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1. Reagents Available

Reagents	Size	Cat. no.	Storage
LanthaScreen™ Tb-pATF2 [pThr71] Antibody	25 µg	PV4451	–20°C
	1 mg	PV4452	–20°C
GFP-ATF2 (19-96) Substrate (10,000 10-µl reactions @ 200 nM)	20 nmoles	PV4445	–80°C
LanthaScreen™ Tb-pc-Jun [pSer73] Antibody	25 µg	PV4453	–20°C
	1 mg	PV4454	–20°C
GFP-c-Jun (1-179) Substrate (10,000 10-µl reactions @ 200 nM)	20 nmoles	PV4446	–80°C
LanthaScreen™ Tb-p4E-BP1 [pThr46] Antibody	25 µg	PV4757	–20°C
	1 mg	PV4758	–20°C
GFP-4E-BP1 (10,000 10 µL reactions @ 200 nM)	20 nmoles	PV4759	–80°C
LanthaScreen™ Tb-peIF2α [pSer52] Antibody	25 µg	PV4810	–20°C
	1 mg	PV4811	–20°C
GFP-eIF2α Substrate (10,000 10-µl reactions @ 200 nM)	20 nmoles	PV4809	–80°C
LanthaScreen™ Tb-pMAP2K1 [pSer217/221] Antibody	25 µg	PV4813	–20°C
	1 mg	PV4814	–20°C
Fluorescein-MAP2K1 Substrate (10,000 10 µl reactions @ 200 nM)	20 nmoles	PV4812	–80°C
LanthaScreen™ Tb-anti-pSTAT1 [pTyr701] Antibody	25 µg	PV4844	–20°C
	1 mg	PV4845	–20°C
GFP-STAT1 (10,000 10-µl reactions @ 100 nM)	10 nmoles	PV5211	–80°C
LanthaScreen™ Tb-anti-pSTAT3 [pTyr705] Antibody	25 µg	PV4846	–20°C
	1 mg	PV4847	–20°C
GFP-STAT3 (10,000 10-µl reactions @ 100 nM)	10 nmoles	PV5212	–80°C

2. 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 proximity of one another, excitation of the first fluorophore (the donor) results 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 high-throughput screening (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 quenching effects due to colored compounds.

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. When used as a FRET donor, europium is typically paired with the fluorescent protein allophycocyanin (also called APC or XL-665) as the acceptor. APC is a large protein (approximately 100 kD) that is isolated from cyanobacteria or red algae and must be chemically crosslinked (hence the "XL" designation) prior to use to improve stability. Because direct conjugates of APC are time-consuming to prepare, APC is typically prepared and used as a streptavidin conjugate to indirectly label biotinylated biomolecules such as proteins, peptides, or DNA.

Terbium offers unique advantages over europium when used as the donor 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, as there are fewer independent variables that require optimization in a directly labeled system.

A complete guide to commonly asked questions and answers regarding the LanthaScreen™ technology can be found at www.invitrogen.com/lanthascreen.

3. Instrument Settings

The excitation and emission spectra of terbium and fluorescein are shown below 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, 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 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 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. 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 from BMG Instruments.

