



# LanthaScreen™ Reagents for Facile TR-FRET Assay Development

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## Introduction

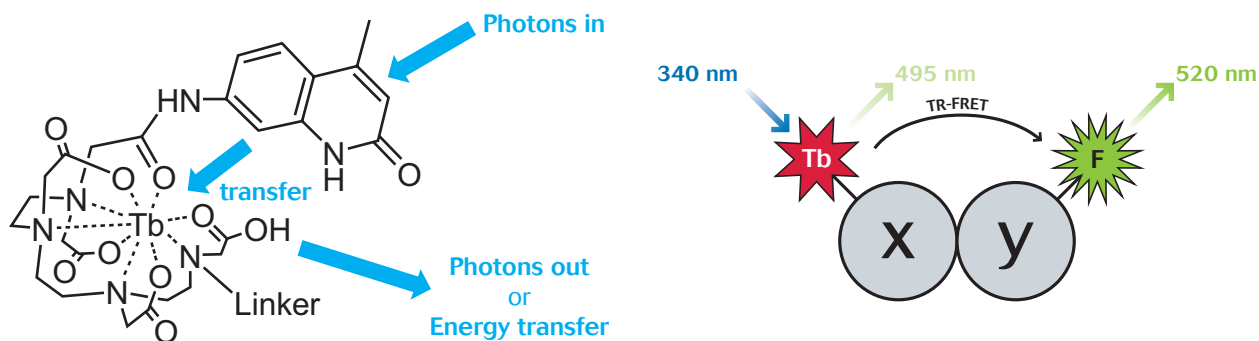


TR-FRET assays have traditionally used europium as the long-lifetime donor label and the fluorescent protein allophycocyanin (also known as APC or XL-665) as the acceptor species. Due to its size (>100 kD), APC is typically used as a streptavidin conjugate to indirectly label a biotinylated substrate. In contrast, the LanthaScreen™ TR-FRET platform from Invitrogen Drug Discovery Solutions uses terbium in place of europium as the long-lifetime donor species, and fluorescein as the acceptor species.

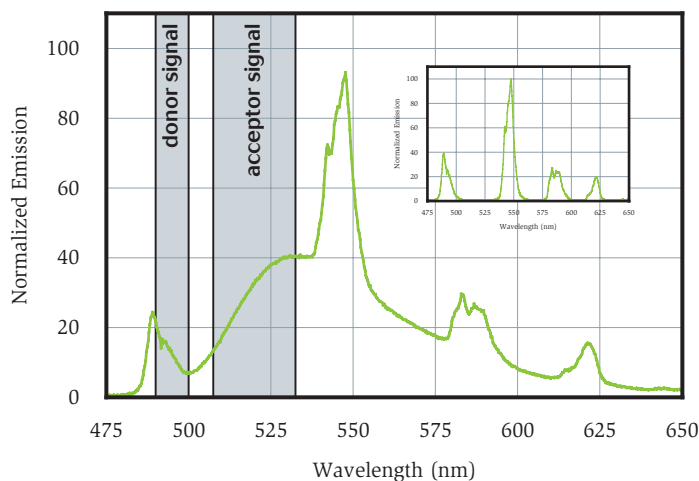
The terbium-based LanthaScreen™ configuration has several advantages over the trimolecular donor/biotinylated substrate/streptavidin-APC format, including simpler assay optimization, faster kinetics of complex formation, avoidance of steric problems associated with the large streptavidin-APC moiety, as well as the cost and lot-to-lot consistency of fluorescein relative to streptavidin-APC.

We have successfully applied this technology to a variety of target classes such as kinases, proteases, and nuclear receptors, and have demonstrated the resistance of the readout format to interference from color quenchers, light scatterants, or fluorescent compounds.

