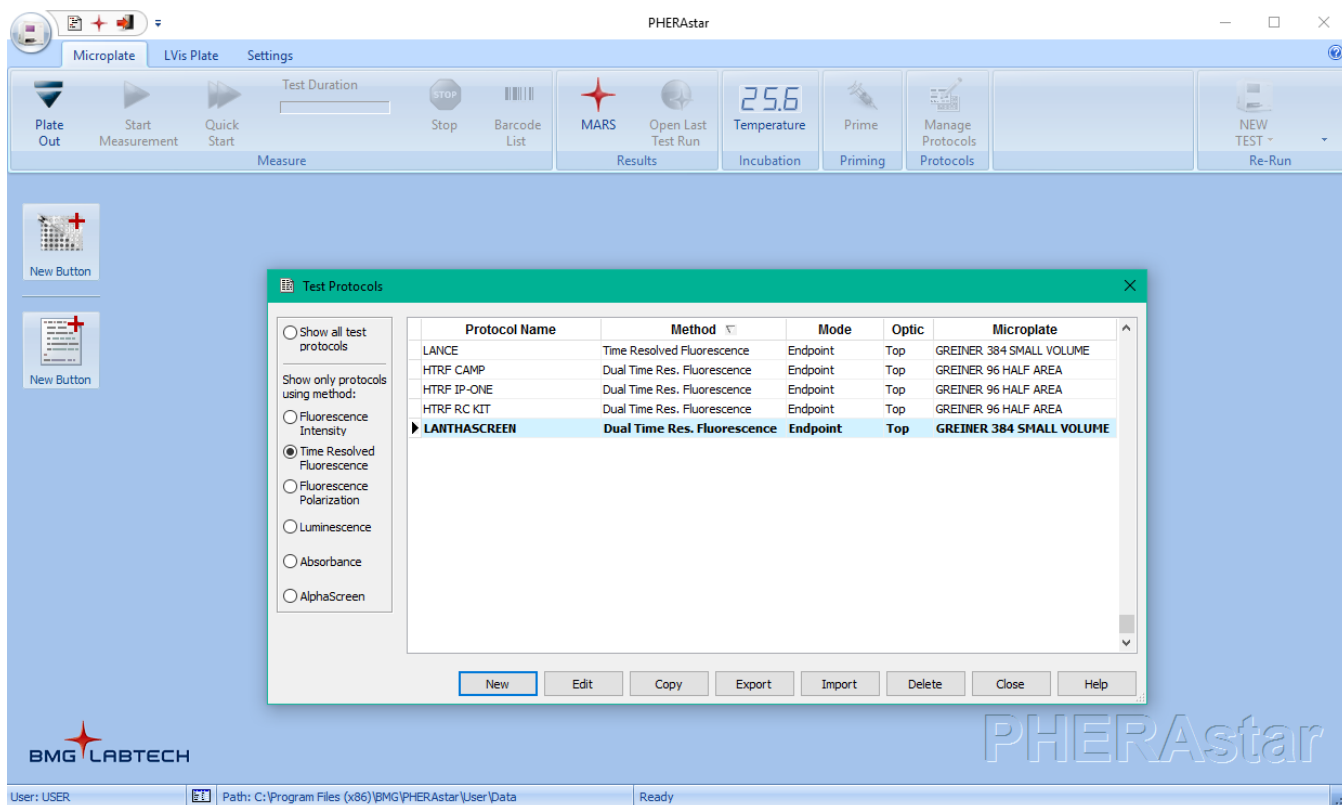
Version No.:
May 2016

Page 2 of 18

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

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.
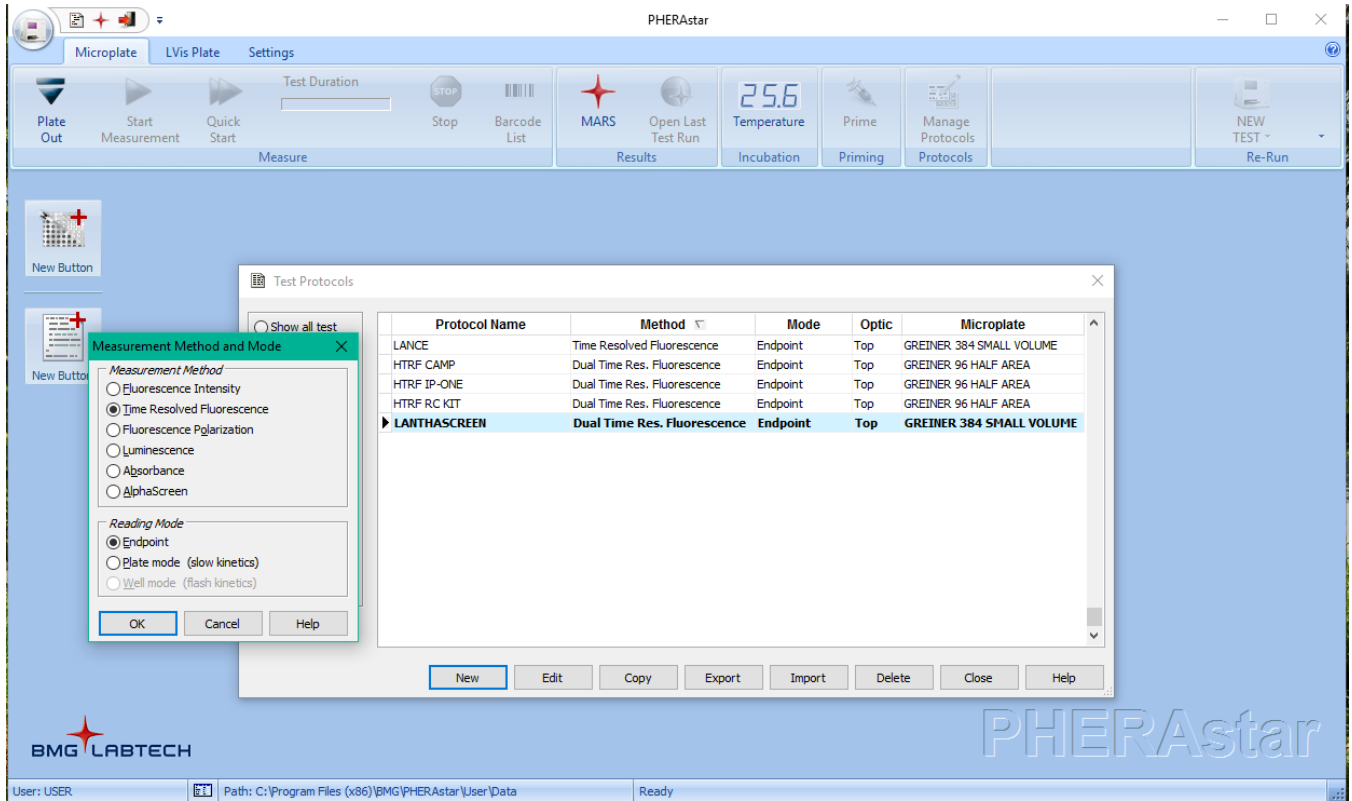
