LanthaScreen™ TR-FRET Labeling Reagents Protocol

Part # PV3578, PV3579, PV3580, PV3581, PV3582, and PV3583

Lit. # PV357883.pps Revision Date: 053105

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1.0 EQUIPMENT AND MATERIALS

1.1 Reagents

Part No.	Name	Form Supplied	Function
PV3583	LanthaScreen™ Amine Reactive Tb Chelate	10 µg, lyophilized	labeling amino groups
PV3582		100 µg, lyophilized	
PV3581		1 mg, lyophilized	
PV3580	LanthaScreen™ Thiol Reactive Tb Chelate	10 µg, lyophilized	labeling thiol groups
PV3579		100 µg, lyophilized	
PV3578		1 mg, lyophilized	

1.2 Required Equipment, Recommended Filters, and Suitable Acceptor Fluorophores

A fluorescence plate reader capable of detecting Time-Resolved Fluorescence Resonance Energy Transfer (TR-FRET), with the appropriate filter sets installed for detecting energy transfer to acceptor dyes is necessary. In general, monochromator-based instruments are not sufficiently sensitive for TR-FRET assays. A 340-nm (30-nm bandpass) filter is used for excitation.

The settings in the table below are recommended for detecting energy transfer with fluorescein, rhodamine, or other suitable acceptor fluorophores (*i.e.*, RPE, GFP, or Alexa Fluor® 488 or 555 dyes) by monitoring either an increase in acceptor intensity or a decrease in donor intensity.

Note that the 520/25 and 570/10 filters are used to detect signals in the "silent" regions in the terbium (Tb) chelate emission spectrum; only filters that meet these specifications or that have narrower bandpass ranges should be used.

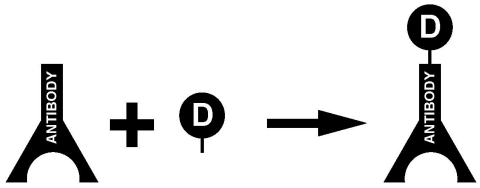
Acceptor	Filter Setting	Purpose
Fluorescein, Alexa Fluor® 488 dye, GFP, etc.	495 nm (10-nm bandpass)	Decrease in donor intensity
	520 nm (25-nm or less bandpass)	Increase in acceptor intensity
Rhodamine, Alexa Fluor® 555 dye, RPE, etc.	546 nm (10-nm bandpass)	Decrease in donor intensity
	570 nm (10-nm or less bandpass)	Increase in acceptor intensity

2.0 INTRODUCTION

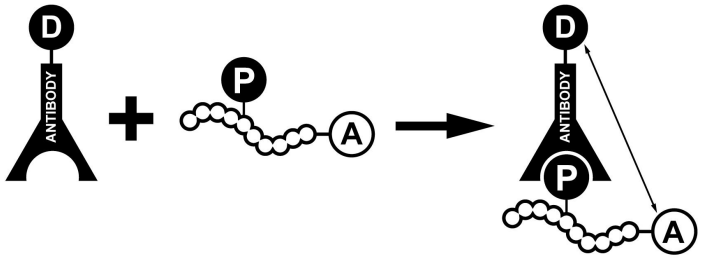
TR-FRET-based assays measure interactions between molecules that are in proximity to one another (typically less than 100 Å apart), such as protein-protein, protein-peptide, or DNA-protein interactions (1). In a TR-FRET assay, an excited-state Tb ion (donor) transfers its energy to a nearby acceptor molecule, often a fluorescent molecule such as fluorescein or rhodamine. The transferred energy is then emitted as a detectable fluorescence signal.

