LanthaScreen™ Terbium Labeled TR-FRET Secondary Antibody Reagents-User Guide

Cat. nos. PV3765, PV3767, PV3769, PV3771, PV3773, PV3775, PV3777, and PV3779

O-062132-r1 US 0405

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1.0 REAGENTS AVAILABLE

Reagents	Size	Cat. no.
LanthaScreen™ Tb-anti-Mouse Antibody	25 µg	PV3765
	1 mg	PV3767
LanthaScreen™ Tb-anti-Goat Antibody	25 µg	PV3769
	1 mg	PV3771
LanthaScreen™ Tb-anti-Rabbit Antibody	25 µg	PV3773
	1 mg	PV3775
LanthaScreen™ Tb-anti-Human Antibody	25 µg	PV3777
	1 mg	PV3779

2.0 INTRODUCTION

When 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 are brought within close 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, and 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 and europium. Terbium offers unique advantages over europium when used as the donor species in a TR-FRET assay. In contrast to europium based systems that employ APC as the acceptor, terbium-based TR-FRET assays can use common fluorophores such as fluorescein as the acceptor. Because it is straightforward (and inexpensive) to label a molecule such as a peptide with fluorescein, directly labeled biomolecules may be used in terbium-based TR-FRET assays, rather than biotinylated molecules that must then be indirectly labeled via streptavidin-mediated recruitment of APC. The use of directly labeled molecules in a terbium-based TR-FRET assay reduces costs, improves kinetics, avoids problems due to steric interactions involving large APC conjugates, and simplifies assay development, since there are fewer independent variables requiring optimization in a directly labeled system.

3.0 INSTRUMENT SETTINGS

The excitation and emission spectra of terbium and fluorescein are shown in Figure 1. 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 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 the figure, the terbium emission spectrum is characterized by four sharp emission peaks, with silent regions between each peak. The first terbium emission peak (centered between approximately 485 and 505 nm) overlaps 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 as well. 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 instrument) may necessitate the use of a 495 nm filter. The effect on the quality of the resulting measurements is minimal in either case. Filters suitable for LanthaScreen™ assays are available from Chroma (www.chroma.com) as filter set PV001, or from other vendors. A LanthaScreen™ filter module for the BMG PheraStar is available direct from BMG Instruments.

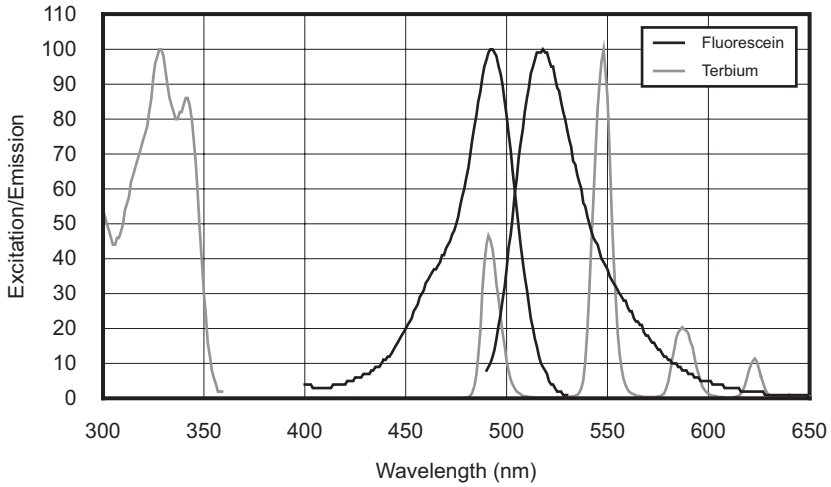

Figure 1—Excitation and Emission spectra of fluorescein and terbium.

Aside from filter choices, instrument settings are typical to the settings used with europium-based technologies. 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™ 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™ assays can be run on any filter-based instrument capable of time-resolved FRET, such as the Tecan Ultra, BMG PheraStar, Molecular Devices Analyst, or PE Envision. LanthaScreen™ assays have also been performed successfully on the Tecan Safire^2 monochromator-based instrument. Contact Invitrogen Technical Services for instrument-specific setup guidelines.