LanthaScreen® Eu Compatible
Microplate Reader Documentation

Version No.:
May 2016

Page 3 of 18
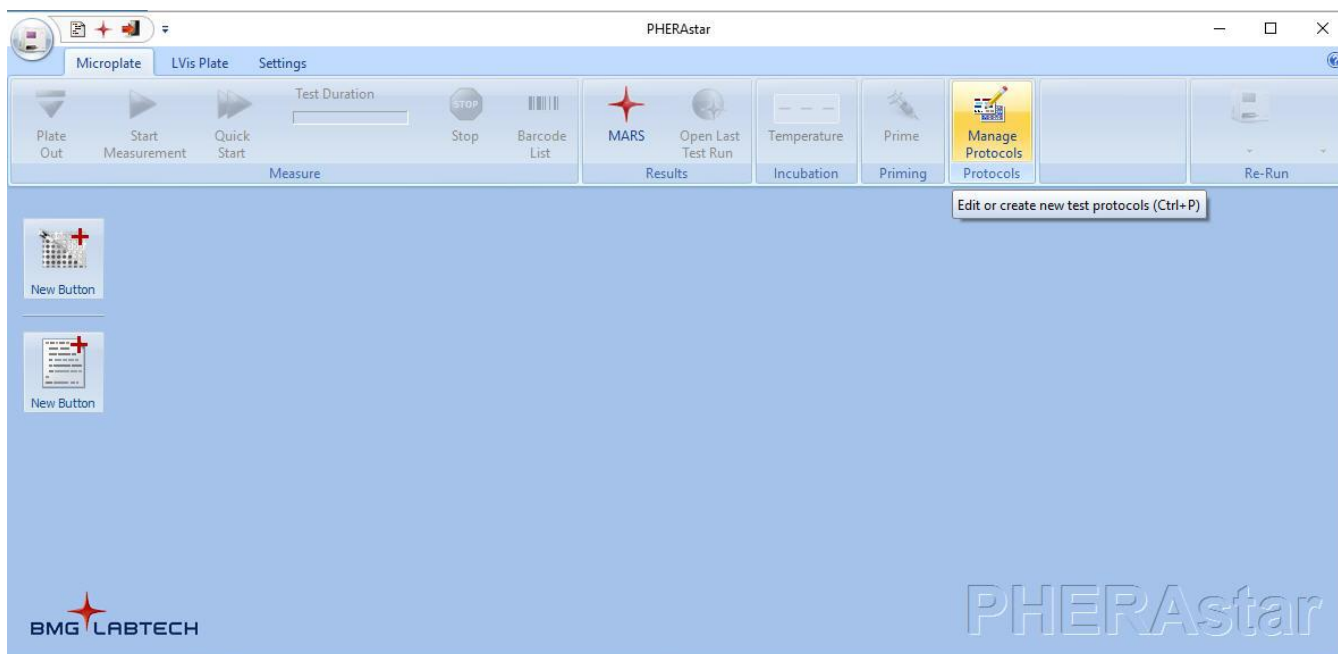
Setup Guide on the BMG LABTECH PHERAstar *FSX*® Microplate Readers