Schematic Example of a TR-FRET Assay

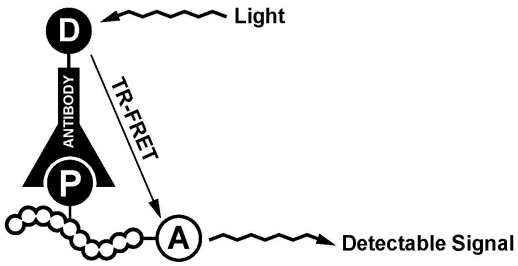
1 Label anti-phosphopeptide antibody with donor Tb chelate (D)



2 Design assay using Tb chelate-labeled antibody and a phosphopeptide (P) labeled with a suitable acceptor dye (A).



3 Excitation and emission of detectable fluorescence signal via TR-FRET.



3.0 LABELING GUIDELINES

3.1 Reagent Concentrations

The rate of the labeling reaction is directly proportional to the concentrations of the reaction components. Therefore, the minimum recommended protein concentration is 1 mg/mL. Better labeling results can be achieved with protein concentrations of up to 10 mg/mL. Because the optimal protein concentration and reaction time can vary considerably, users must experimentally determine the optimal conditions for their particular application.

3.2 Solvent Considerations

Unlike many fluorescent dyes, Invitrogen's amine- and thiol-reactive Tb chelates are water-soluble. Therefore, it is not necessary to make a stock solution of the Tb chelate in DMSO or DMF before the reaction. The amine- and thiol-reactive Tb chelates can be reconstituted in water or buffer and then diluted in the buffer of choice.

3.3 Buffer Considerations

3.3.1 General Buffer Considerations

Avoid using buffers containing metal-chelating compounds, such as ethylene diamine tetraacetic acid (EDTA).

3.3.2 Buffer Considerations for Amino Groups

A basic buffer (pH > 9.0) is recommended for labeling amino groups, because the reaction proceeds most efficiently with uncharged amino groups. The labeling reaction will still proceed at lower pH, however, but less efficiently. Generally, a sodium carbonate buffer (pH 9.5) is appropriate for labeling amino groups on proteins, while a borate buffer (pH 9.5) is appropriate for labeling peptides, amine-modified oligonucleotides, or other small molecules (see table below). Avoid using buffers containing primary or secondary amine groups, such as ethanolamine or Tris, because competing amine groups will interfere with the amine-labeling reaction.

3.3.3 Buffer Considerations for Thiol Groups

Thiol groups are most effectively labeled in buffers with a near neutral pH (pH 7.0–8.5 is acceptable). Non-specific labeling of other functional groups can potentially occur in more basic buffers. Avoid buffers containing β-mercaptoethanol (βME), dithiothreitol (DTT), or other thiol-containing compounds, because these groups can interfere with the labeling reaction.

Material to Be Labeled	Recommended pH	Recommended Buffers	Buffers to Avoid	Additives to Avoid
Proteins with primary or secondary amino groups	9.5	50–100 mM Sodium Carbonate	Buffers containing Tris, ethanolamine, or other primary or secondary amine groups	EDTA and other metal-chelating compounds
Peptides, amine-modified oligonucleotides, and other small molecules	9.5	50–100 mM Sodium Tetraborate	Buffers containing Tris, ethanolamine, or other primary or secondary amine groups	EDTA and other metal-chelating compounds
Proteins with thiol groups	7.0–8.5	Buffers containing HEPES, MOPS, Phosphate, or Tris	- -	EDTA and other metal-chelating compounds; □-mercaptoethanol, DTT, and other thiol-containing compounds

3.4 Pre-labeling Purification of Oligonucleotides

Commercially available amine-modified oligonucleotides are often contaminated with amine-containing impurities that can interfere with the labeling reaction. Remove these contaminants by gel filtration using a standard "desalting" column or HPLC. Standard ethanol precipitation will not remove these contaminants.

3.5 Post-labeling Purification of Oligonucleotides, Peptides, and Proteins

3.5.1 HPLC purification of small molecules (e.g., peptides and oligonucleotides)

Caution: Water-methanol or water-acetonitrile gradients used in HPLC often contain small amounts of trifluoroacetic acid (TFA). The Tb ion will immediately dissociate from the chelate under acidic conditions (pH < 5.0), but can readily be reconstituted under less acidic conditions. Because unchelated Tb^{3+} forms insoluble precipitates with phosphate, however, avoid using phosphate buffers for the reconstitution step. Once the Tb chelate is reconstituted, however, it is stable in phosphate buffers.

