LanthaScreen® TR-FRET Phospho-specific Antibody Reagents and Substrates User Guide

Shipping: Dry Ice **Storage: –20°C**

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1. Reagents Available

For a complete list of available LanthaScreen® activity assays and reagents, visit www.invitrogen.com/lanthascreenkinase.

2. Principles of TR-FRET

For screening libraries of compounds, time-resolved FRET (TR-FRET) is a recognized method for overcoming interference from compound autofluorescence or light scatter from precipitated compounds. The premise of a TR-FRET assay is the same as that of a standard FRET assay: when a suitable pair of fluorophores is brought within proximity of one another, excitation of the first fluorophore (the donor) can result in energy transfer to the second fluorophore (the acceptor). This energy transfer is detected by an increase in the fluorescence emission of the acceptor, and a decrease in the fluorescence emission of the donor. In HTS assays, FRET is often expressed as a ratio of the intensities of the acceptor and donor fluorophores. The ratiometric nature of such a value corrects for differences in assay volumes between wells, as well as corrects for quenching effects due to colored compounds.

In contrast to standard FRET assays, TR-FRET assays use a long-lifetime lanthanide chelate as the donor species. Lanthanide chelates are unique in that their excited state lifetime (the average time that the molecule spends in the excited state after accepting a photon) can be on the order of a millisecond or longer. This is in sharp contrast to the lifetime of common fluorophores used in standard FRET assays, which are typically in the nanosecond range. Because interference from autofluorescent compounds or scattered light is also on the nanosecond timescale, these factors can negatively impact standard FRET assays. To overcome these interferences, TR-FRET assays are performed by measuring FRET after a suitable delay, typically 50 to 100 microseconds after excitation by a flashlamp excitation source in a microtiter plate reader. This delay not only overcomes interference from background fluorescence or light scatter, but also avoids interference from direct excitation due to the non-instantaneous nature of the flashlamp excitation source.

The most common lanthanides used in TR-FRET assays for HTS are terbium (Tb) and europium (Eu). The emission spectrum of the europium reagents are red shifted in comparison to the terbium emission. See Figure 1. As a result, the europium LanthaScreen® labeled reagents are ideally suited for pairing with red shifted dyes (including Alexa Fluor® 647), while the terbium LanthaScreen® labeled reagents are used with common green fluorophores (such as fluorescein) or Green Fluorescent Protein.

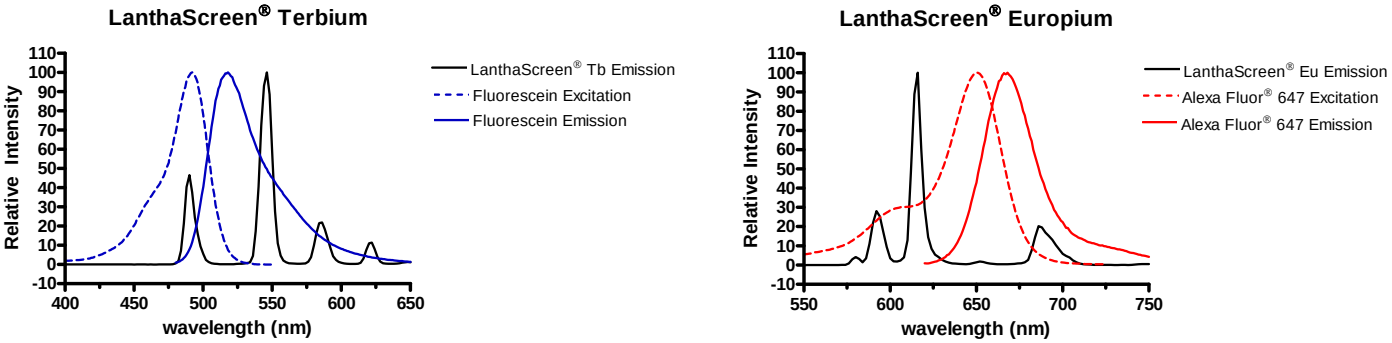

Figure 1. Emission and excitation spectra of the LanthaScreen® terbium and europium reagents and their matched fluorophores.

3. LanthaScreen® Kinase Activity Assay Principle

The principle of a LanthaScreen® Tb Kinase Activity Assay is shown in Figure 2. The assay itself can be divided into two phases: the reaction phase and the detection phase. In the reaction phase, all components required for the kinase reaction are added to the well, including fluorescein-labeled substrate, and the reaction is allowed to incubate for a set period of time, typically 30–60 minutes.