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.

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LanthaScreen® Eu Compatible
Microplate Reader Documentation

Version No.:
May 2016

Page 4 of 18

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

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

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LanthaScreen® Eu Compatible
Microplate Reader Documentation

Version No.:
May 2016

Page 5 of 18

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

5. A new protocol window will open automatically. Enter a test name, select plate type. We recommend Corning or Greiner 384 well small volume plates for LanthaScreen® Europium 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. When finished, select the "Layout" tab at the top of the Protocol window.
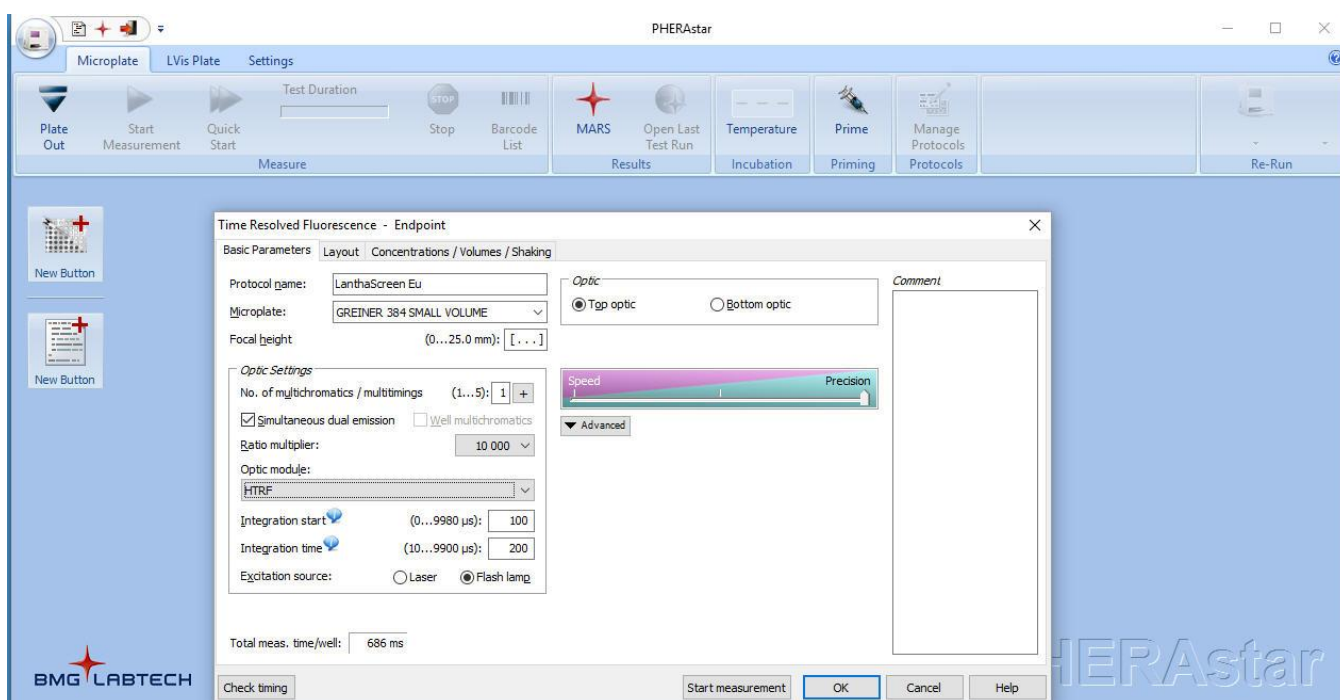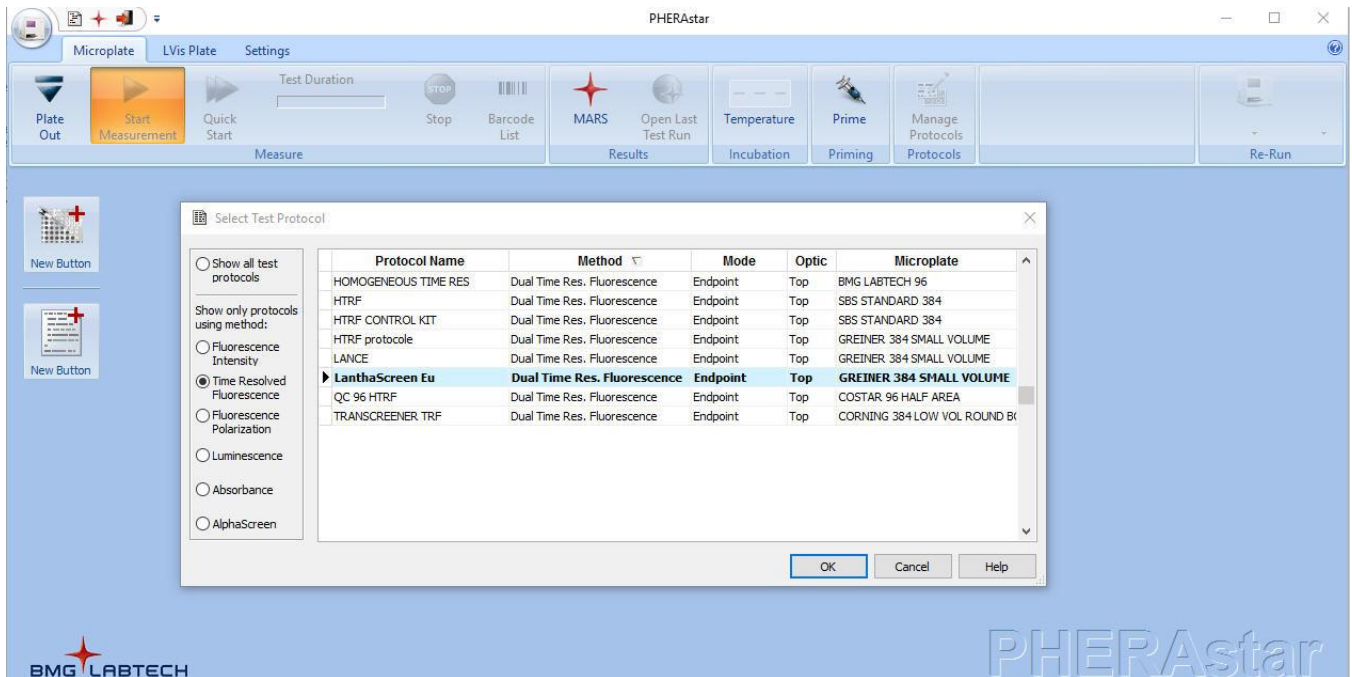

NOTE:

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).
ii)   The HTRF 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.