Acidic HPLC purification

After acidic HPLC purification:

1. Remove the solvent containing TFA by speed vacuum or lyophilization;
2. Resuspend the labeled compound in the buffer of choice (pH 5.5–8.5, avoiding phosphate buffers as noted above); and
3. Add 1 to 1.1 equivalents of $TbCl_3$ to the purified, labeled compound to reconstitute the Tb center of the chelate. At micromolar concentrations or greater, the reformation of the Tb chelate is nearly instantaneous.

Non-acidic HPLC purification

Alternatively, use a non-acidic, aqueous phase HPLC solvent, such as 100 mM triethylammonium-acetate (TEAA) buffer (pH 6.5).

3.5.2 Purification of labeled proteins

Purify labeled proteins by dialysis or gel filtration chromatography, using a buffer with pH 5.5–8.5.

4.0 APPLICATION EXAMPLES

The following four sections provide general protocols for using Invitrogen's Tb chelates to label peptides, amine-modified oligonucleotides, and thiol groups on peptides or proteins. A more detailed description of using the amine-reactive Tb chelate to label amino groups on an IgG antibody is provided in **Section 4.3**.

4.1 Peptides Containing Free Amino Groups

1. Perform peptide labeling at 37°C (the reaction proceeds more slowly at room temperature). Typically with a 5:1 Tb chelate/peptide ratio (2.5 mM Tb chelate, 0.5 mM peptide), more than 95% of the peptide will be labeled after a 6-h reaction. Agitation (rocking or intermittent light vortexing) can assist in driving the reaction forward.
2. Separate the labeled peptide from excess unreacted or hydrolyzed Tb chelate by HPLC. Use a diode-array spectrophotometer to identify the Tb chelate and the Tb chelate-labeled peptide by their characteristic absorbance peaks at 328 nm and 343 nm, respectively.

Caution: Water-methanol or water-acetonitrile gradients used in HPLC often contain small amounts of trifluoroacetic acid (TFA). The Tb ion will immediately dissociate from the chelate under acidic conditions (pH < 5.0), but can readily be reconstituted under less acidic conditions. Because unchelated Tb^{3+} forms insoluble precipitates with phosphate, avoid using phosphate buffers for the reconstitution step. Once the Tb chelate is reconstituted, however, it is stable in phosphate buffers.

Acidic HPLC purification

After acidic HPLC purification:

1. Remove the solvent containing TFA by speed vacuum or lyophilization;
2. Resuspend the labeled compound in the buffer of choice (pH 5.5–8.5, avoiding phosphate-containing buffers as noted above); and
3. Add 1 to 1.1 equivalents of $TbCl_3$ to the purified labeled compound to reconstitute the Tb center of the chelate. At micromolar concentrations or greater, the reformation of the Tb chelate is nearly instantaneous.

Non-acidic HPLC purification

Alternatively, use a non-acidic aqueous phase HPLC solvent, such as 100 mM TEAA buffer (pH 6.5).

4.2 Amine-modified Oligonucleotides

Amine-modified oligonucleotides can be effectively labeled with similar efficiency by following the peptide protocol, with the modifications listed below. As noted in **Pre-labeling Purification of Oligonucleotides** (**Section 3.4**), commercially available amine-modified oligonucleotides often contain undesired amine-containing impurities that standard ethanol precipitation procedures do not remove. These impurities, if not removed, will participate in undesired side-reactions and reduce the labeling efficiency.

1. Before proceeding with the labeling reaction, purify amine-modified oligonucleotides using a G-25 spin column.
2. Perform amine-modified oligonucleotide labeling at 37°C (the reaction proceeds more slowly at room temperature). Typically with a 5:1 Tb chelate/oligonucleotide ratio (2.5 mM Tb chelate, 0.5 mM oligonucleotide), more than 95% of the oligonucleotide will be labeled after a 6-h reaction. Agitation (rocking or intermittent light vortexing) can assist in driving the reaction forward.
3. If desired, separate labeled from unlabeled DNA by reverse-phase HPLC, ion-exchange chromatography, or gel electrophoresis.