4.0 APPLICATIONS OF ANTI-SPECIES ANTIBODIES TO KINASE ASSAYS

LanthaScreen™ assays may be performed with reagents that are directly labeled with a terbium chelate, or by using reagents that are "indirectly" labeled through association with another moiety that is itself labeled with a terbium chelate. For example, in antibody-based kinase assays, one can utilize a phosphospecific antibody that is directly labeled with terbium, or an unlabeled phosphospecific primary antibody that is then "indirectly" labeled with terbium through association with a terbium-labeled secondary antibody that specifically binds to the phosphospecific primary antibody. Although this strategy is not as straightforward as using a directly labeled primary antibody, it can be advantageous when the directly labeled primary antibody is not readily available. For example, labeling of a primary antibody typically requires larger amounts of purified, concentrated antibody that may be expensive or time-consuming to obtain. The suitability of such an antibody in a particular assay application can be readily evaluated through the use of a Tb-labeled secondary antibody using the approach described below. Depending on the results of such an evaluation, one may then decide whether or not to examine such an assay using a directly labeled antibody.

The principle of a LanthaScreen™ kinase assay using a Tb-labeled secondary antibody is shown below in Figure 2. The assay itself can be divided into three phases: the kinase reaction phase, primary antibody incubation phase, and the detection phase. In the kinase reaction phase, all components required for the kinase reaction are added to the well, including fluorescein labeled substrate, and the kinase reaction is allowed to incubate for a set period of time, typically 60 to 90 minutes. After the reaction, EDTA is added to stop the kinase reaction and the primary anti-phosphospecific antibody is added to bind phosphorylated product. The incubation is conducted for 30 minutes after which the Tb-labeled secondary antibody is added to detect the complex. Binding of the Tb-labeled secondary antibody to the primary antibody that is associated with the fluorescein-labeled phosphorylated product brings the terbium and fluorescein into close 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.

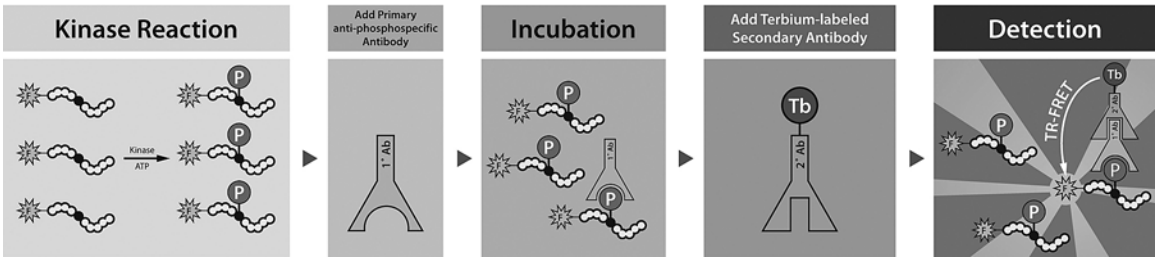

Figure 2—Schematic illustration of the use of a terbium labeled secondary antibody in a kinase assay format.

There are a range of variables associated with developing a TR-FRET assay utilizing Tb-labeled secondary antibodies. Optimized reagent concentrations, order of antibody additions (*i.e.* primary first, secondary first, or premixed primary and secondary), incubation times, etc., must be determined by the end user for a particular application.

4.1 Antibody concentration determination

It is also important to determine the optimal concentration of secondary antibody to be used in the assay. While too little can cause a lack of appreciable signal generation, too much can increase the background such that dynamic range can become compromised. Too much or too little secondary antibody may result in a smaller "read window". We recommend determining the optimal concentration of the Tb-labeled secondary by using the secondary antibody at 0.5X, 1X, 2X, and 4X concentrations of the primary antibody. From an experiment such as this, a user can balance the size of the read window versus the amount of antibody used in order to determine optimal amounts of secondary antibody for their particular application. The optimal amount of secondary antibody used will depend on the amount of primary antibody used, and would need to be determined for a given concentration of primary antibody used in the assay.

