# LanthaScreen® Terbium (Tb) Assay Setup Guide on the Berthold Technologies Tristar² S LB 942 Microplate Reader

The Berthold Technologies Tristar² S LB 942 Microplate Reader was tested for compatibility with LanthaScreen® Activity Assay, a TR-FRET assay from Thermo Fisher Scientific, 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 Thermo Fisher Scientific 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 | 320/40 (Id. Nr 52733[1]) | Id. Nr. 62772 |
| **Emission 1** | 520 | 520/10uv (Id. Nr. 38836[1]) | Id. Nr. 68492 or 62772 |
| **Emission 2** | 495 | 495/10uv (Id. Nr. 39798[1]) | Id. Nr. 68492 |

[1] Although filters 52733, 38836 and 39798 are available separately, they need to be mounted in a special way to be suitable for LanthaScreen®. Use only the filters provided in filter packages 68492 or 62772. Do not disassemble, change or move the filters included in the LanthaScreen® 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 |

Filter Package 68492 includes

| | |
|---|---|
| Excitation slide: | 340/26, Id. Nr. 54083* |
| Emission slide: | 520/10uv, Id. Nr. 38836 |
| | 495/10uv, Id. Nr. 39798 |

* Not used in this application.

For this assay the excitation slide from filter package 62772 and the emission slide from filter package 68492 are used.

**Note**: Eu-based TR-FRET and Tb-based TR-FRET use different excitation filters for optimal performance.
**Note:** Monochromator based detection is not recommend for TR-FRET assays.

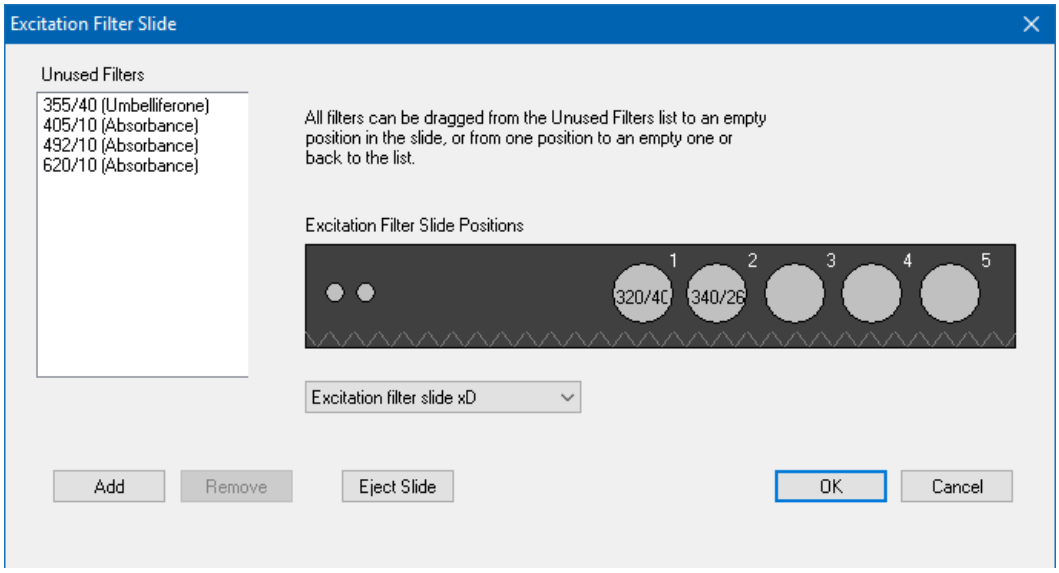
## B. Instrument Setup

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.