**Figure 1—Principle of LanthaScreen™ TR-FRET Format**

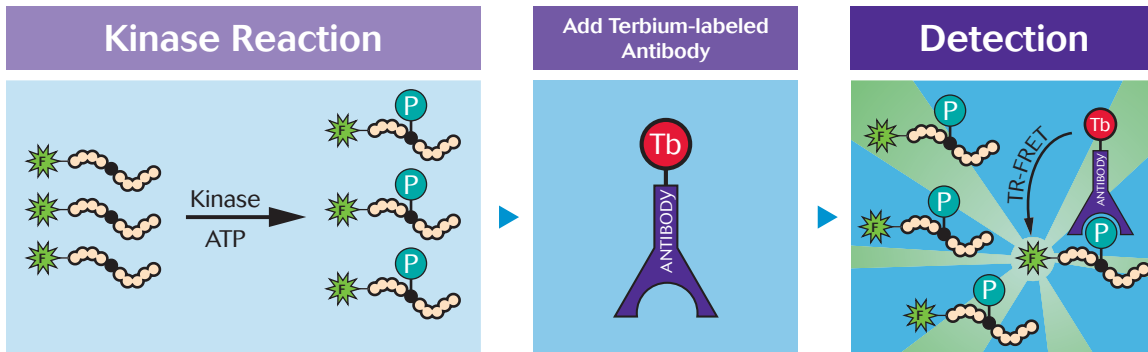


The LanthaScreen™ format is based on the use of a long-lifetime terbium chelate as the donor species and fluorescein as the acceptor species. When terbium and fluorescein labeled molecules are brought into proximity, energy transfer takes place, which can be read in a time-resolved manner to reduce assay interference and increase data quality.

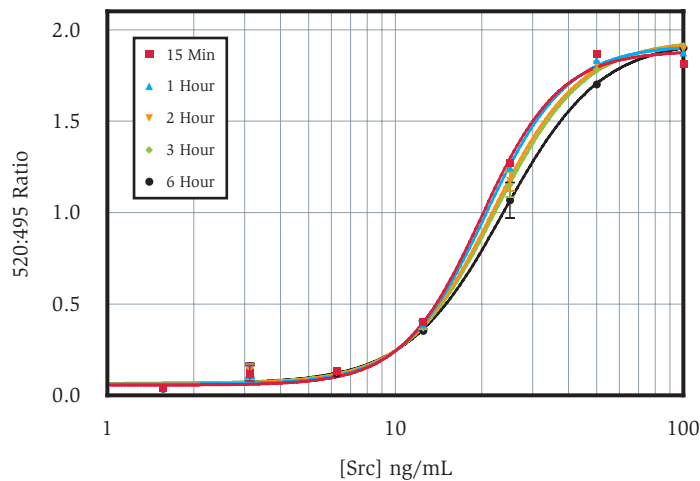


The time-resolved spectra at right illustrates energy transfer occurring when terbium and fluorescein are brought into proximity via biomolecular interactions. The TR-FRET value is determined as a ratio of the FRET-specific signal measured with a 520 nm filter to that of the signal measured with a 495 nm filter, which is specific to terbium. The inset shows the time-resolved spectra in the absence of energy transfer.

Figure 2—Applications to Kinase Assays

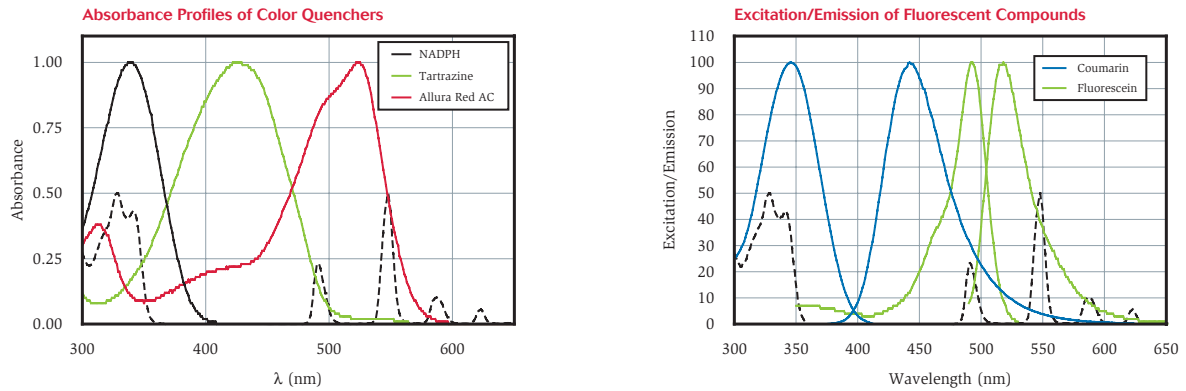


Schematic of the LanthaScreen™ TR-FRET kinase assay platform. A fluorescein-labeled kinase substrate peptide is incubated with kinase and ATP. Terbium-labeled antibody is then added and phosphorylation detected by an increase in the TR-FRET ratio. Because the substrate is directly labeled, there is no need to add streptavidin-APC. Additionally, unlike some europium based systems, there is no requirement to add high concentrations of KF, which can potentially disrupt antibody/product interactions.