4.2 Assay stability and read window

For a given assay system, signal stability and read window should be assessed. In general, many assays will reach equilibrium within 2 hours, and will show a stable signal for at least 6 hours. However, depending on assay configuration and the specific demands of the assay, these times may vary and should be determined experimentally for the given assay system.

4.3 Cross-reactivity

In all cases, the species-specific antibodies bind to their corresponding species IgG with low nM apparent K_d's, and show negligible cross reactivity with IgG's from other species. The Tb-anti-Goat antibody exhibits this highest level of cross-reactivity with the Fluorescein-Human IgG, but this is minor in comparison to the signal from its corresponding IgG (data not shown).

Additionally, the Tb-anti-Mouse antibody has been demonstrated to react with Fluorescein-Mouse IgM antibody for use in assays that employ mouse antibodies of the IgM isotype (data not shown).

5.0 FIRST TIME USERS

Each Tb-labeled anti-species antibody from Invitrogen is provided with a corresponding fluorescein-labeled IgG as a positive control. To verify that instrument parameters are properly set-up to run the LanthaScreen™ assay format, it is suggested that a dilution series of the fluorescein-labeled IgG should be titrated against a fixed concentration of Tb-labeled antibody in order to generate a binding curve. The certificate of analysis provided with each antibody kit contains a binding curve that is representative of the data that should be obtained in conducting such an experiment. As an example, Figure 3 shows the results of a titration series of Fluorescein-Mouse IgG against 2 nM Tb-labeled Anti-Mouse Antibody. The plate was read on a Tecan Ultra plate reader after a 2 hour room temperature incubation.

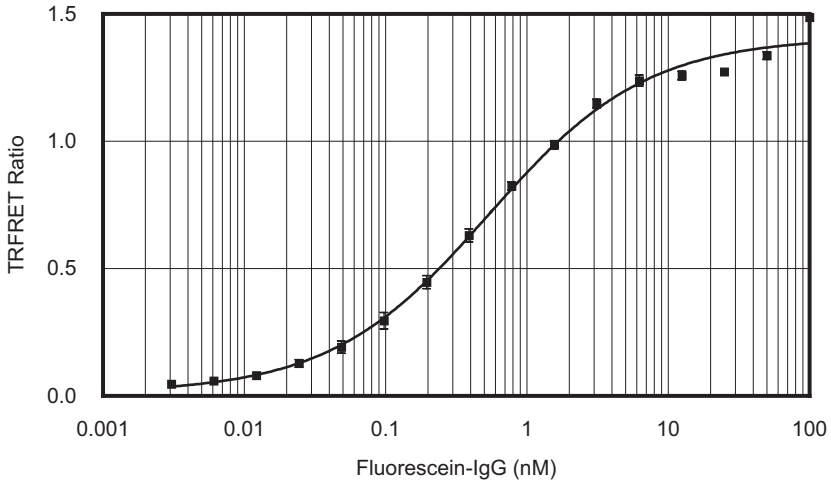

Figure 3—Titration of Fluorescein-Mouse IgG into Tb-Anti-Mouse antibody on the Tecan Ultra.

6.0 ASSESSING DATA QUALITY IN RATIOMETRIC MEASUREMENTS

The TR-FRET value is a unitless ratio derived from the underlying donor and acceptor signals. 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. Figure 4 is instructive in demonstrating the pitfalls of simply relying on the assay window as a measure of data quality. The ratiometric data on the left is all identical in quality (despite vastly different assay windows), as is evident when the curves are normalized and re-plotted in the graph at right. 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 (*J Biomol Screen* 1999: 4(2) pp 67-73), which takes these factors into account, is the correct way to assess data quality in a TR-FRET assay. Typically, our assays have Z' values of greater than 0.70.