LanthaScreen® Eu Compatible
Microplate Reader Documentation

Version No.:
May 2016

Page 6 of 18

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

6.   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".
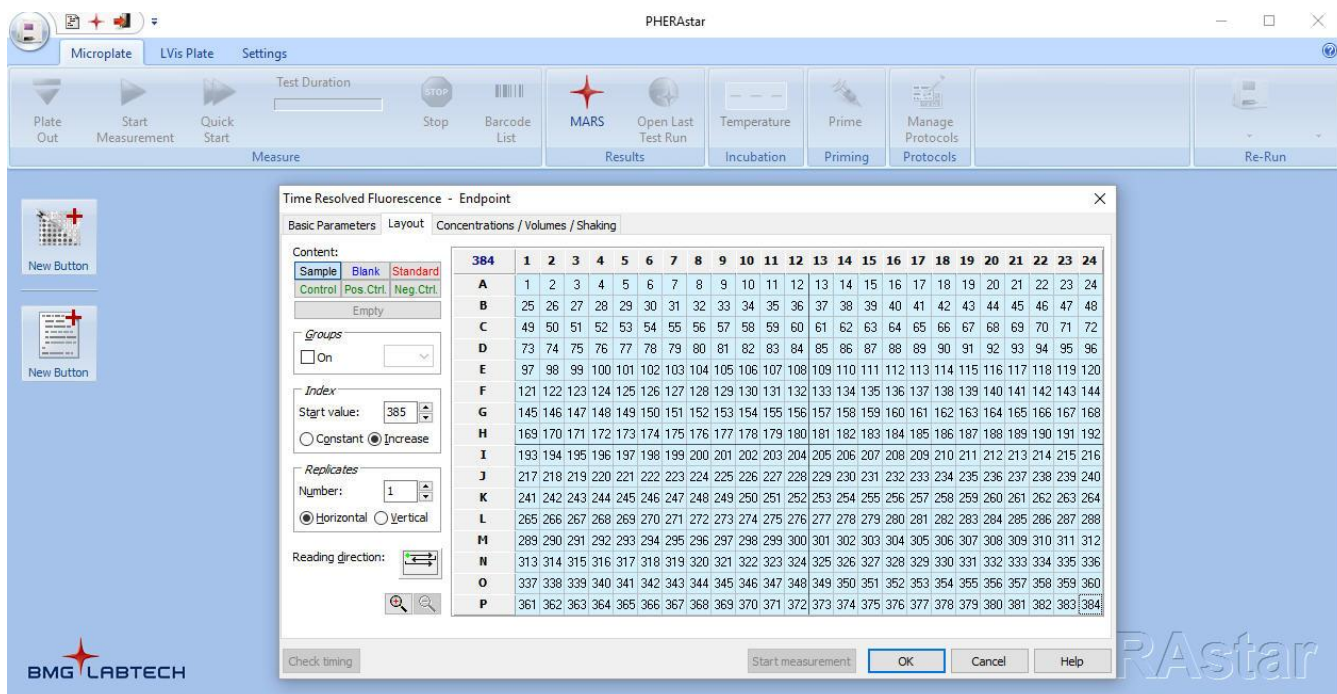
7. You will return to the main window. Then press the "Start Measurement" button. A