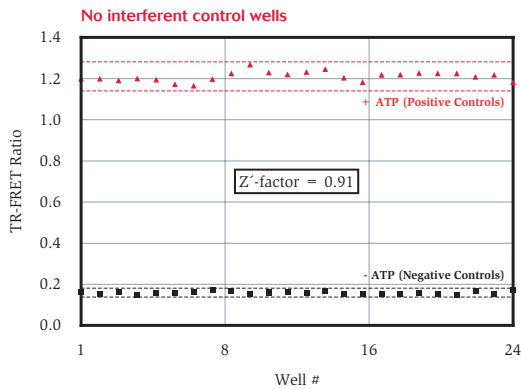


Tb-labeled PY-20 was used to assay Src kinase activity. A two-fold serial dilution of Src (Invitrogen P3044) was assayed against 200 nM fluorescein-YIYGSFK (Invitrogen PV3513) and 100  $\mu$ M ATP in 50 mM HEPES pH 7.5, 10 mM MgCl<sub>2</sub>, 1 mM EGTA, and 0.01% Brij35 in a total reaction volume of 10  $\mu$ L. After 75 minutes, EDTA and Tb-labeled PY-20 were added to a final concentration of 10 mM and 2 nM, respectively, for a final assay volume of 20  $\mu$ L. After a brief equilibration the plate was read on a BMG PheraStar. All data-points were performed in triplicate. The detection reaction was seen to rapidly come to equilibrium, and the curve was stable for over 6 hours. The “assay window” showed over a 30-fold increase in response between fully phosphorylated and non-phosphorylated substrate.

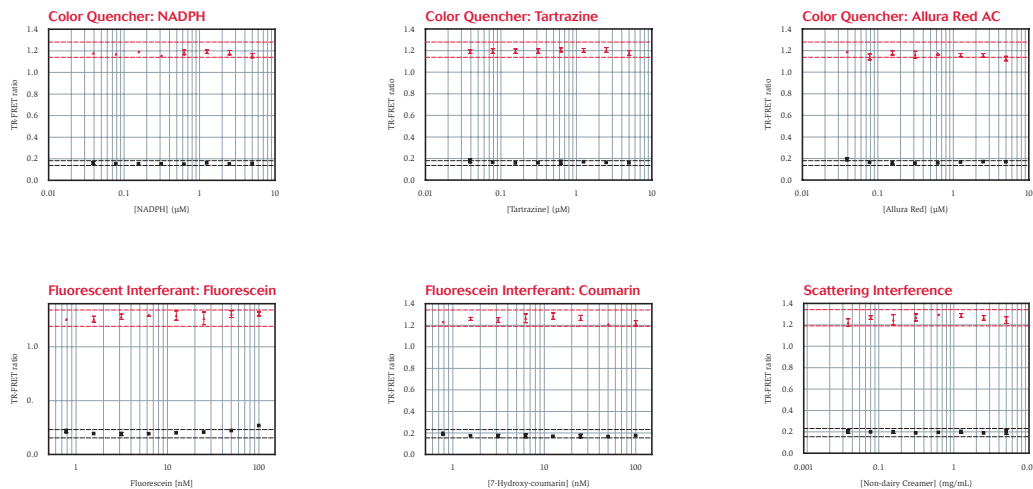
**Figure 3—LanthaScreen™ assays are resistant to interference from color quenchers, light scatterants, and fluorescent compounds**



To demonstrate the resistance of the LanthaScreen™ format to interference from color quenchers, fluorescent compounds, or light scatterants, kinase assays were read in the presence of such compounds. The color quenchers and fluorescent compounds were chosen to overlap with the excitation and emission spectra of the terbium chelates, in order to provide a “worst case scenario” for interference. The absorbance or fluorescent spectra of these compounds is shown in the graphs at left

