LanthaScreen™ Terbium-labeled Biotin Binding Reagents User Guide

invitrogen™

Catalog nos. PV3965, PV3966, PV4749, PV4750

Shipping Condition: Dry Ice **Storage: –20°C**

Protocol part no. PV396X.pps

Rev. date: 3 May 2007

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1. REAGENTS AVAILABLE

REAGENTS	Size	Cat. No.
LanthaScreen™ Tb-Streptavidin, 1 mg/ml (17.9 µM)	50 µg	PV3965
	1 mg	PV3966
LanthaScreen™ Tb-Anti-Biotin antibody, 1 mg/ml (6.7 µM)	25 µg	PV4749
	1 mg	PV4750

2. INTRODUCTION

For screening compound libraries, time-resolved fluorescence resonance energy transfer (TR-FRET) is a recognized method for overcoming interference from compound autofluorescence or light scattering 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 close 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, and corrects for quenching effects because of 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) lasts a millisecond or longer. This contrasts sharply with the nanosecond-long lifetimes of fluorophores commonly used in standard FRET assays. Because interference from autofluorescent compounds or light scattering is also on the nanosecond timescale, these factors negatively affect standard FRET assays. TR-FRET assays overcome these interferences 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 greatly reduces interference

from background fluorescence or light scattering, but also avoids interference from direct excitation because of the non-instantaneous nature of the flashlamp excitation source.

Although the terbium chelate donor directly attaches to a biomolecule such as a protein or peptide, it can also be "indirectly" attached to a target molecule through a biotin-mediated association with terbium-labeled streptavidin or anti-biotin antibody. Invitrogen's terbium-labeled biotin binding reagents are therefore universal reagents that easily label biotinylated biomolecules with terbium for use in TR-FRET assays. This guide discusses such a labeling strategy applied to a protease assay using a peptide substrate that is labeled at one terminus with biotin and on the other with fluorescein.

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. The exact specifications of the excitation filter are not critical; filters with similar specifications will work well. In general, excitation filters that are compatible with europium-based TR-FRET systems will perform well with the LanthaScreen™ terbium chelates.

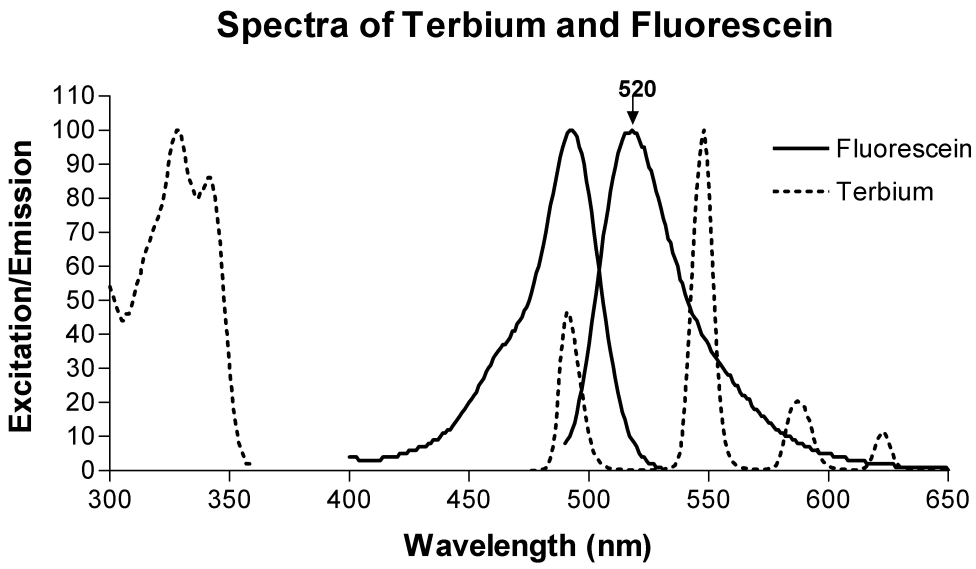

Figure 1: Excitation and emission spectra of terbium and fluorescein.