new window will appear allowing you to select which test protocol(s) you wish to run. Select the protocol you created for LanthaScreen®, and then press "OK."

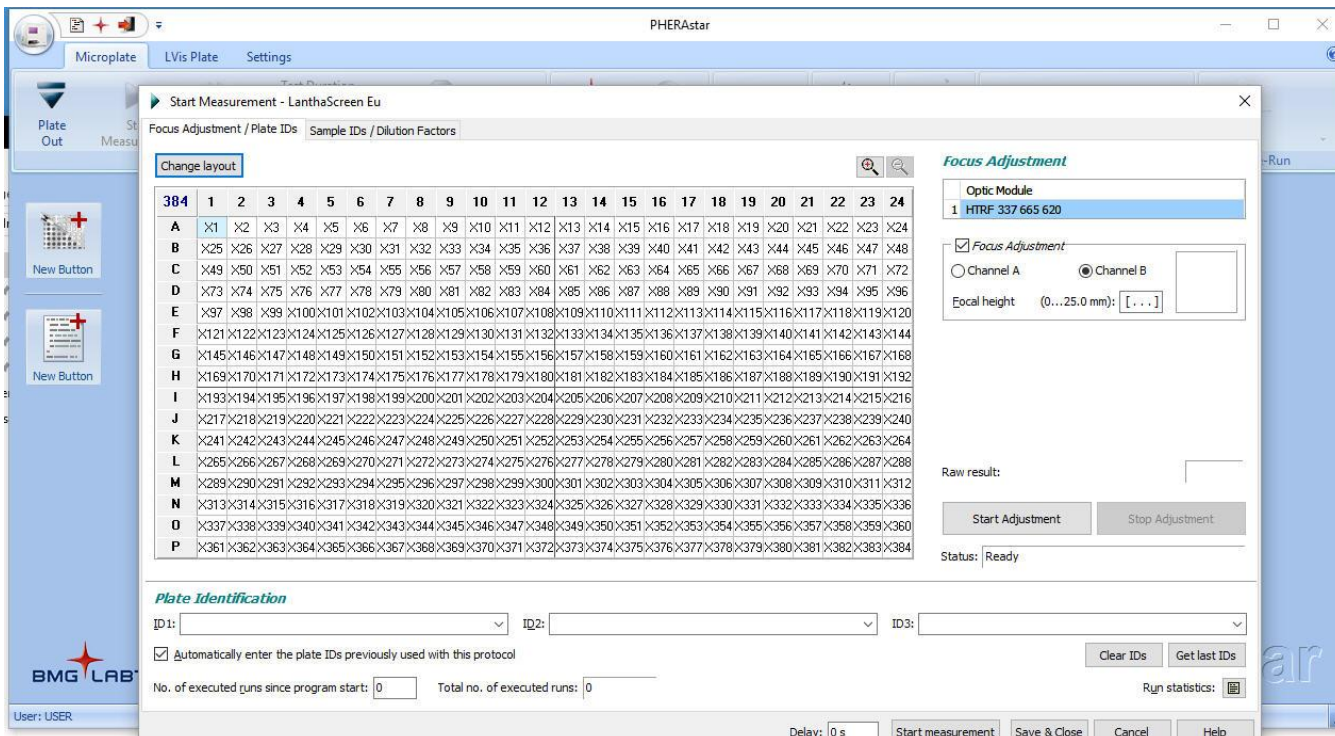| ThermoFisher SCIENTIFIC	LanthaScreen® Eu Compatible Microplate Reader Documentation	Version No.: May 2016	Page 7 of 18