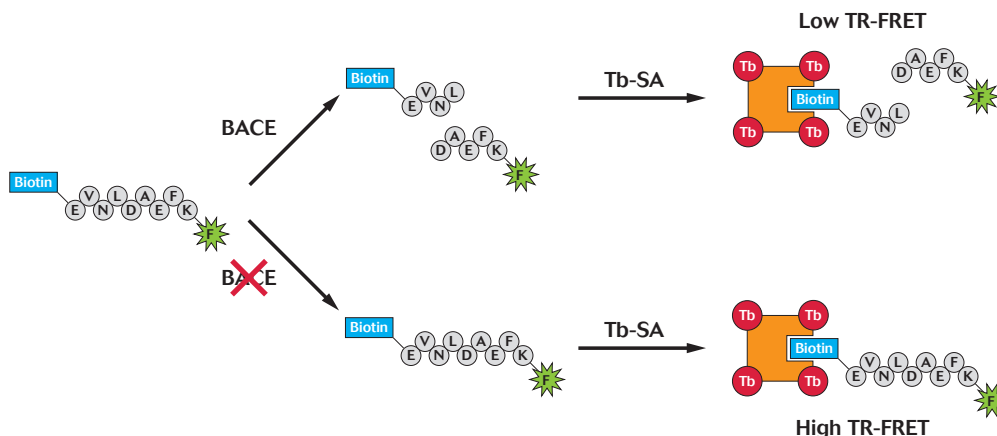


Src kinase was first assayed in the absence of interfering compounds. The results of 24 positive and 24 negative control wells produced a Z'-factor of 0.91. Dashed lines represent ± 3 standard deviations from the average control value.

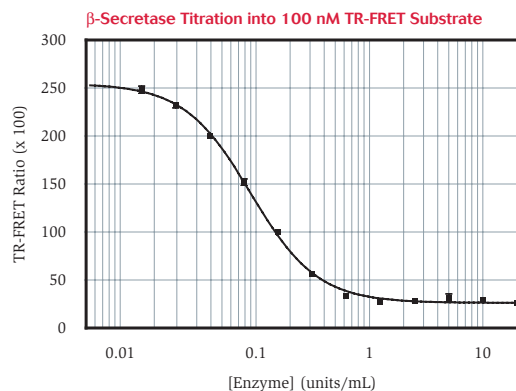


Src kinase assays were read in the presence of interfering compounds. Dashed lines represent ± 3 standard deviations from the average control values in the “no interferant” control plate shown above. Interference seen in the raw data of the color-quenched samples was compensated for by the ratiometric nature of the readout. No interference was seen in the raw data of the samples containing light scatterant or fluorescent compounds.

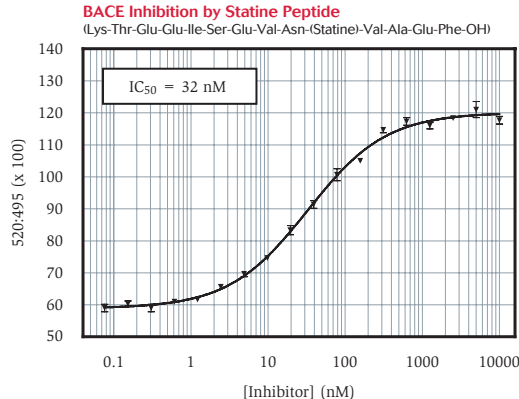
Figure 4—Applications to Protease Assays



Terbium labeled streptavidin is useful as a “generic” labeling reagent for biotinylated biomolecules such as proteins, peptides, or oligonucleotides. In protease assays, Invitrogen’s terbium-based LanthaScreen™ technology allows TR-FRET protease assays to be performed using peptides that contain biotin- and fluorescein- modified termini.

