

Sensitive, Versatile Assays for Lipid Kinase/Phosphatase Drug Discovery

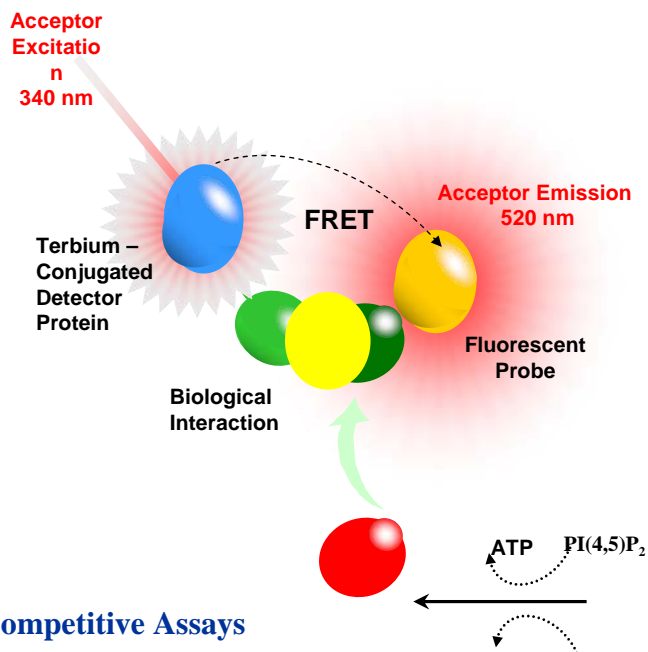
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Abstract

Lipid products of phosphoinositide kinase and phosphatase enzymes act as cell-signaling molecules essential in multiple aspects of cell division, growth, and survival. These include PI(3,4,5)P₃ the product of class I PI3-Ks and PI(3,4)P₂ the product of SHIP2, which are established drug targets for cancer, diabetes, and inflammatory disorders. Traditional assays are nonspecific, have poor sensitivity, are radioactive or involve laborious HPLC/TLC separations. Here we describe the development of a family of competitive fluorescent assays and reagents based on energy transfer from terbium-complex conjugated lipid recognition proteins to BODIPY-FL™-labeled phosphoinositide tracers. We call these products TRueFRET™ for Time Resolved Universally Enhanced Fluorescence Resonance Energy Transfer. TRueFRET™ reagents utilize highly luminescent terbium chelates with 60% quantum yields making them the brightest lanthanide complexes available. TRueFRET™ assays are homogeneous, sensitive, and suitable for HTS and drug development applications in the field of lipid kinases/phosphatases.

Introduction



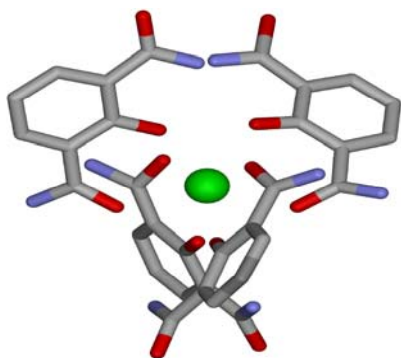
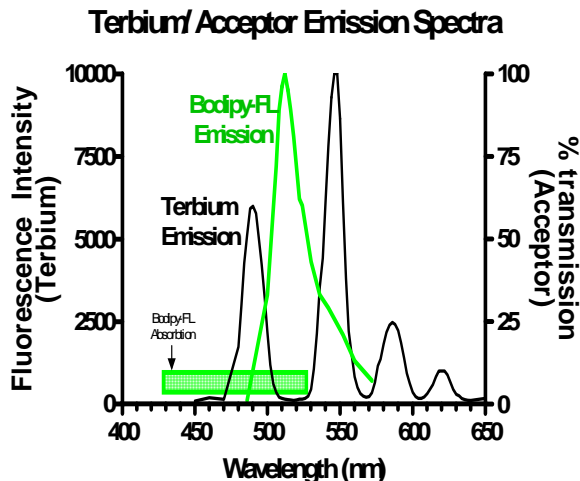
Competitive Assays

TRueFRET™ PI3-K activity assay is a competitive time-resolved fluorescence resonance energy transfer (TR-FRET) assay measuring PI3-K activity through its product, PI(3,4,5)P₃. As enzymatic reactions progress, PI(3,4,5)P₃ levels increase and compete with the fluorescent probe for binding to the Detector (see PI3-K TRueFRET Assay Curve). Lower TRueFRET signal indicates higher PI3-K activity.

TR-FRET

TR-FRET Principle

TRueFRET™ assay are based on time-resolved fluorescence resonance energy transfer (TR-FRET). When the donor fluorophore is in close proximity to the acceptor fluorophore, direct excitation of the donor results in energy transfer to the acceptor. Lanthanide chelates are preferable for FRET assays because the long excited state lifetimes enable detection of energy transfer to an acceptor in a gated mode, which eliminates the high noise levels, sample autofluorescence and light scattering problems often observed with conventional FRET assays.