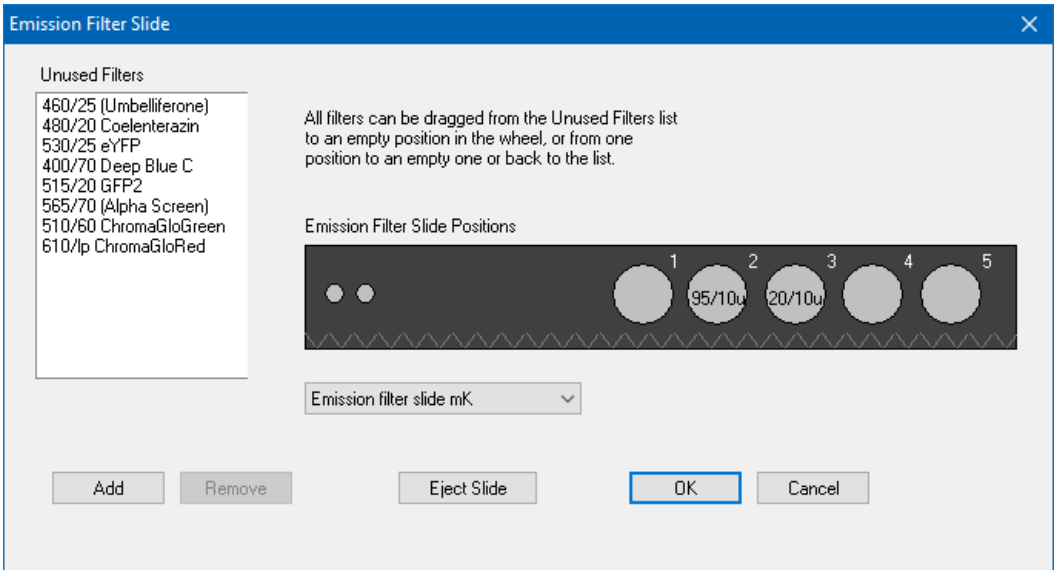
2. Click on Instrument >> Excitation Filter Slide. Check if the right filters are assigned to the right positions of the filter slides; if they are not, assign each filter to the corresponding position in the filter slide. Please follow the example below:

   Excitation slide xD (from filter package 62772)
   Slot 1: 320/40 (HTRF Eu cryptate); usage: TRFluorescence
   Slot 2: 340/26 (HTRF Tb cryptate); usage: TRFluorescence



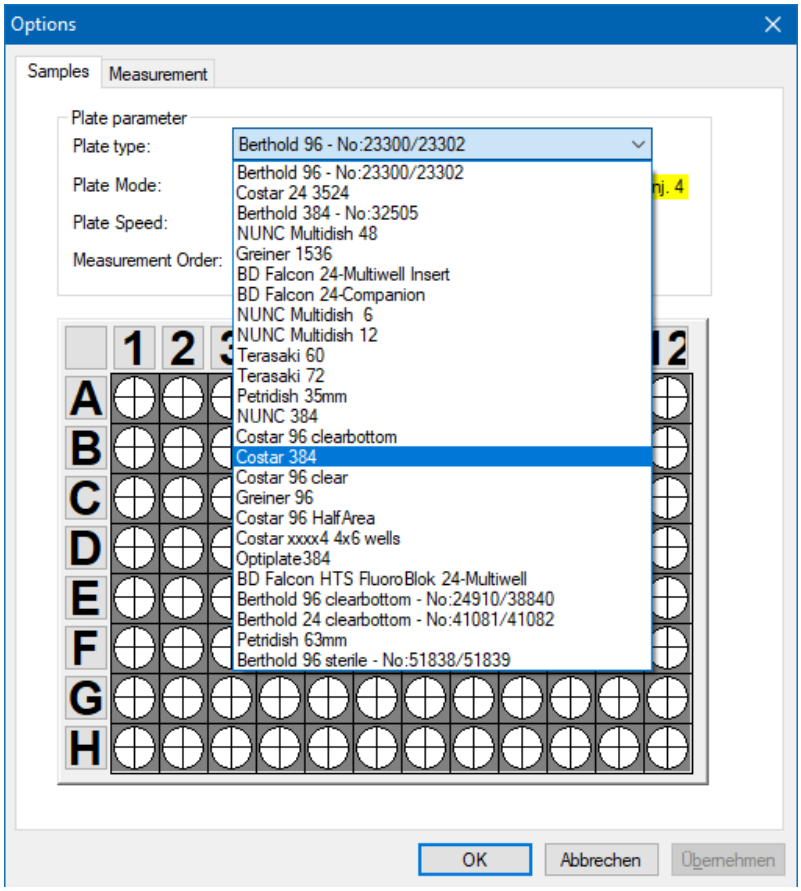
3. Click on Instrument >> Emission Filter Slide. Check if the right filters are assigned to the right positions of the filter slides; if they are not, assign each filter to the corresponding position in the filter slide (add new filters and enter the settings below if needed). Please follow the example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