As shown in the figure, four sharp peaks characterize the terbium emission spectrum, with silent regions between each peak. The first terbium emission peak (between approximately 485 and 505 nm) overlaps the fluorescein maximum excitation peak. The fluorescein maximum emission peak is centered in the silent region between the first and second terbium emission peaks. Thus, energy transfer to fluorescein is measured in this region without interference from terbium by using a filter centered at 520 nm with a 25-nm bandpass. The specifications of this filter are more critical than those of the excitation filter. In general, standard "fluorescein-specific" filters will not perform well, because they also pass light associated with the terbium emission spectrum. The emission of fluorescein because of FRET is referenced (or ratioed) to the emission of the first terbium peak, using a filter that isolates this peak, typically 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 a 495-nm filter may be necessary with some instrument dichroic mirror choices (such as those on the Tecan Ultra Evolution™ instrument). 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. BMG Instruments offers a LanthaScreen™ filter module for the BMG PHERAStar fluorescence plate reader.

Aside from filter choices, instrument settings are similar to those used with europium-based technologies. In general, the instrument manufacturer's guidelines serve as a good starting point for optimization. A LanthaScreen™ assay would

typically use a delay time of 100 µs, followed by a 200-µs integration time. The number of flashes or measurements per well is highly instrument-dependant and should be set as recommended by your instrument manufacturer. In general, LanthaScreen™ assays are run on any filter-based instrument capable of TR-FRET, such as the Tecan Ultra Evolution™, BMG PHERAStar, Molecular Devices Analyst®, or PerkinElmer EnVision™ fluorescence plate readers. LanthaScreen™ assays have also been performed successfully on the Tecan Safire² monochromator-based instrument. Contact the Invitrogen Corporation Technical Services group for instrument-specific setup guidelines.

4. APPLICATIONS OF TERBIUM-LABELED BIOTIN BINDING REAGENTS TO PROTEASE ASSAYS

Terbium-labeled biotin binding reagents are useful as universal labeling reagents for biotinylated biomolecules such as proteins, peptides, or oligonucleotides. In protease assays, Invitrogen's terbium-based LanthaScreen™ technology enables TR-FRET protease assays to be performed using peptides that contain biotin- and fluorescein-modified termini. Such peptides are readily prepared by standard peptide synthesis methods, and are inexpensively available from a variety of vendors. The following describes the use of such reagents in an assay for β-secretase (BACE1) activity.

BACE1 is a key enzyme involved in the production of amyloid β-peptides found in the extracellular amyloid plaques of Alzheimer's disease. In some cases, early-onset familial Alzheimer's disease is attributed to a "Swedish" mutation in the amyloid precursor protein (APP), which dramatically enhances cleavage of this protein by BACE1. This and other genetic and pathological evidence led to therapeutic approaches that focus on inhibiting BACE1 and other APP-cleaving enzymes.

To assay BACE1-catalyzed cleavage of the Swedish mutant of APP, a peptide corresponding to the cleavage site, EVNL∧DAEF) (cleavage between residues L and D) was synthesized with an N-terminal biotin and a C-terminal fluorescein. The fluorescein was attached to the ε-amino group of a lysine appended to the C-terminus of the peptide sequence. This peptide was incubated with BACE1 in the presence or absence of an inhibitor for 1 hour at room temperature, after which the reaction was stopped by adding Tb-SA in a pH 8.0 buffer (BACE1 is inactive at high pH) and the plate read on a BMG PHERAStar fluorescence plate reader using the LanthaScreen™ filter module available from BMG. The assay is shown schematically below in Figure 2.