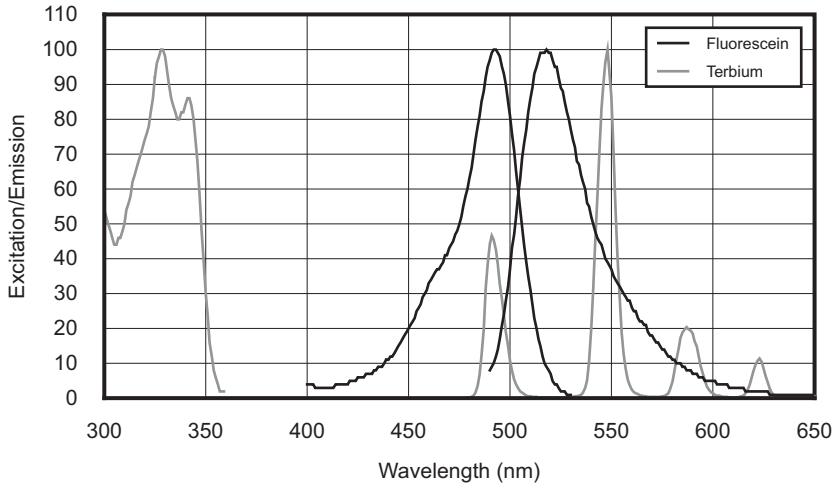

Figure 1. Spectra of Terbium and fluorescein.

Aside from filter choices, instrument settings are similar 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 Evolution™, BMG PHERAstar, Molecular Device's Analyst™, or PerkinElmer's EnVision™. Ask your Invitrogen representative for instrument-specific setup guidelines.

4. Assay Principle

Invitrogen offers a variety of Tb-labeled phospho-specific antibodies and fluorescein-labeled peptide substrates. However, a number of kinases preferentially or exclusively act on protein substrates rather than small peptides. To address this need, we have developed a set of TR-FRET reagents that utilize physiologically relevant substrates for certain kinases. These products are protein substrates labeled with an acceptor fluorophore (Green Fluorescent Protein or fluorescein) and paired with a corresponding Tb-labeled phospho-specific antibody. The principle of these kinase assays is shown below 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 the labeled protein substrate. The 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 terbium-labeled antibody is added to bind phosphorylated product. Because the terbium chelate is stable at the EDTA concentrations used to stop a kinase assay, the antibody and EDTA can be pre-mixed prior to addition to minimize pipetting steps. Binding of the terbium labeled antibody to the fluorophore-labeled phosphorylated product brings the terbium and GFP or 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.

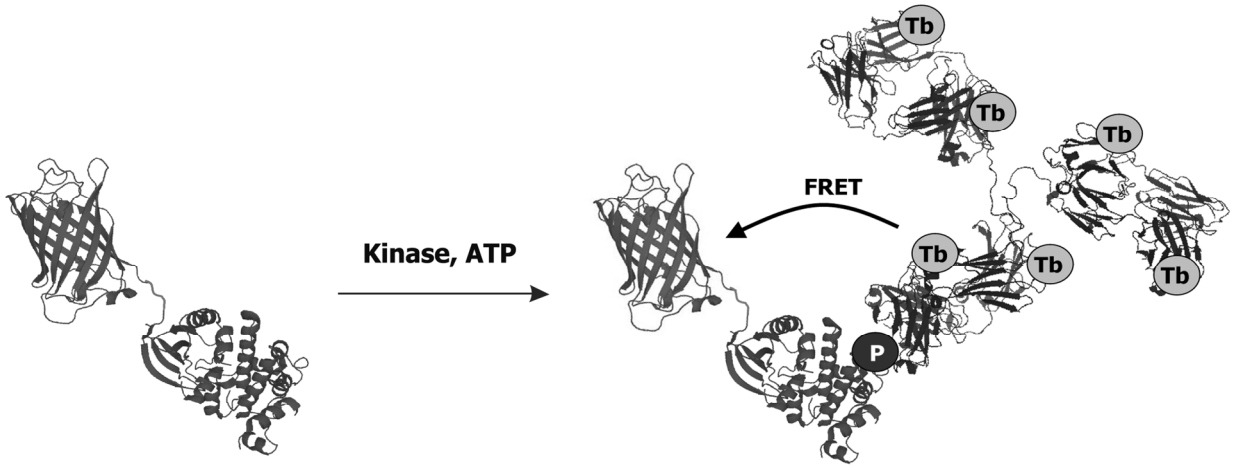

Figure 2. Principle of LanthaScreen™ TR-FRET kinase assay using a protein substrate.