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

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LanthaScreen® Eu Compatible
Microplate Reader Documentation

Version No.:
May 2016

Page 8 of 18

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

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 europium signal is read through channel B. Select "Focus Adjustment" and "Channel B", then select a well containing a sample with europium labeled antibody to use for adjusting focal height. Calibrate the height by clicking the "Start Adjustment" button.

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LanthaScreen® Eu Compatible
Microplate Reader Documentation

Version No.:
May 2016

Page 9 of 18

Setup Guide on the BMG LABTECH PHERAstar _FSX_® Microplate Readers

9. In a moment, the instrument will have calculated the optimal focal height. When finished, click on the "Start Measurement" tab to read.

LanthaScreen® Eu Compatible
Microplate Reader Documentation

Version No.:
May 2016

Page 10 of 18
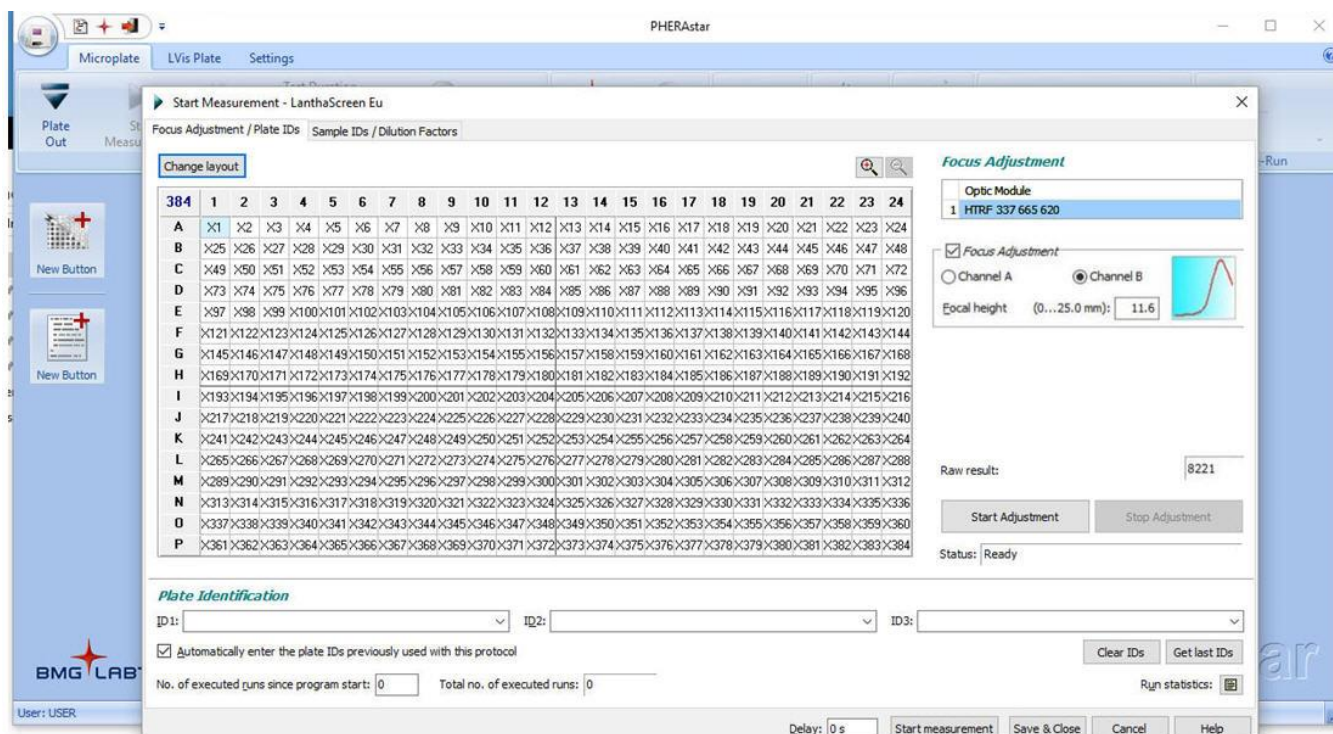
Setup Guide on the BMG LABTECH PHERAstar *FSX*® Microplate Readers