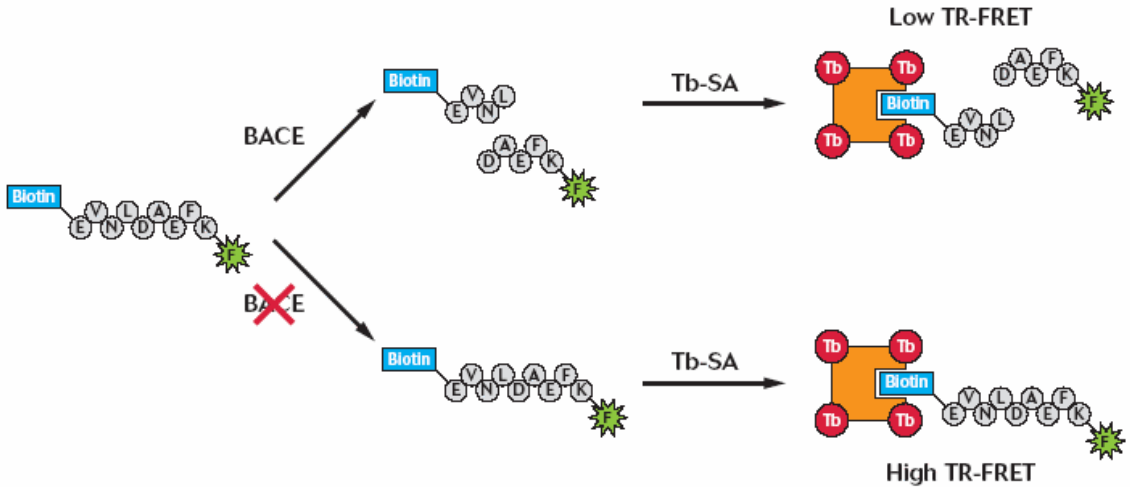

Figure 2: Schematic of a LanthaScreen™ protease assay using Tb-SA.

4.1 BACE1 Assay Using Tb-labeled Streptavidin

To determine the amount of BACE1 required for the assay, 100 nM of peptide substrate was incubated with a dilution series of BACE1 in a 10-µL reaction volume of 50-mM sodium acetate buffer, pH 4.5. Each reaction was performed in triplicate. After 1 hour, a 10-µL solution containing 10-nM Tb-SA and 300-mM Tris buffer, pH 8, in TR-FRET dilution buffer (Invitrogen part number PV3574) was added. The high concentration of Tris buffer was used to ensure that the final pH was slightly basic to stop the BACE1 reaction. After a short incubation (approximately 10 minutes), the plate was read in TR-FRET mode on a Tecan Ultra Evolution™ fluorescence plate reader. The data are presented below in Figure 3A. Error bars are shown, but are extremely small.

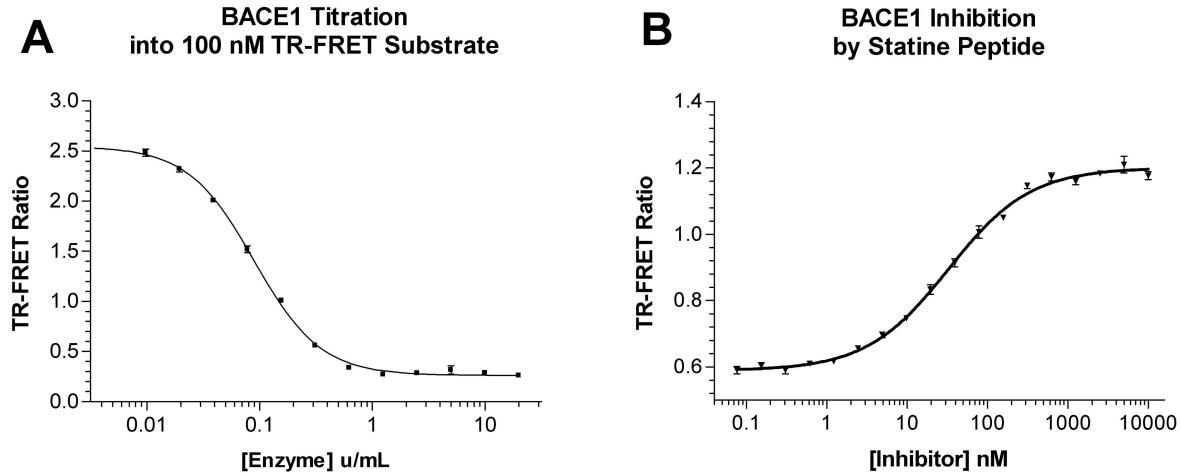

Figure 3: (A) Titration of BACE1 against 100-nM peptide substrate. (B) Inhibition of BACE1 by a statine containing peptide.