Caution: Water-methanol or water-acetonitrile gradients used in HPLC often contain small amounts of trifluoroacetic acid (TFA). The Tb ion will immediately dissociate from the chelate under acidic conditions (pH < 5.0), but can readily be reconstituted under less acidic conditions. Because unchelated Tb^{3+} forms insoluble precipitates with phosphate, however, avoid using phosphate buffers for the reconstitution step. Once the Tb chelate is reconstituted, however, it is stable in phosphate buffers.

Acidic HPLC purification

After acidic HPLC purification:

1. Remove the solvent containing TFA by speed vacuum or lyophilization;
2. Resuspend the labeled compound in the buffer of choice (pH 5.5–8.5, avoiding phosphate-containing buffers as noted above); and
3. Add 1 to 1.1 equivalents of $TbCl_3$ to the purified labeled compound to reconstitute the Tb center of the chelate. At micromolar concentrations or greater, the reformation of the Tb chelate is nearly instantaneous.

Non-acidic HPLC purification

Alternatively, use a non-acidic aqueous phase HPLC solvent, such as 100 mM TEAA buffer (pH 6.5).

4.3 IgG Antibodies

The following example is a general protocol for labeling IgG antibodies. The isothiocyanate functional group of the amine-reactive Tb chelate is identical to that found on other isothiocyanate labeling reagents, such as fluorescein isothiocyanate (FITC). The principles of the TR-FRET labeling reaction are similar to those of a FITC labeling reaction.

This example describes the labeling of 1 mg of mouse IgG. The quantities listed in the table below provide for a 14-fold molar excess of Tb chelate, sufficient to label 1 mg of IgG in a 2- to 12-h reaction. The labeling reaction is not 100% efficient; this protocol typically yields 3–10 Tb chelates per IgG antibody. Varying the amount of Tb chelate used, the concentration of the protein being labeled, and the duration of the labeling reaction will affect the amount of bound Tb chelates per antibody.

Note: In some cases, excessively labeled antibody (more than 10 Tb chelates/antibody) will negatively affect antibody affinity or specificity.

Compound	Mass	Amounts Used in Labeling Protocol
IgG	150 kDa	1 mg = 6.7 nmol
Amine-Reactive Tb Chelate	1051 g/mol	100 µg = 95 nmol

1. Equilibrate the antibody to be labeled via dialysis or gel filtration to 100 mM sodium carbonate buffer (pH 9.5).
2. After equilibration, use a spin concentrator to concentrate the antibody to 3–10 mg/mL.
3. Verify the antibody concentration spectrophotometrically. The extinction coefficient of IgG is approximately 210,000 $M^{-1}cm^{-1}$ at 280 nm.
4. Dissolve 100 µg of amine-reactive Tb chelate in 20 µL of 100 mM sodium carbonate buffer (pH 9.5) to prepare a 5.5 mM amine-reactive Tb chelate solution. Adding this concentration of Tb chelate to 1 mg antibody gives an approximately 14-fold molar excess of chelate to antibody in the labeling reaction. The Tb chelate/antibody ratio can be increased or decreased to optimize labeling for a particular protein or

application (3–10 Tb chelates/antibody is a typical target ratio). However, using an excessive number of Tb chelates/antibody can negatively affect the affinity of the antibody for its target sequence.

5. Allow the reaction to proceed between 2–12 hours at room temperature. Agitation (rocking or intermittent light vortexing) can assist in driving the reaction forward.
6. Separate the labeled antibody from excess unreacted or hydrolyzed Tb chelate by dialysis or gel filtration into the buffer of choice (pH 5.5–8.5).
7. Determine the extent of labeling (the number of Tb chelates per antibody) by measuring the absorbance at 280 and 343 nm. The molar concentration of Tb chelate is determined spectrophotometrically. The extinction coefficient of the amine reactive Tb chelate at 343 nm is 12,570 $M^{-1}cm^{-1}$ and, when conjugated to an amine, its extinction coefficient at 280 nm is 1.1 times its value at 343 nm. The extinction coefficient of the IgG at 280 nm is 210,000 $M^{-1}cm^{-1}$.