   Emission Slide mK (from filter package 68492)
   Slot 2: 495/10uv; usage: TRFluorescence
   Slot 3: 520/10uv; usage: TRFluorescence

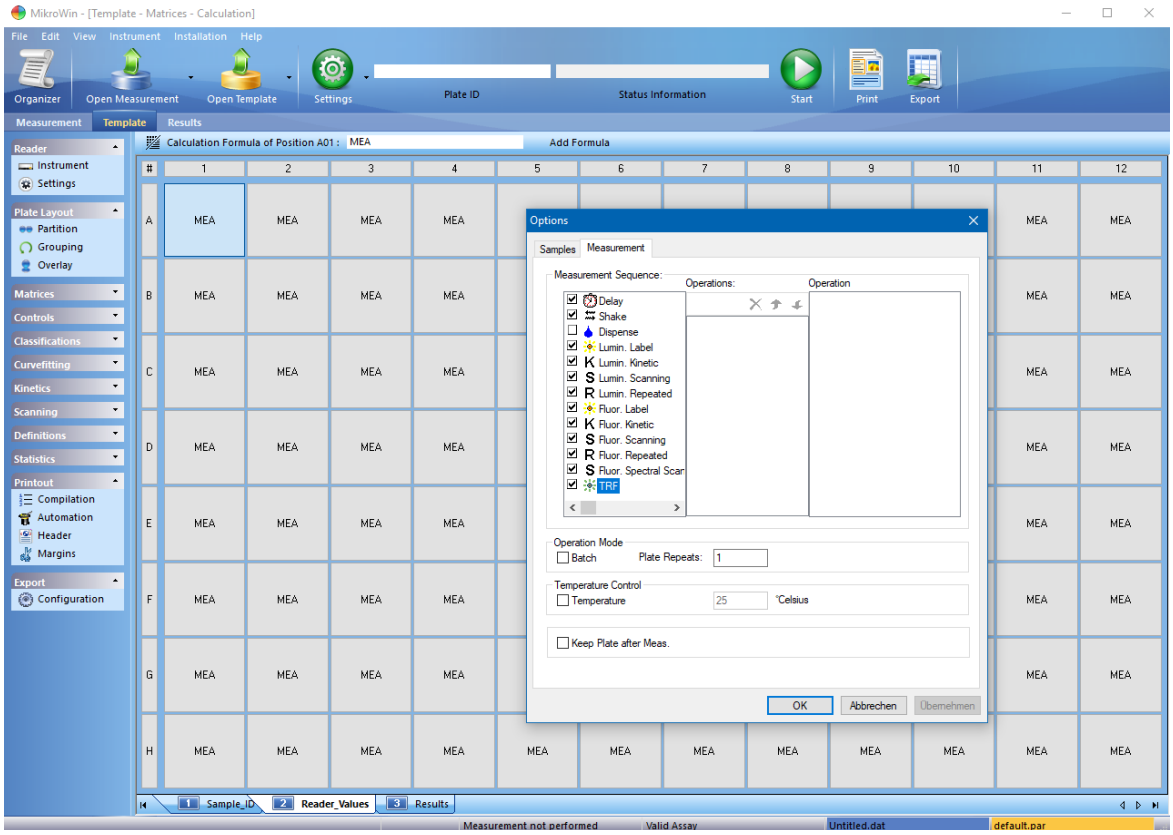



4. If you already have a pre-existing template for LanthaScreen®, open it and use this document to review your settings; if you don't have yet any suitable template, click on Settings in the menu bar at the top portion of the window to start creating a new template.

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.