The enzyme titration experiment indicated that 0.1 units/mL BACE1 were appropriate for the assay. At this enzyme concentration, approximately 50% of the substrate will be cleaved in the absence of inhibitor. The inhibitor was a statine containing peptide (Lys-Thr-Glu-Glu-Ile-Ser-Glu-Val-Asn-(Statine)-Val-Ala-Glu-Phe-OH) that was titrated against BACE1 in a two-fold dilution series beginning at 10 µM. Each reaction was performed in triplicate. After 60 minutes, the reaction was stopped as described previously by the addition of Tb-SA and Tris buffer, pH 8, to a final concentration of 5 nM Tb-SA and 150 mM Tris buffer. The plate was read on a Tecan Ultra Evolution™ fluorescence plate reader using standard instrument settings and the results are shown in Figure 3B. Error bars are shown, but are extremely small. The statine-containing peptide inhibited BACE1 with an IC_{50} value of 32 nM, which agrees closely with the published value of 30 nM.

4.2 BACE1 Assay Using Tb-Anti-Biotin Antibody

To determine the amount of BACE1 required for the assay, 200 nM of peptide substrate was incubated with a dilution series of BACE1 in a 15-µL reaction volume of 50-mM sodium acetate buffer, pH 4.5. Each reaction was performed in triplicate. After 1 hour, a 5-µL solution containing 20-nM Tb-anti-Biotin antibody in LanthaScreen™ BACE1 Assay Stop Solution was added. After a 1-hour incubation, the plate was read in TR-FRET mode on a Tecan Ultra Evolution™ fluorescence plate reader. The data are presented below in Figure 4A. Error bars are shown, but are extremely small.

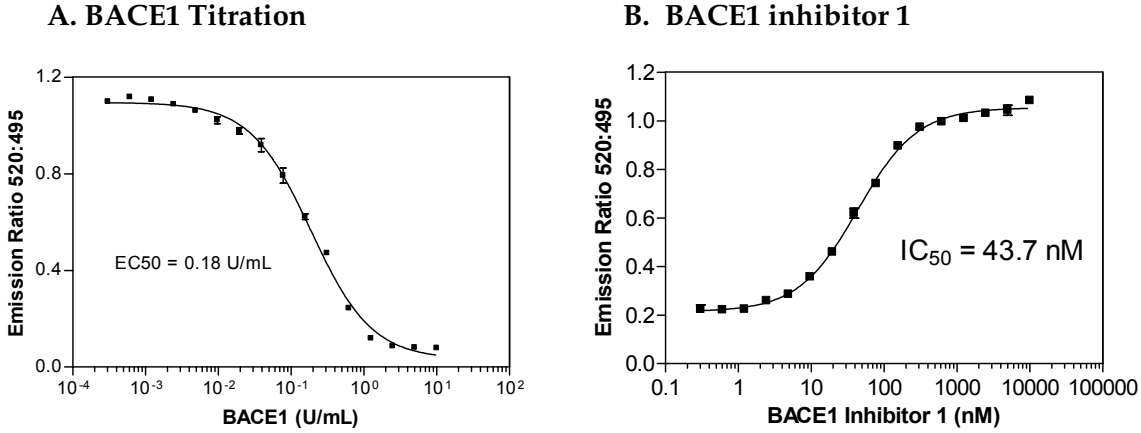

Figure 4: (A) Titration of BACE1 against 200-nM peptide substrate. (B) Inhibition of BACE1 by a statine-containing peptide.

The enzyme titration experiment indicated that 0.7 units/mL BACE1 were appropriate for the assay. At this enzyme concentration, approximately 80% of the substrate will be cleaved in the absence of inhibitor. The inhibitor was a statine containing peptide (Lys-Thr-Glu-Glu-Ile-Ser-Glu-Val-Asn-(Statine)-Val-Ala-Glu-Phe-OH) that was titrated against BACE1 in a two-fold dilution series beginning at 10 µM. Each reaction was performed in triplicate. After 60 minutes, the reaction was stopped as described previously by the addition of Tb-Anti-Biotin Antibody in LanthaScreen™ BACE1 Assay Stop Solution, to a final concentration of 5-nM Tb-anti-Biotin antibody. The plate was read on a Tecan Ultra Evolution™ fluorescence plate reader using standard instrument settings and the results are shown in Figure 4B. Error bars are shown, but are extremely small.