5. First-Time Users: Terbium-Labeled Antibody Reagents

The LanthaScreen™ Tb-labeled phospho-specific antibodies and labeled physiologically relevant substrates provide a unique and sensitive way to assay kinases that preferentially or exclusively function on protein rather than peptide substrates. With the acceptor fluorophore directly attached to a protein, we have been able to eliminate the use of a biotin-mediated (or a substrate-specific antibody-mediated) acceptor fluorophore labeling system. Since the physiologically relevant substrate reagents provide such sensitive detection of kinase activity, it is important to perform some optimization experiments prior to beginning any screening campaign. Determining the appropriate concentration of kinase and ATP is very important. We typically recommend that the kinase be used at a concentration that generates 80% of the maximal signal (EC_{80}) in the presence of ATP equal to the K_m apparent (EC_{50}).

These conditions are very important to maximize the potency of any potential inhibitors. If ATP is in excess relative to the K_m, it is less likely that an inhibitor competing for the ATP binding pocket will exhibit efficient inhibition of the kinase. After determining these values, it is recommended that a Z' experiment be performed to determine the robustness of the conditions and that the assay be run against a known inhibitor titration to verify that inhibitor potency is consistent with previous data.

For simplicity, optimization experiments for the phosphorylation of GFP-ATF2 with JNK1 are outlined in the following examples. To find specific assay conditions and representative data for your particular kinase and physiological protein substrate, please visit http://www.invitrogen.com/lanthascreenkinase.

5.1 Kinase Titration

To determine the proper amount of kinase to use in the assay, start by performing a two-fold serial dilution of the kinase. Calculate the EC_{80} concentration of the kinase.

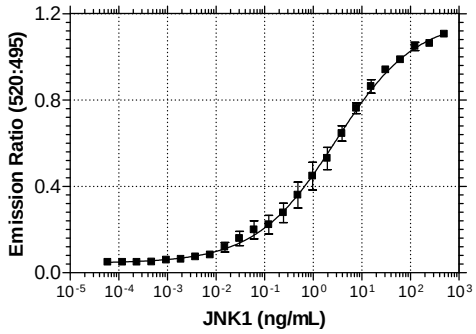

Figure 3. In triplicate 10-µl reactions, a dilution series of JNK1 (PV3319) was assayed against 200 nM GFP-ATF2 in the presence of 100 µM ATP, in 50 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 0.01% Brij-35, and 1 mM EGTA. After 1 hour, a 10-µl solution of terbium-labeled anti p-ATF2 (pThr 71) and EDTA in TR-FRET Dilution Buffer (PV3574) was added to each well, for a final concentration of 2 nM antibody and 10 mM EDTA. After a 1-hour incubation, the plate was read and TR-FRET values calculated. These values were then graphed against the concentration of JNK1 per well and the EC_{80} concentration of JNK1 was determined to be 40 ng/ml.

5.2 ATP Optimization

Once the concentration of kinase has been determined, it may be advisable to determine the ATP K_m apparent for that particular kinase on the substrate being used. To do this, serial dilute ATP and assay against the EC_{80} concentration of kinase determined in the kinase titration.

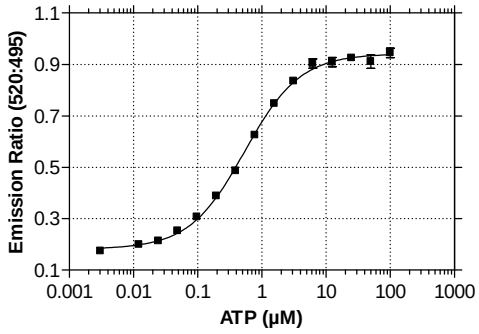

Figure 4. In triplicate 10-µl reactions, a dilution series of ATP was assayed against 200 nM GFP-ATF2 in the presence of 40 ng/ml JNK1, in 50 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 0.01% Brij-35, and 1 mM EGTA. After 1 hour, a 10-µL solution of terbium-labeled anti p-ATF2 (pThr 71) and EDTA was added to each well, for a final concentration of 2 nM antibody and 10 mM EDTA. After a 1-hour incubation, the plate was read and TR-FRET values calculated. These values were then graphed against the ATP concentration per well and the K_m apparent (EC_{50}) was determined to be 0.5 µM.