After the reaction, EDTA is added to stop the kinase reaction, and terbium-labeled antibody is added to bind phosphorylated product. Because the terbium chelate is relatively stable in EDTA, the antibody and EDTA can be pre-mixed to minimize pipetting steps. Binding of the terbium-labeled antibody to the fluorescein-labeled phosphorylated product brings the terbium and fluorescein into proximity, resulting in an increase in TR-FRET. In the presence of an inhibitor, formation of phosphorylated product is reduced, and the TR-FRET value is decreased.

While LanthaScreen® Tb reagents were specifically discussed in this example, the same theory can be applied to the LanthaScreen® Eu activity assays.

A growing list of Invitrogen kinases have been validated for use with LanthaScreen® Kinase Activity Assay reagents. Please visit www.invitrogen.com/lanthascreenkinase to see if your kinase of interest has been validated.

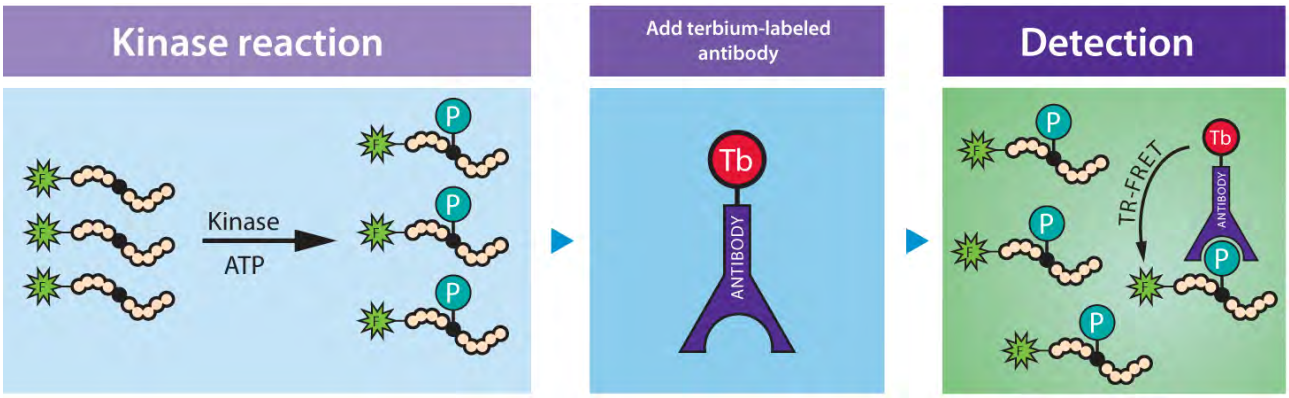

Figure 2. Mechanism for LanthaScreen® Tb Kinase Activity Assay.

4. Instrument Settings

To aid in instrument set-up, Instrument Control Kits are available for the LanthaScreen® assay kits and reagents. Please contact Invitrogen Drug Discovery technical support at 800-955-6288 (select option 3 and enter 40266) or email drugdiscoverytech@invitrogen.com for assistance.

Instrument Control Kit	Unit	Catalog no.
LanthaScreen® Tb Instrument Control Kit	Each	PV5591
LanthaScreen® Eu Instrument Control Kit	Each	Please contact technical support for details

4.1 LanthaScreen® Tb Kinase Activity Assay Filter Selection

As with other TR-FRET systems, the terbium donor is excited using a 340 nM excitation filter with a 30 nm bandpass. However, the exact specifications of the excitation filter are not as critical, and filters with similar specifications will work well. In general, excitation filters that work with europium-based TR-FRET systems will perform well with the LanthaScreen® terbium chelates.

As is shown in Figure 1, four sharp emission peaks characterize the terbium emission spectrum, with silent regions between each peak. The first terbium emission peak (centered between approximately 485 and 505 nm) overlaps nearly perfectly with the maximum excitation peak of fluorescein. Energy transfer to fluorescein is then measured in the silent region between the first two terbium emission peaks.

Because it is important to measure energy transfer to fluorescein without interference from terbium, a filter centered at 520 nm with a 25 nm bandpass is used for this purpose. The specifications of this filter are more critical than those of the excitation filter. In general, **standard "fluorescein" filters may not be used**, because such filters also pass light associated with the terbium spectra. The emission of fluorescein due to FRET is referenced (or "ratioed") to the emission of the first terbium peak, using a filter that isolates this peak. This is typically accomplished with a filter centered at 490 or 495 nm, with a 10 nm bandpass.