5. FIRST-TIME USERS

Invitrogen provides users with an aliquot of biotinylated, fluorescein labeled peptide (200 nM Fluorescein-Biotin Positive Control in TR-FRET Dilution Buffer) as a positive control. To verify that instrument parameters are properly set for the LanthaScreen™ assay format, titrate a dilution series of the peptide against a fixed concentration of Tb-labeled reagents to generate a binding curve. The certificate of analysis provided shows a binding curve representative of data expected from such an experiment.

6. ASSESSING DATA QUALITY IN RATIOMETRIC MEASUREMENTS

The TR-FRET ratio is a unitless value derived from the ratio of the underlying donor and acceptor signals. Because these underlying signals depend 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 5 demonstrates the pitfalls of relying solely on the assay window to determine data quality. The ratiometric data in Panel A is identical in quality (despite vastly different assay windows), as demonstrated in Panel B, after the data have been normalized and re-plotted. The important factor in determining the robustness of an assay is the magnitude of errors in the data compared to the difference between the maximum and minimum values, not the size of the instrument's assay window. The Z´-factor value proposed by Zhang and colleagues (Zhang *et al.*, 1999), which takes these factors into account, offers a more accurate assessment of data quality in a TR-FRET assay. Typically, our assays have Z´-factor values of greater than 0.70 (a Z´-factor value of 1 is the ideal; an assay exhibiting a Z´-factor value greater than 0.5 is considered highly reliable).

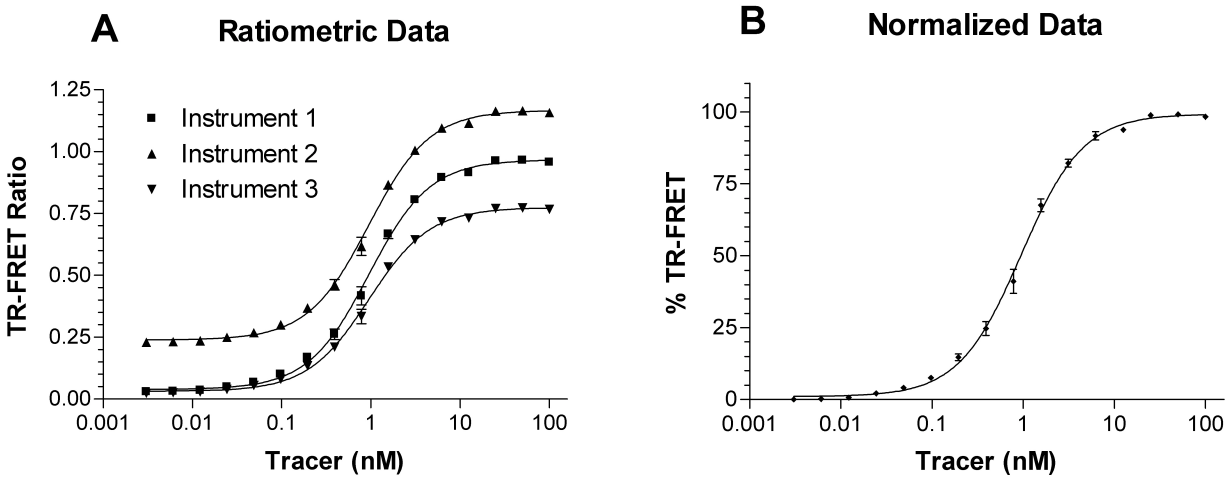

Figure 5: Assay window variability due to instrument type does not affect the resulting data. A. Data obtained from three different instruments with different assay windows. B. Upon normalization, the three curves in Panel A are identical.

7. RELATED PRODUCTS

REAGENTS	Volume	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 Substrate) Antibody	25 µg	PV3560
	1 mg	PV3561
LanthaScreen™ Tb-IκB 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-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
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 (YIYGSFK), 1 mg/mL	1 mg	PV3513
Fluorescein-PTK Substrate 2 (CDC2 $^{(6-20)}$), 1 mg/mL	1 mg	PV3511
Fluorescein-Poly GT, 30 µM	1 mL	PV3610
Fluorescein-Poly GAT, 30 µM	1 mL	PV3611
LanthaScreen™ Thiol Reactive Tb Chelate	10 µg	PV3580
	100 µg	PV3579
	1 mg	PV3578
LanthaScreen™ Amine Reactive Tb Chelate	10 µg	PV3583
	100 µg	PV3582
	1 mg	PV3581

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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