   Determine the Tb chelate concentration using the following equation:

$$\textbf{[Tb chelate] (M)} = \frac{A_{343}}{12{,}570} \times \textbf{dilution factor}$$

   Determine the antibody concentration using the following equation:

$$\textbf{[antibody] (M)} = \frac{A_{280} - (1.1 \times A_{343})}{210{,}000} \times \textbf{dilution factor}$$

   The Tb chelate/antibody ratio can be determined from these two concentrations.

   Note: To quantitate the degree of labeling of proteins other than IgG, use the same calculations to determine the Tb chelate concentration. Calculate the protein concentration similarly, substituting the protein's extinction coefficient at 280 nm for that of IgG at 280 nm.

4.4 Cysteine-containing Peptides or Proteins

Proteins or peptides that contain reduced, exposed cysteine groups can be readily labeled by reacting them with the maleimide moiety of the thiol-reactive Tb chelate. As noted in **Section 3.3**, various buffers are acceptable and the reaction proceeds cleanly at pH 7.0–8.5. The success of the reaction relies upon the cysteine to be labeled being both accessible and reduced.

1. Use at least a 10-fold excess of DTT (2) or Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (3) to reduce disulfide bonds in the peptide or protein. After reduction, it is critical to remove excess DTT or TCEP before the labeling reaction via dialysis or gel filtration with the appropriate buffer.

   Note: If desired, determine the number of accessible, reduced cysteines. Ellman's reagent, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (4), can reveal the number of accessible, reduced cysteines available for labeling, because only reduced, accessible cysteines react with DTNB. Reacting a thiol-containing compound with excess DTNB at pH 8.0 will yield a product with an extinction coefficient of 13,600 at 412 nm. However, DTNB reacts with both DTT and TCEP, so these reducing agents must be removed before the DTNB reaction. If the peptide or protein of interest does not react with DTNB, this indicates that the cysteine(s) cannot be labeled, because they are either oxidized or inaccessible (i.e., sterically blocked).

2. Allow the labeling reaction to proceed for 1–12 h at room temperature. Agitation (rocking or intermittent light vortexing) can assist in driving the reaction forward. The reaction proceeds more rapidly when higher concentrations of reactants are used. For example, when labeling cysteine-containing peptides at concentrations of 1 mM peptide and 1.2 mM Tb chelate, the reaction will be 97% complete in only one h. A two- to 10-fold excess of Tb chelate and longer incubation times are often necessary when labeling proteins at lower (micromolar) concentrations.
3. Separate labeled protein from excess unreacted or hydrolyzed Tb chelate by dialysis or gel filtration into a buffer of choice (pH 7.0–8.5). Separate labeled peptide from excess unreacted or hydrolyzed Tb chelate by HPLC as described in **Section 3.5.1**. Use a diode-array spectrophotometer to identify he Tb chelate and the Tb chelate-labeled peptide or protein by the characteristic absorbance peaks at 328 nm and 343 nm, respectively.
4. For proteins, determine the extent of labeling either with DTNB or spectrophotometrically. DTNB will react with any accessible, unlabeled cysteine that did not react with the Tb chelate. Alternatively, the molar concentration of Tb chelate can be determined spectrophotometrically by measuring the absorbance at 343

nm. The extinction coefficient of the Tb chelate at 343 nm is 12,570 $M^{-1}cm^{-1}$ and, when conjugated to a thiol on a peptide or a protein, its extinction coefficient at 280 nm is 75% of its value at 343 nm.