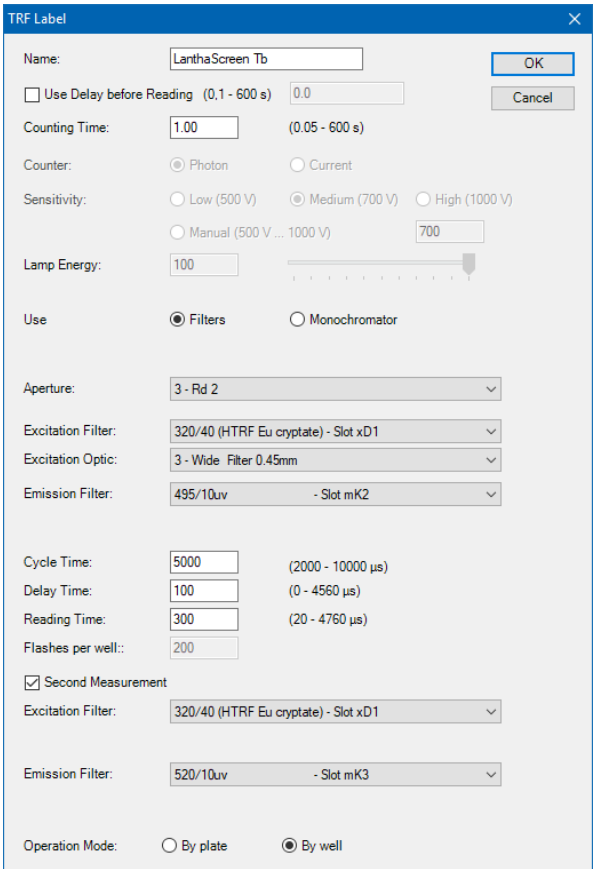


7. Double click on TRF to insert a TRF measurement operation. A new window will appear. If desired, enter a Name for the measurement operation. Configure the settings as shown in the screenshot below:

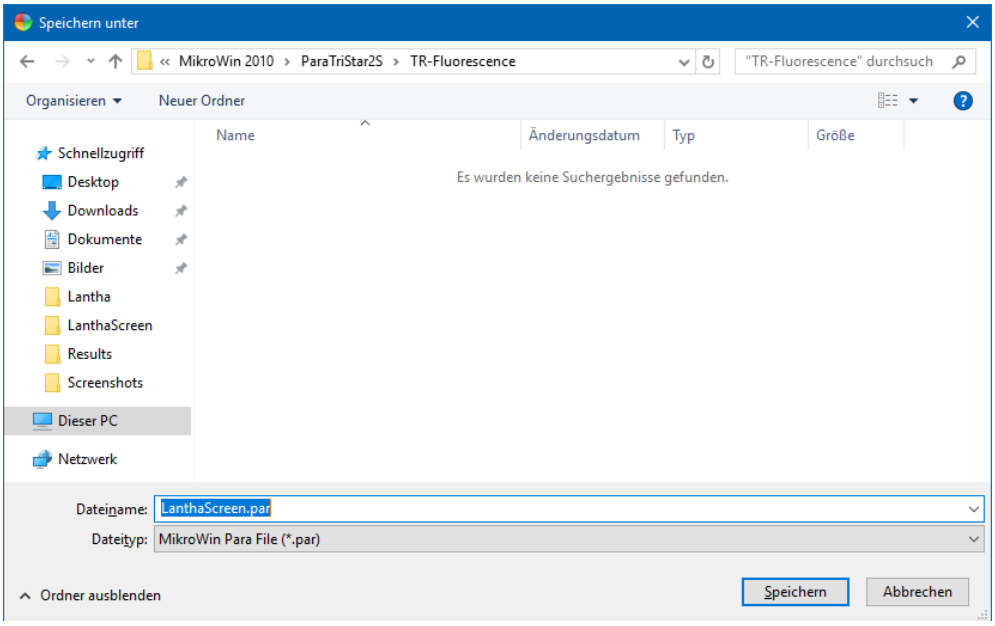
- Enter Counting Time: 1.00
- Select Aperture: 3 (Rd 2)
- Select Excitation Filter: 320/40 (HTRF Eu cryptate)*
- Select Excitation Optic: 3 – Wide Filter 0.45mm
- Select Emission Filter: 495/10uv*
- Enter Timing settings: Cycle Time 5000, Delay Time 100, Reading Time 300
- Check Second Measurement
- Select Excitation Filter: 320/40 (HTRF Eu cryptate)*
- Select Emission Filter: 520/10uv*

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.



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.