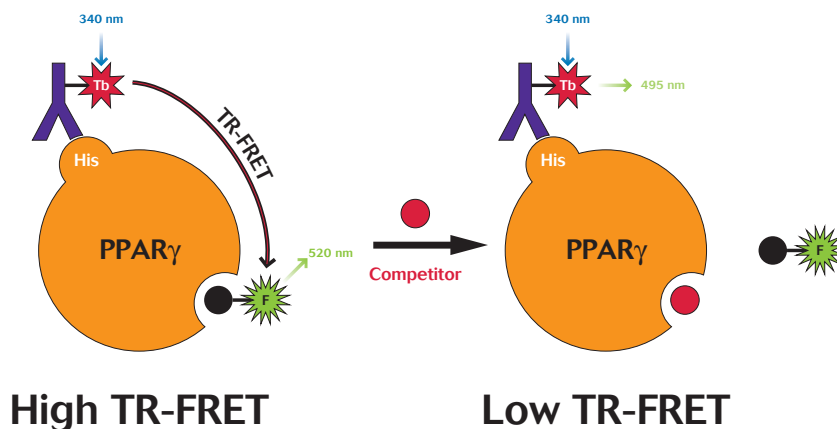


To assay β-secretase (BACE1) catalyzed cleavage of a peptide corresponding to the cleavage site in amyloid precursor protein, a peptide corresponding to the cleavage site was synthesized with an N-terminal biotin and a C-terminal fluorescein. This peptide was incubated with a dilution series of BACE1 enzyme for 1 hour at room temperature, after which the reaction was stopped by adding Tb-labeled streptavidin in a pH 8.0 buffer and the plate read on a BMG PheraStar plate reader.

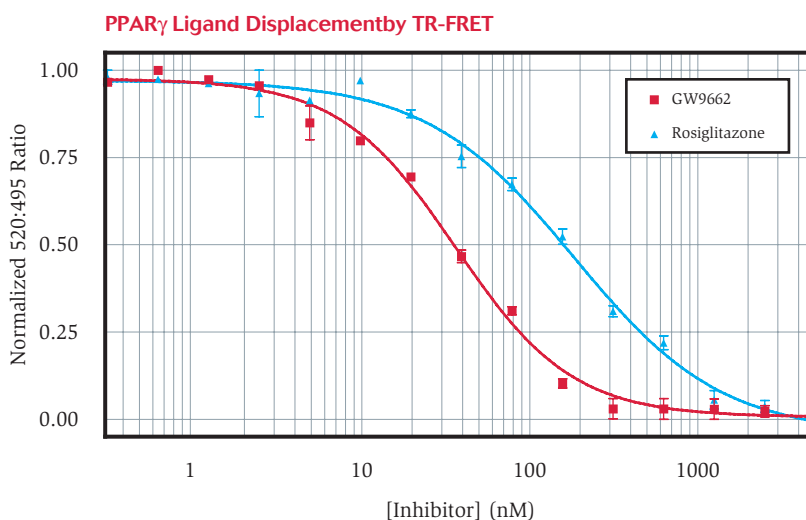


For inhibition experiments, a reaction concentration of 0.1 units/mL BACE1 was chosen for the assay. This concentration of enzyme is expected to give approximately 50% cleavage of substrate in the absence of inhibitor. The inhibitor was a statine containing peptide that was titrated against enzyme in a two-fold dilution series beginning at 10 μM. The assay was performed as described previously. Each data point represents the average of three wells. The statine containing peptide was shown to inhibit BACE1 with an  $IC_{50}$  of 32 nM, a value in agreement with the previously reported 30 nM.

**Figure 5—Applications to Nuclear Receptor Assays**



Terbium labeled anti-“tag” antibodies are useful as “generic” labeling reagents for epitope tagged proteins. Such reagents are especially useful in assays containing partially purified protein sources. In the nuclear receptor ligand-displacement assay shown schematically above, TR-FRET takes place between a terbium-labeled antihistag antibody bound to histagged PPAR $\gamma$  ligand binding domain and a fluorophore-labeled ligand for PPAR $\gamma$ . In the presence of compounds that displace the fluorophore labeled ligand, FRET is disrupted.



The graph shows displacement of fluorophore-labeled ligand from recombinant human PPAR $\gamma$  ligand binding domain by known competitors. The observed EC<sub>50</sub> values for GW9662 and rosiglitazone were 36 and 182 nM, respectively. These EC<sub>50</sub> values are in accord with values determined by a similar fluorescence polarization assay (21 and 109 nM, respectively)

## Results and Conclusions

The terbium-based LanthaScreen™ format offers several advantages over traditional europium-based formats. The ability to use fluorescein rather than APC as an acceptor simplifies assay development and greatly reduces assay cost. Although a move to “redder” fluorophores has been shown to reduce assay interference in other formats, the time-resolved and ratiometric nature of the LanthaScreen™ format allows for the use of fluorescein without the associated drawbacks.

Complete reagent sets (Tb-labeled antibodies and fluorescein labeled substrates) for assaying a broad range of kinases are available from Invitrogen Drug Discovery Solutions. In addition, “generic” reagents such as streptavidin or anti-“tag” antibodies are available for easily developing assays against a range of diverse target classes. Custom labeling services are also available to provide solutions to assay problems.



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