Determine the Tb chelate concentration using the following equation:

$$\textbf{[Tb chelate] (M)} = \frac{A_{343}}{12{,}570} \times \textbf{dilution factor}$$

Determine the peptide or protein concentration similarly to the method shown in **Section 4.3**, substituting the protein's extinction coefficient at 280 nm for that of IgG at 280 nm, as shown in the following equation:

$$\textbf{[peptide or protein] (M)} = \frac{A_{280} - (0.75 \times A_{343})}{\textbf{Extinction coefficient of peptide or protein at 280 nm}} \times \textbf{dilution factor}$$

The Tb chelate/peptide or protein ratio can be determined from these two concentrations.

5.0 TROUBLESHOOTING GUIDE

Problem	Possible Solution
Low or no TR-FRET fluorescence signal	Donor and acceptor are too far apart, so resonance energy transfer (RET) is inefficient. The characteristic Tb emission spectrum will remain detectable. Re-engineer labeling sites to bring the donor and acceptor closer together.
	Donor and acceptor are too close. RET occurs, but within the "gating time" of the assay, so very little or no fluorescence signal is detected. The characteristic Tb emission spectrum will be severely decreased, because its signal will be quenched. If the donor and acceptor are on the same molecule, the characteristic Tb emission spectrum will again become detectable if the acceptor is removed. Re-engineer labeling sites to situate the donor and acceptor farther apart.
	Too few sites have been labeled. The rate of the labeling reaction is directly proportional to the concentration of the reaction components and the length of the reaction. Repeat the labeling reaction, but use conditions that will result in more label binding, such as increasing the concentration of reagents or the reaction time. The appropriate reagent concentrations and reaction time can vary considerably for different proteins or peptides and each user must experimentally determine the exact conditions for their particular application.
	Fluorescence plate reader is insufficiently sensitive; filter-based fluorescence plate readers are generally 50-fold more sensitive than monochromator-based fluorescence plate readers.
No Tb emission spectrum detected	The Tb center has been lost during the reaction, because of an incompatible buffer. Equilibrate the labeled compound in an appropriate buffer (see **Section 3.3, Buffer Considerations**) and add 1 to 1.1 equivalents of $TbCl_3$ to the purified labeled compound to reconstitute the Tb center of the Tb chelate. At micromolar concentrations or greater, the reformation of the Tb chelate is nearly instantaneous.
	Donor and acceptor are too close. RET occurs, but within the "gating time" of the assay, so very little or no fluorescence signal is detected. The characteristic Tb emission spectrum will be severely decreased, because its signal is quenched. If the donor and acceptor are on the same molecule, the characteristic Tb emission spectrum will again become detectable if the acceptor is removed. Re-engineer labeling sites to situate the donor and acceptor farther apart.
Spurious fluorescence signal detected (interaction known to be disrupted or not to exist)	Non-specific binding has occurred. Adding detergents or salts to the buffer may reduce non-specific binding.
	Concentrations of labeled binding partners are too high. At micromolar concentrations, diffusion-enhanced RET can occur. Repeat labeling reaction, but reduce the concentration of either the donor or the acceptor molecule.
Labeled compound has reduced solubility	Too many sites have been labeled with the Tb chelate. Repeat labeling reaction, but use conditions that will result in less label binding, such as a lower concentration of Tb chelate or a shorter reaction time.
Tb-labeled antibody exhibits reduced affinity or specificity	Too many sites have been labeled with the Tb chelate. Repeat labeling reaction, but use conditions that will result in less label binding, such as a lower concentration of Tb chelate or a shorter reaction time.

6.0 STORAGE AND STABILITY

The amine- and thiol-reactive chelates are shipped desiccated and should be stored desiccated at –20°C. After reconstitution, the Tb chelates should be stored on ice and used within the same day.

7.0 REFERENCES

1. Selvin, P.R. (2002) *Annu. Rev. Biophys. Biomol. Struct.* **31:**275-302.
2. Cleland, W.W. (1964) *Biochemistry.* **3**:480-2.
3. Getz, E.B., Xiao, M. *et al.* (1999) *Anal. Biochem.* **273**:73-80.
4. Ellman, G.L. (1959) *Arch. Biochem. Biophys.* **82**:70-7.
5. Li, M. and Selvin, P.R. (1997) *Bioconjug. Chem.* **8:**127-32.
6. Chen, J. and Selvin, P.R. (1999) *Bioconjug. Chem.* **10:**311-5.