In general, a 490 nm filter will reduce the amount of fluorescein emission that "bleeds through" into this measurement, although instrument dichroic mirror choices (such as those on the Tecan Ultra Evolution instrument) may necessitate the use of a 495 nm filter. The effect on the quality of the resulting measurements is minimal in either case. Excitation and emission filters for most microplate readers are available directly from Invitrogen at www.invitrogen.com/LanthaScreen. A LanthaScreen® Tb filter module for the BMG PHERAstar is available from BMG Instruments.

Filter-based Instruments: General Settings for LanthaScreen® Tb Kinase Activity Assays	
Excitation:	340 nm filter (30 nm bandwidth)
Fluorescein Emission:	520 nm filter (25 nm bandwidth)
Terbium Emission:	490 or 495 nm filter (10 nm bandwidth)
Dichroic Mirror:	Fluorescein (Tecan Ultra, GENios, Infinite®, GENios Pro) LANCE/TRF (Perkin Elmer EnVision®, Victor™ *X*) 380 nm (preferred) or 400 nm (Molecular Device Analyst®) Built In (BMG Labtech PHERAstar)
Delay Time:	100 µs
Integration Time:	200 µs

Monochromator-based Instruments: General Settings for LanthaScreen® Tb Kinase Activity Assays	
Excitation:	332 nm (20 nm bandwidth)
Fluorescein Emission:	515 nm (20 nm bandwidth)
Terbium Emission:	486 nm (20 nm bandwidth)
Delay Time:	100 µs
Integration Time:	200 µs

Some monochromator-based instruments, such as the Tecan Infinite® M1000, allow each emission wavelength to be individually set. In this case, match the settings to those listed for the filter-based instruments above. When the bandwidths of the two emission wavelengths cannot be individually set, use the general settings for monochromator-based instruments listed in the table above as a guide.

Note that not all monochromator-based instruments are capable of a 20-nm bandwidth; smaller bandwidth settings may be used, but with a decrease in assay performance. Additionally, we have found that while some monochromator based instruments (Tecan Safire^{2}™, Tecan Infinite® M1000) give satisfactory performance in LanthaScreen® Tb activity assays, other monochromator-based instruments (*e.g.*, the Tecan Safire™ and Molecular Devices Gemini series) may not be optimal for detection, although some change in signal and a ratio may still be observed. We have also found that with some assays, white plates give better assay performance than black plates when using monochromator-based instruments. In filter-based instruments, the difference is typically negligible.

4.2 LanthaScreen® Eu Kinase Activity Assay Filter Selection

Filter sets that are used with other europium-based technologies may also be used with the LanthaScreen® Eu reagents.

In general, excitation filters that work with other europium-based TR-FRET systems will perform well with LanthaScreen® Eu reagents. Typically, the europium donor is excited using a 340 nM excitation filter with a 30 nm bandwidth. However, the exact specifications of the excitation filter are not critical, and filters with similar specifications will work well. Emission from the FRET acceptor (Alexa Fluor® 647) is divided by (or ratioed) to the emission of the FRET donor (europium chelate) using filters at 665 nm and 620 nm, respectively, each with a 10 nm bandpass.

Filter-based Instruments: General Settings for LanthaScreen® Eu Kinase Activity Assays	
Excitation:	340 nm filter (30 nm bandwidth)
Alexa Fluor® 647 Emission:	665 nm filter (10 nm bandwidth)
Europium Emission:	620 nm filter (10 nm bandwidth)
Dichroic Mirror:	Automatic (Tecan Infinite®) LANCE/TRF (Perkin Elmer EnVision®) Built In (BMG Labtech PHERAstar)
Delay Time:	100 µs
Integration Time:	200 µs

For monochromator-based instruments, the same excitation and emission wavelengths are used. However, it is recommended that the bandwidths be increased to 20 nm to increase the amount of light reaching the detector.

Monochromator-based Instruments: General Settings for LanthaScreen® Eu Kinase Activity Assays	
Excitation:	332 nm (20 nm bandwidth)
Fluorescein Emission:	515 nm (20 nm bandwidth)
Terbium Emission:	486 nm (20 nm bandwidth)
Delay Time:	100 µs
Integration Time:	200 µs

4.3 Other Instrument Settings

Aside from filter choices, instrument settings are similar to the settings used with other TR-FRET based technologies and are identical if using LanthaScreen® Tb or Eu reagents. In general, guidelines provided by the instrument manufacturer can be used as a starting point for optimization. A delay time of 100 µs, followed by a 200 µs integration time, would be typical for a LanthaScreen® activity assay. The number of flashes or measurements per well is highly instrument dependant and should be set as advised by your instrument manufacturer. In general, LanthaScreen® activity assays can be run on any filter-based instrument capable of time-resolved FRET, such as the Tecan Infinite® F500, BMG LABTECH PHERAstar, Molecular Device Analyst®, or PerkinElmer EnVision®. Monochromator-based instruments such as the Tecan Safire^{2}™ or Molecular Devices SpectraMax® M5 are also capable of reading TR-FRET, but with data quality that is often less than that seen with filter-based instruments.