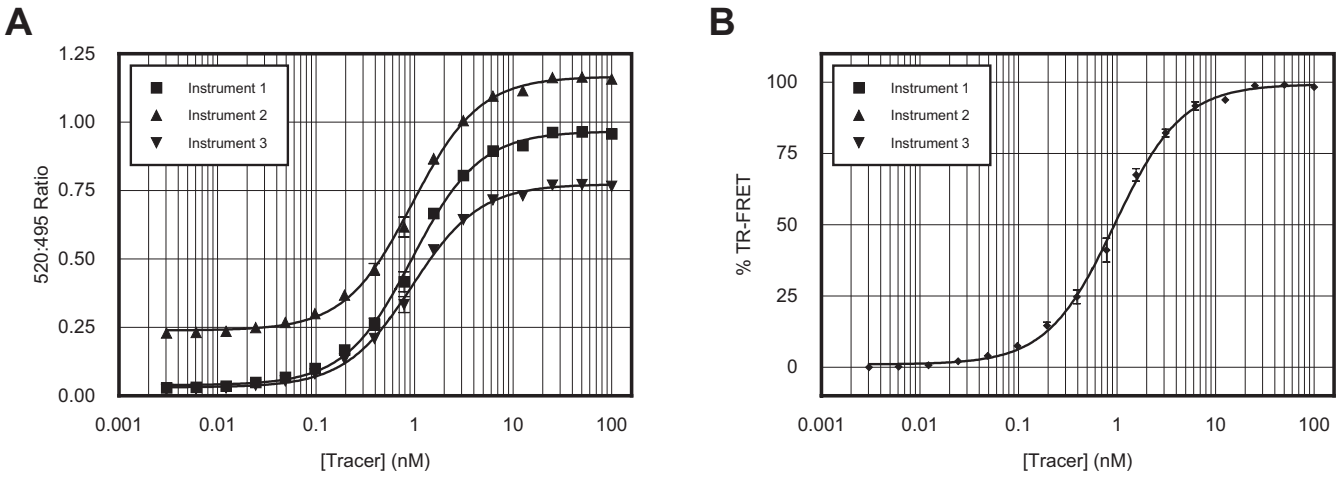

Figure 4—Assay window variability due to instrument type does not affect the resulting data and upon normalization, the above three curves are identical.

7.0 RELATED PRODUCTS

Reagents	Size	Cat. no.
LanthaScreen™ Tb-PY20 Antibody	25 µg	PV3552
	1 mg	PV3553
LanthaScreen™ Tb-PY72 Antibody	25 µg	PV3554
	1 mg	PV3555
LanthaScreen™ Tb-PY100 Antibody	25 µg	PV3556
	1 mg	PV3557
LanthaScreen™ Tb-PT66 Antibody	25 µg	PV3558
	1 mg	PV3559
LanthaScreen™ Tb-pSer (PKC) Antibody	25 µg	PV3560
	1 mg	PV3561
LanthaScreen™ Tb-IκBα pSer32 Antibody	25 µg	PV3562
	1 mg	PV3563
LanthaScreen™ Tb-pCrosstide Antibody	25 µg	PV3564
	1 mg	PV3565
LanthaScreen™ Tb-CREB pSer133 Antibody	25 µg	PV3566
	1 mg	PV3567
LanthaScreen™ Tb-Streptavidin, 1 mg/ml	50 µg	PV3965
	1 mg	PV3966
Fluorescein-PKC Substrate, 1 mg/ml	1 mg	PV3506
Fluorescein-IKK Substrate, 1 mg/ml	1 mg	PV3507
Fluorescein-CREBtide Substrate, 1 mg/ml	1 mg	PV3508
Fluorescein-Crosstide Substrate, 1 mg/ml	1 mg	PV3509
Fluorescein-PTK Substrate 1, 1 mg/ml	1 mg	PV3513
Fluorescein-PTK Substrate 2, 1 mg/ml	1 mg	PV3511
Fluorescein-Poly GT, 30 µM	1 ml	PV3610
Fluorescein-Poly GAT, 30 µM	1 ml	PV3611
LanthaScreen™ Amine Reactive Tb Chelate	10 µg	PV3583
	100 µg	PV3582
	1 mg	PV3581

8.0 NOTICE TO PURCHASER

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