8.0 TECHNICAL BACKGROUND

LanthaScreen™ assays employ a Time-Resolved Fluorescence Resonance Energy Transfer (TR-FRET)-based detection method to measure interactions between various binding partners. Examples of binding partners include protein-protein, protein-peptide, or DNA-protein interactions.

TR-FRET assays rely upon non-radiative energy transfer from an excited-state lanthanide ion (donor) to an acceptor molecule when the two components are close to one another, typically less than 100 Å apart. If the acceptor is a fluorescent molecule, such as fluorescein or rhodamine, then the transferred energy is emitted as a detectable fluorescence signal. The primary advantage of TR-FRET over standard, non-TR-FRET is that the excited-state lifetime of a lanthanide chelate is on the millisecond-time scale, while that of most small-molecule fluorophores is on the nanosecond-time scale (1). Consequently, waiting 100 µs before measuring the fluorescence intensity of either the donor or the acceptor species will "gate out" interfering fluorescence arising from matrix components, library compounds, or laboratory plastics. Further, when measuring energy transfer to an acceptor fluorophore, this gated detection method reduces spurious fluorescence signals arising from direct excitation of the acceptor fluorophore. Therefore, the signal-to-background ratio is typically several fold higher than is generally seen with standard, shorter lifetime FRET pairs. Additionally, the lanthanide chelates' large Stokes shift (> 200 nm) helps to decrease background fluorescence levels. Further utility arises from the characteristic shape of the Tb emission spectrum, which has four sharp peaks with large baseline separation. These "silent regions" between the four peaks can be used to detect emissions from an acceptor fluorophore (see **Figure 1**).

Invitrogen's LanthaScreen™ Tb chelates are readily excited with a flashlamp using a 340-nm (30-nm bandpass) filter. As shown in **Figure 1**, the emission spectrum displays four sharp, distinct peaks centered at 490, 546, 585, and 620 nm, with clear baseline separation and "silent" regions between the peaks. With appropriate filters and using the settings and acceptors described in **Required Equipment, Recommended Filters and Suitable Acceptor Fluorophores** (**Section 1.2**), energy transfer to a suitable acceptor, such as fluorescein or rhodamine, can be readily detected in these otherwise silent regions by monitoring an increase in acceptor fluorescence intensity. This signal may be referenced to the Tb (donor) signal at either 490 nm (fluorescein) or 546 nm (rhodamine).