Once the ATP K_m apparent has been determined, we recommend repeating the kinase titration to verify that the EC_{80} concentration of kinase has not shifted too much due to the limiting amount of ATP now present.

5.3 Z´ Data

To examine the robustness of the assay, a Z′ experiment should be performed.

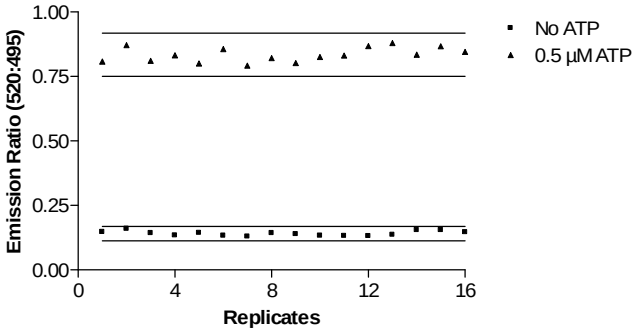

Figure 5. Sixteen positive control wells (containing 0.5 µM ATP) and 16 negative control wells (no ATP) were assayed against 200 nM GFP-ATF2 in the presence of 40 ng/ml JNK1, in 50 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 0.01% Brij-35, and 1 mM EGTA. After 1 hour, a 10-µL solution of terbium-labeled anti p-ATF2 (pThr 71) and EDTA was added to each well, for a final concentration of 2 nM antibody and 10 mM EDTA. After a 1-hour incubation, the plate was read and TR-FRET values calculated and the Z′ value was determined to be 0.84.

5.4 Inhibitor Titration

We also recommend performing an inhibitor titration to confirm inhibitor potency with the conditions determined.

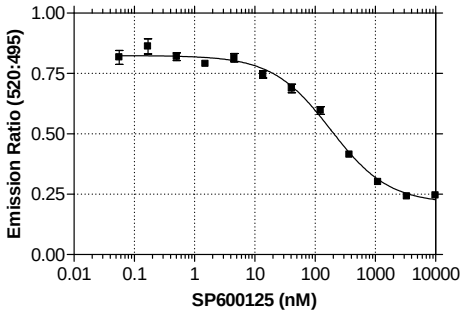

Figure 6. The JNK inhibition study was performed against a three-fold dilution series of inhibitor SP600125. JNK1 for this assay was at 40 ng/ml in 200 nM GFP-ATF2 substrate and 0.5 µM ATP, which was determined to be the concentration of ATP that gave half maximal assay response when the assay was performed against a dilution series of ATP. The resulting IC_{50} value was determined to be 180 nM.

6. 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 7 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. For this reason, the "Z prime" value proposed by Zhang and colleagues (Zhang *et al.*, 1999), which takes these factors into account, is the correct way to assess data quality in a TR-FRET assay.