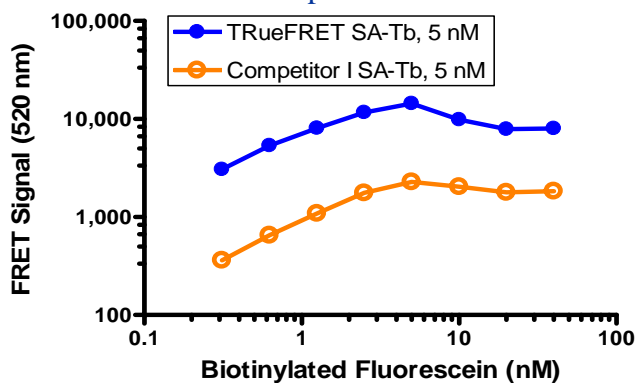
At the heart of TRueFRET™ technology is Lumi4™-Tb, a terbium complex that offers unparalleled brightness which may lower limits of detection in assays by as much as 10X (see Streptavidin assay at right) or may allow using less reagent in already existing assays.

The highly luminescent Lumi4™-Tb reagents exhibit 60% quantum yields, the highest available, while also offering high stability in bioassay matrices.

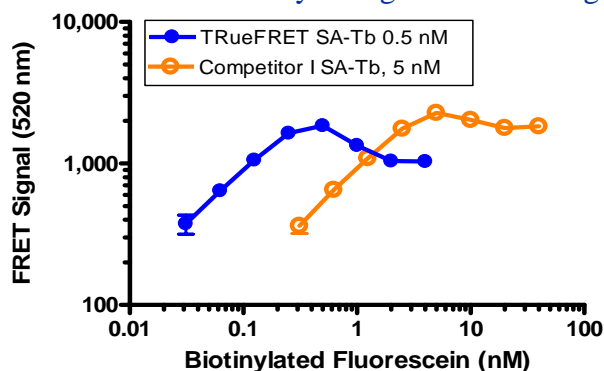
Product Comparison

Streptavidin-Tb Complexes: Using Biotinylated Fluorescein as FRET Acceptor

Greater FRET Signal using Equivalent Amounts of Streptavidin

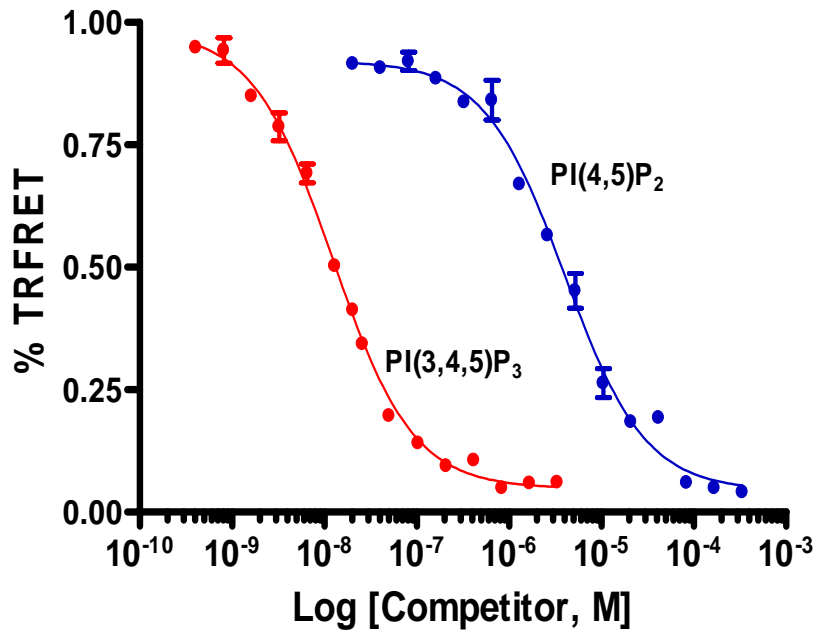


10X Greater Sensitivity Using 10X Less Reagent



PI3-Kinase

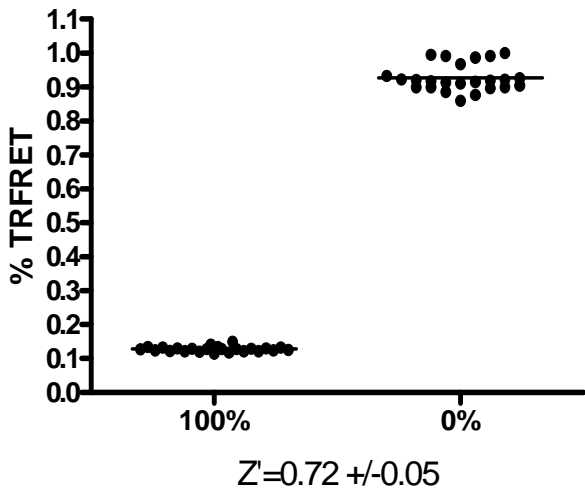
TRueFRET™ PI3-Kinase Assay: Specific & Sensitive