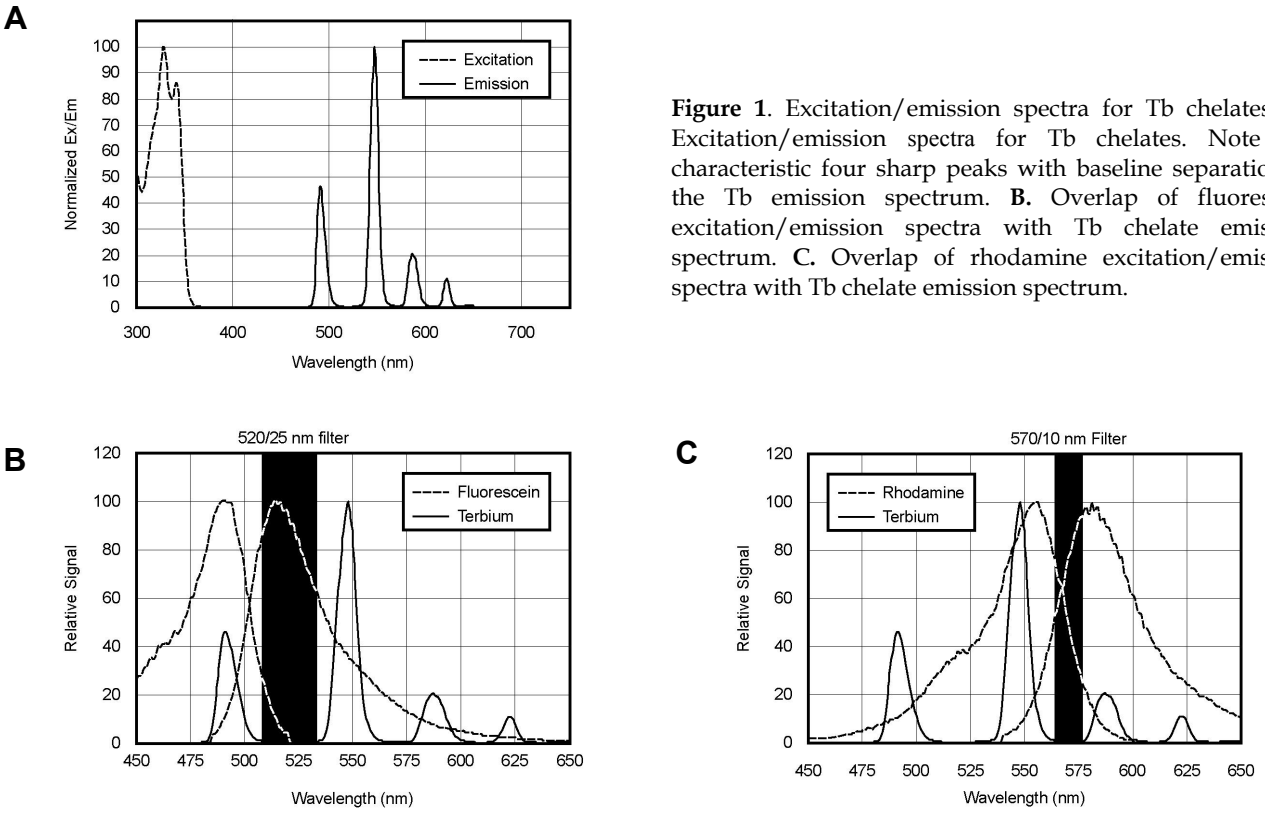


Figure 1. Excitation/emission spectra for Tb chelates. **A.** Excitation/emission spectra for Tb chelates. Note the characteristic four sharp peaks with baseline separation in the Tb emission spectrum. **B.** Overlap of fluorescein excitation/emission spectra with Tb chelate emission spectrum. **C.** Overlap of rhodamine excitation/emission spectra with Tb chelate emission spectrum.

To take advantage of the long fluorescent lifetime of the lanthanide chelates in reducing background fluorescence, TR-FRET measurements are performed using gated detection after excitation by the flashlamp. Setting the measurement window to begin after undesired background signals have completely decayed eliminates background fluorescence and luminescence from matrix components, library compounds, or laboratory plastics, and from the non-instantaneous nature of the flashlamp pulse. The delay time between excitation and the beginning of measurement is typically set to 100 µs, and the integration time (the time during which energy transfer is measured) is set for 100–1000 µs. The exact values must be experimentally determined for the particular application under investigation. Increasing the number of flashes per read can minimize measurement errors. These principles are shown schematically in **Figure 2**.

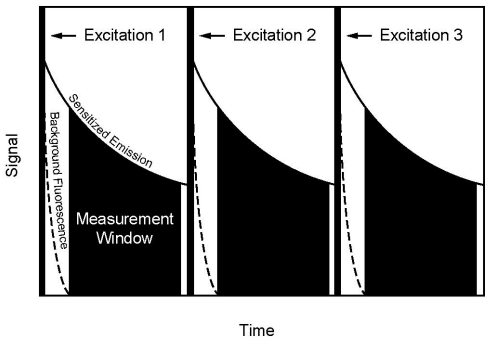


Figure 2. Schematic of measurement of TR-FRET-based energy transfer using flashlamp excitation. This figure shows data collected from three excitations; typically measurements from 10 or more excitations are collected. The measurement window begins after the undesired background signals have completely decayed, and closes before the next excitation.

9.0 PURCHASER NOTIFICATION

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