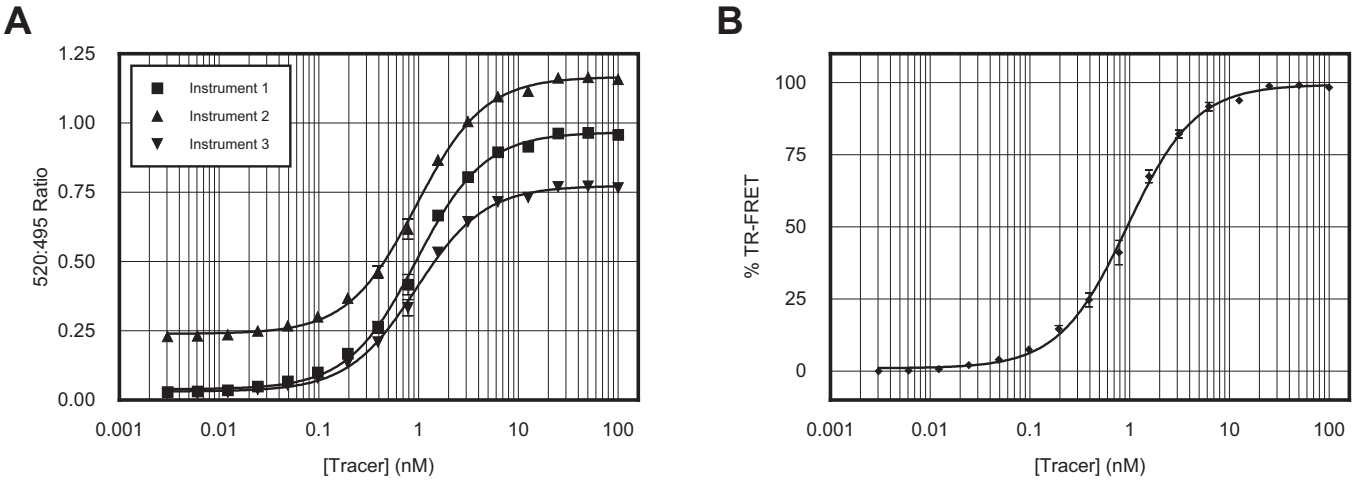

Figure 7. Ratiometric data (*A*) is identical in quality to normalized data (*B*), as is evident when the curves are normalized and replotted.

7. Related Products

Reagents	Size	Cat. no.
LanthaScreen™ Tb-PY20 Antibody	25 mg 1 mg	PV3552 PV3553
LanthaScreen™ Tb-PY72 Antibody	25 mg 1 mg	PV3554 PV3555
LanthaScreen™ Tb-PY100 Antibody	25 mg 1 mg	PV3556 PV3557
LanthaScreen™ Tb-PT66 Antibody	25 mg 1 mg	PV3558 PV3559
LanthaScreen™ Tb-pSer (PKC Substrate) Antibody	25 mg 1 mg	PV3560 PV3561
LanthaScreen™ Tb- IκBα pSer32 Antibody	25 mg 1 mg	PV3562 PV3563
LanthaScreen™ Tb-pCrosstide Antibody	25 mg 1 mg	PV3564 PV3565
LanthaScreen™ Tb-CREB pSer133 Antibody	25 mg 1 mg	PV3566 PV3567
Fluorescein-PKC Substrate, 1 mg/ml	1 ml	PV3506
Fluorescein-IKK Substrate, 1 mg/ml	1 ml	PV3507
Fluorescein-CREBtide Substrate, 1 mg/ml	1 ml	PV3508
Fluorescein-Crosstide Substrate, 1 mg/ml	1 ml	PV3509
Fluorescein-PTK Substrate 1 (CDC2 $^{(6\text{-}20)}$), 1 mg/ml	1 ml	PV3513
Fluorescein-PTK Substrate 2 (YIYGSFK), 1 mg/ml	1 ml	PV3511
Fluorescein-Poly GT, 30 µM	1 ml	PV3610
Fluorescein-Poly GAT, 30 µM	1 ml	PV3611
LanthaScreen™ Amine Reactive Tb Chelate	10 µg 100 µg 1 mg	PV3583 PV3582 PV3581
LanthaScreen™ Thiol Reactive Tb Chelate	10 µg 100 µg 1 mg	PV3580 PV3579 PV3578
LanthaScreen™ Tb-anti-Mouse Antibody	25 µg 1 mg	PV3765 PV3767
LanthaScreen™ Tb-anti-Goat Antibody	25 µg 1 mg	PV3769 PV3771
LanthaScreen™ Tb-anti-Rabbit Antibody	25 µg 1 mg	PV3773 PV3775
LanthaScreen™ Tb-anti-Human Antibody	25 µg 1 mg	PV3777 PV3779
LanthaScreen™ Tb-Streptavidin, 1 mg/ml	50 µg 1 mg	PV3965 PV3966

8. 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

9. Notice to Purchaser

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