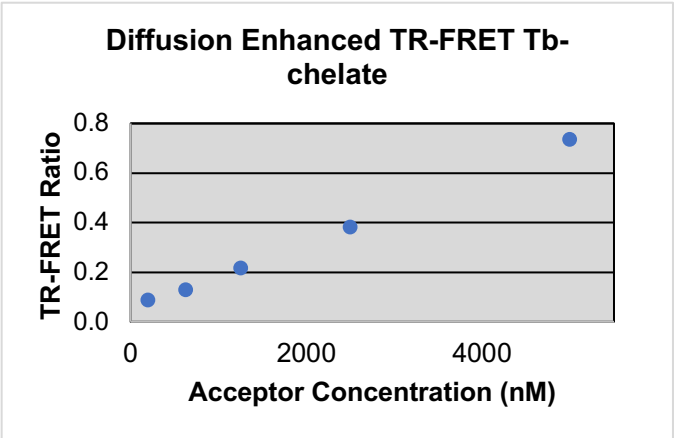

9. To start the measurement, enter the desired Plate ID to identify the measurement. If you want to edit the wells to be measured, click on Settings and select the desired wells (see point 3). When you are ready, click Start. The plate tray will open; insert the plate and click OK to start the measurement.

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

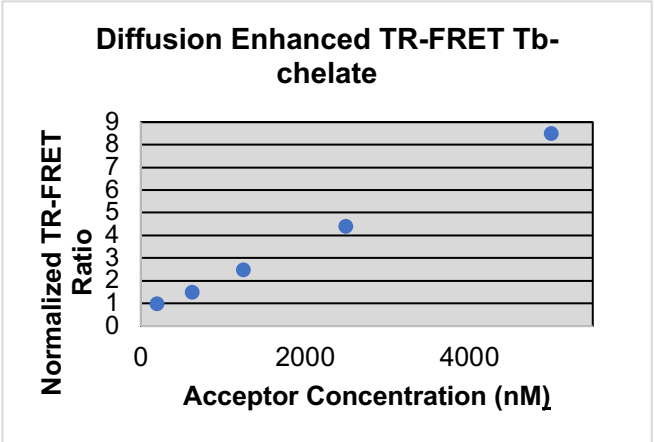
| Donor signal (495 nm) | | | | | |
|---|---|---|---|---|---|
| [Acceptor] | 5000 nM | 2500 nM | 1250 nM | 625 nM | 200 nM |
| A | 72080 | 114730 | 138331 | 154378 | 166714 |
| B | 84620 | 110952 | 167138 | 164687 | 172279 |
| C | 84353 | 119646 | 163230 | 158120 | 179057 |
| D | 93596 | 119719 | 133115 | 178583 | 181955 |
| E | 81901 | 116300 | 159747 | 170857 | 175962 |
| F | 78187 | 121427 | 151868 | 173419 | 176770 |
| G | 90046 | 109680 | 153657 | 173173 | 183518 |
| H | 75351 | 125453 | 155489 | 171273 | 163278 |
| I | 76437 | 118729 | 149655 | 167632 | 176547 |
| J | 72668 | 109586 | 140843 | 165270 | 177934 |
| | | | | | |
| Acceptor signal (520 nm) | | | | | |
| [Acceptor] | 5000 nM | 2500 nM | 1250 nM | 625 nM | 200 nM |
| A | 59800 | 47679 | 31795 | 20861 | 14986 |
| B | 58916 | 44836 | 35194 | 21540 | 14895 |
| C | 58518 | 45205 | 33611 | 20813 | 16441 |
| D | 66299 | 41950 | 29785 | 22536 | 15696 |
| E | 59961 | 40919 | 34110 | 22562 | 15093 |
| F | 54247 | 46138 | 32433 | 22146 | 16135 |
| G | 59240 | 40088 | 32845 | 21755 | 15922 |
| H | 58345 | 47410 | 31741 | 21392 | 14084 |
| I | 58952 | 46061 | 31982 | 21586 | 14125 |
| J | 57458 | 45350 | 31469 | 21430 | 14488 |

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® 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 Fluormones. 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 10.

**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 pages 11 - 12 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 14.

## 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 Fluormone 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) | -20ºC | Various | PV3550 |
| LanthaScreen® fluorescein-labeled substrate (acceptor) | -20ºC | 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 5 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.

***Note:*** This application is NOT suited for LanthaScreen® GFP-tagged physiological substrates or Nuclear Receptor Fluormones. Instead, use Fluorescein-poly GT (Cat no. PV3610) or other Fluorescein-peptides as the acceptor.