10. 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.

LanthaScreen® Eu Compatible
Microplate Reader Documentation

Version No.:
May 2016

Page 11 of 18
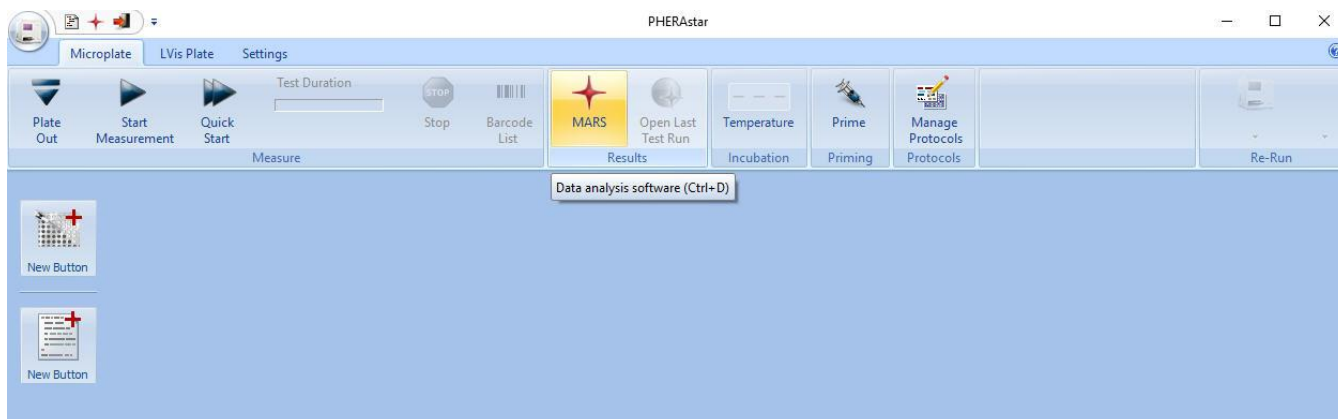
Setup Guide on the BMG LABTECH PHERAstar *FSX*® Microplate Readers

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

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LanthaScreen® Eu Compatible
Microplate Reader Documentation

Version No.:
May 2016

Page 12 of 18

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

12. Plots of these values are shown here.

A. Ratio Data

B. Normalized Data
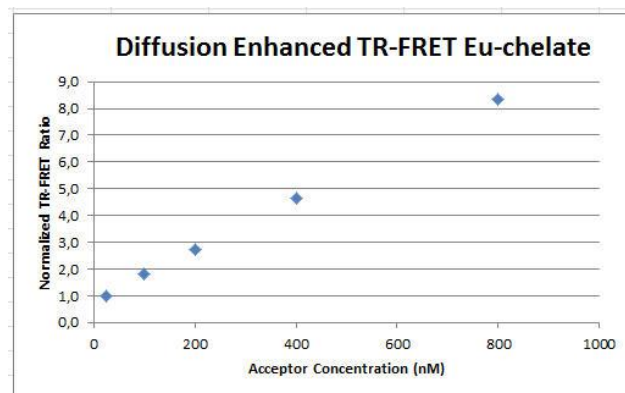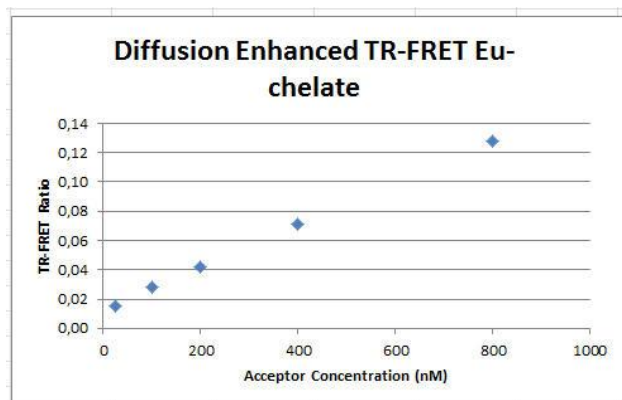
13. These test values were obtained using the following procedure.