Ask your Invitrogen sales representative for instrument-specific setup guidelines, or contact Invitrogen Drug Discovery technical support at 800-955-6288 (select option 3 and enter 40266), or email drugdiscoverytech@invitrogen.com for more information on performing LanthaScreen® activity assays on your particular instrument.

5. Standard Assay Conditions

Specific assay conditions should be optimized by the end user to achieve maximal assay performance. However, we have found a standard set of reagent concentrations to be optimal for a majority of LanthaScreen® activity assays:

Reagent	Final 1X Concentration in Assay	Cat. no.
LanthaScreen® Antibody	2 nM	varies
Fluorescein or Alexa Fluor® 674 Labeled Substrate	200–400 nM	varies
Kinase Quench Buffer (500 mM EDTA)	10 mM	P2825

A growing list of Invitrogen kinases have been validated for use with LanthaScreen® Kinase Activity Assay reagents. To see specific assay conditions for your kinase of interest, please visit www.invitrogen.com/lanthascreenkinase for detailed validation packets.

6. Data Analysis

Typically a TR-FRET value (or ratio) is calculated by dividing the acceptor signal by the donor signal. For LanthaScreen® Tb reagents, this would be the 520 nm signal divided by the 495 nm signal (i.e. 520nm/495nm). Alternatively, for the LanthaScreen® Eu reagents, this would be the 665 nm signal divided by the 620 nm signal (*i.e.*, 665nm/620nm). Because the underlying donor and acceptor signals are dependant on instrument settings (such as instrument gain), the TR-FRET ratio, and the resulting "top" and "bottom" of an assay window will depend on these settings as well, and will vary from instrument to instrument. What is important in determining the robustness of an assay is not the size of the window as much as the size of the errors in the data relative to the difference in the maximum and minimum values. It is for this reason that the "Z prime" value proposed by Zhang and colleagues, which takes these factors into account, is the correct way to assess data quality in a TR-FRET assay (Zhang *et al.*, 1999).

For convenience, titration curves can be normalized by dividing all values in the curve by the ratio obtained at the bottom of the curve. For example, if the bottom of the curve begins at a ratio of 0.014, divide all values in the curve by 0.014. This will normalize the titration curves making data comparison between various instruments and gain settings easier. See Figure 3 below. There is no effect on the IC_{50} values or Z prime.

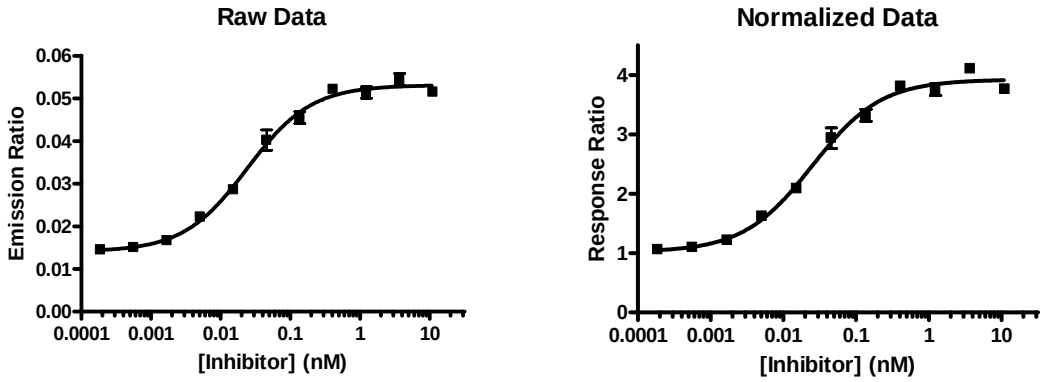

Figure 3. To normalize the data, the raw emission ratios in the left graph were divided by the ratio from the bottom of the curve (*i.e.*, 0.014) to create the normalized data in the right graph. Normalization of the data is useful when comparing data between instruments, or experiments that were run under different instrument settings (*e.g.*, different gain settings).

7. References

Zhang, J. H., Chung, T. D., and Oldenburg, K. R. (1999) A simple statistical parameter for use in evaluation and validation of high-throughput screening assays. *J. Biomol. Screen., 4*, 67-73

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