**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 | 118.4 µL |
| **Volume 30 µM Acceptor (prepared above)** | 40.0 µL | 20.0 µL | 10.0 µL | 5.0 µL | 1.6 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 | $C_2$ |
|---|---|---|---|---|---|---|---|
| | | | **[Stock]** | | | | **[2X]** |
| 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 the 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:

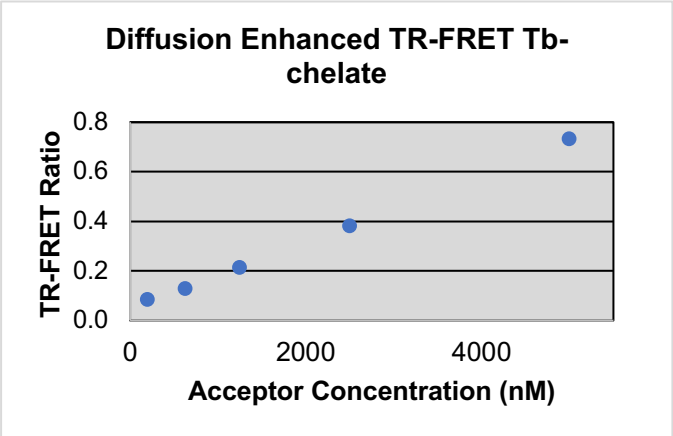
1. Obtain the emission ratios by dividing the acceptor signal (520 nm) by the donor signal (495 nm, exact wavelength varies with instrument) for each well.

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.

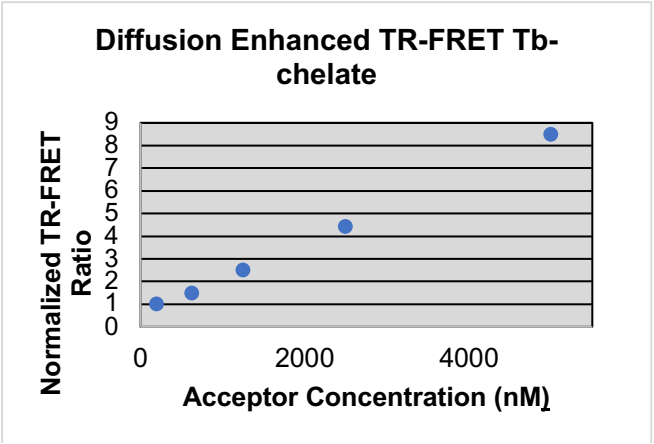
3. Using the data from column 5 (200 nM acceptor) as the bottom of the "assay window," divide the average ratios from the other columns b the average ratio from column 5 to obtain a range of simulated "assay window" sizes. See the example data below. This "normalized" data can be plotted against the 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 |

**Example data:** Ratiometric data obtained on a Berthold Technologies Tristar² LB 942 microplate reader.

| [Acceptor] | 5,000 nM | 2,500 nM | 1,250 nM | 625 nM | 200 nM |
|---|---|---|---|---|---|
| Row A | 0.830 | 0.416 | 0.230 | 0.135 | 0.090 |
| Row B | 0.696 | 0.404 | 0.211 | 0.131 | 0.086 |
| Row C | 0.694 | 0.378 | 0.206 | 0.132 | 0.092 |
| Row D | 0.708 | 0.350 | 0.224 | 0.126 | 0.086 |
| Row E | 0.732 | 0.352 | 0.214 | 0.132 | 0.086 |
| Row F | 0.694 | 0.380 | 0.214 | 0.128 | 0.091 |
| Row G | 0.658 | 0.365 | 0.214 | 0.126 | 0.087 |
| Row H | 0.774 | 0.378 | 0.204 | 0.125 | 0.086 |
| Row I | 0.771 | 0.388 | 0.214 | 0.129 | 0.080 |
| Row J | 0.791 | 0.414 | 0.223 | 0.130 | 0.081 |

**Data Analysis:**

| [Acceptor] | 5,000 nM | 2,500 nM | 1,250 nM | 625 nM | 200 nM |
|---|---|---|---|---|---|
| **Average Ratio** | 0.735 | 0.382 | 0.215 | 0.129 | 0.087 |
| **St dev** | 0.054 | 0.023 | 0.008 | 0.003 | 0.004 |
| **% CV** | 7.4 | 6.1 | 3.8 | 2.5 | 4.4 |
| **Assay Window** | **8.49** | **4.42** | **2.49** | **1.49** | **Reference** |
| **Z'-factor** | 0.73 | 0.73 | 0.72 | 0.50 | |

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