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LanthaScreen® Eu Compatible
Microplate Reader Documentation

Version No.:
May 2016

Page 13 of 18

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

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: This document can be found at www.thermofishernologies.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 Eu chelate (found on the Certificate of Analysis), NOT the molarity of 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 call1-800-955-6288 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

ThermoFisher
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LanthaScreen® Eu Compatible
Microplate Reader Documentation

Version No.:
May 2016

Page 14 of 18

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

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	PV3179	PV3179

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

96-well plate 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 Acceptor (such as 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.

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LanthaScreen® Eu Compatible
Microplate Reader Documentation

Version No.:
May 2016

Page 15 of 18

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

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 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	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 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

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Thermo Fisher
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LanthaScreen® Eu Compatible
Microplate Reader Documentation

Version No.:
May 2016

Page 16 of 18

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

	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 pipettor 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@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 (620 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.

LanthaScreen® Eu Compatible
Microplate Reader Documentation

Version No.:
May 2016

Page 17 of 18

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

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 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
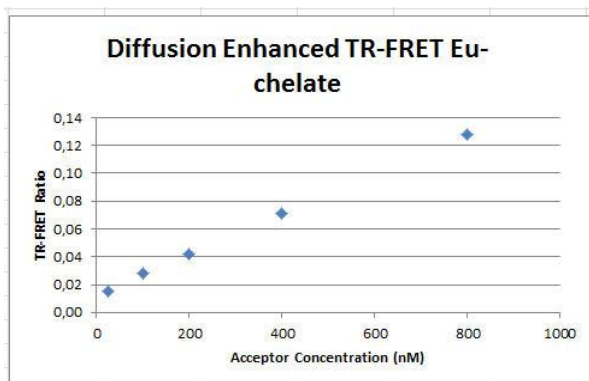
B. Normalized Data
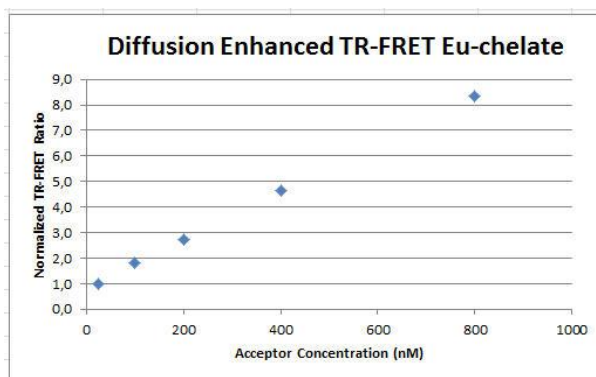
Columns Compared	Description
1 to 5	Largest window
2 to 5	Intermediate window
3 to 5	Small window
4 to 5	Smallest window

LanthaScreen® Eu Compatible
Microplate Reader Documentation

Version No.:
May 2016

Page 18 of 18

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

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

[Eu-chelate]	800 nM	400 nM	200 nM	100 nM	25 nM
Row A	0.123	0.070	0.041	0.027	0.016
Row B	0.126	0.070	0.038	0.033*	0.015
Row C	0.122	0.067	0.042	0.027	0.015
Row D	0.125	0.071	0.046	0.027	0.015
Row E	0.122	0.076	0.040	0.027	0.015
Row F	0.134	0.073	0.042	0.028	0.016
Row G	0.138	0.067	0.041	0.029	0.016
Row H	0.130	0.076	0.046	0.028	0.015
Row I	0.126	0.068	0.042	0.030	0.016

*outlier, not used for data analysis

Data Analysis:

[Acceptor]	800 nM	400 nM	200 nM	100 nM	25 nM
Average Ratio	0.128	0.071	0.042	0.028	0.015
St dev	0.0057	0.0034	0.0024	0.0019	0.0003
Assay Window	**8.5**	**4.7**	**2.8**	**1.9**	**Reference**
Z'-factor	0.84	0.79	0.71	0.67	

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