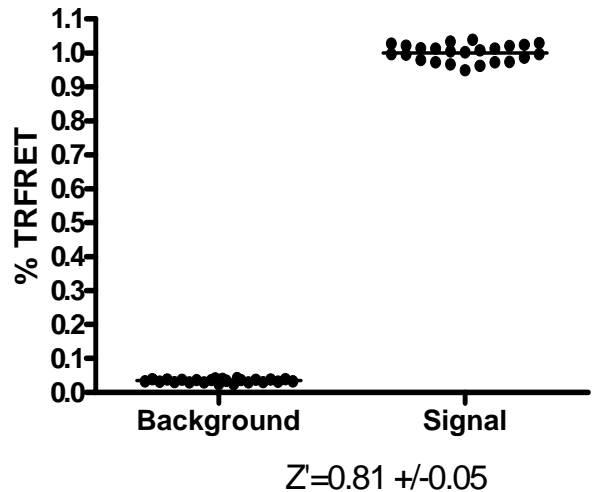
diC₈-PI(3,4,5)P₃ and diC₈-PI(4,5)P₂ compete with a 10 nM BODIPY FL-PI(3,4,5)P₃ acceptor probe for binding to 1 nM Lumi4™-Tb labeled anti-PI(3,4,5)P₃ binding protein.

TRueFRET™ PI3-Kinase Assay: Z' Determination

TRueFRET PI3-K
% conversion Z' Value



TRueFRET PI3-K
Controls Z' Value

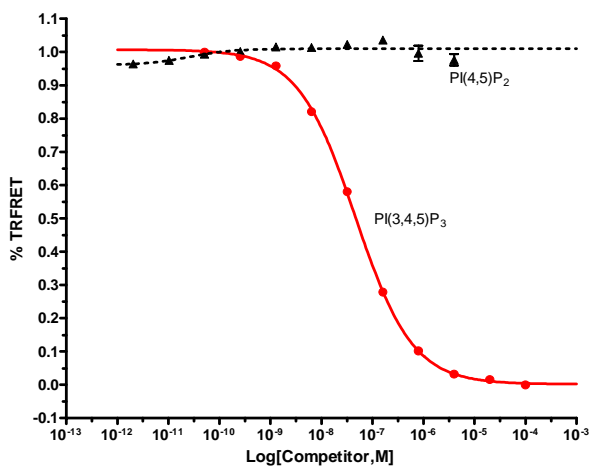


Five independent experiments were averaged to determine Z' values for the TRueFRET PI3-K activity assay. The left graph compares PI3-K enzyme percent conversion values of **100%** and **0%** by competition with 400 nM diC₈-PI(3,4,5)P₃ and diC₈-PI(4,5)P₂ respectively. Z' value was 0.72. In the right graph Lumi4™-Tb labeled binding protein, with no acceptor, (**Background**) and with BODIPY FL-PI(3,4,5)P₃ as acceptor (**Signal**) are compared. Z' value was 0.81.

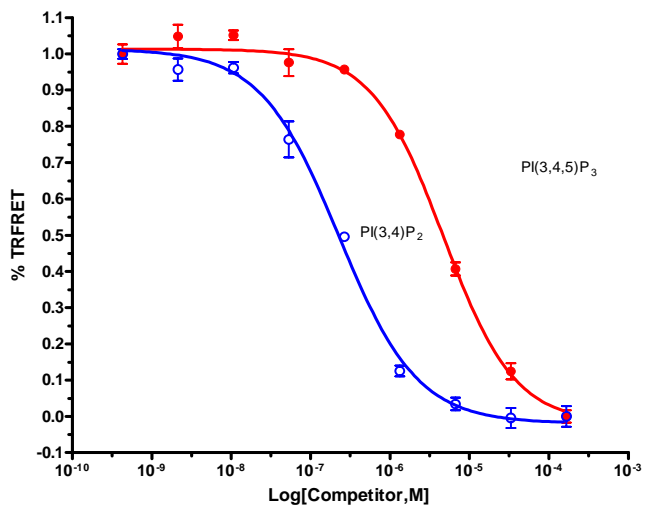
ToolBox Versatility

Competitive PI3-K and SHIP TRueFRET Assays: Biotinylated-binding proteins & Lumi4TM-Tb Streptavidin

TRueFRET PI3-K Activity Assay



TRueFRET SHIP Activity Assay



Competitive PI3-K and SHIP enzyme assays were developed using 6 nM Lumi4TM-Tb labeled Streptavidin as a starting point to ensure a strong FRET signal. The left graph shows competition by diC₈-PI(3,4,5)P₃ and diC₈-PI(4,5)P₂ for interaction with biotin-labeled PIP₃ specific binding protein and 10 nM BODIPY FL-PI(3,4,5)P₃. The right graph shows competition by diC₈-PI(3,4,5)P₃ and diC₈-PI(3,4)P₂ for interaction with biotin-labeled PI(3,4)P₂ binding protein and 10 nM BODIPY FL-PI(3,4)P₂.

Conclusions

Phosphoinositide kinases and phosphatases are important regulators of cell signaling pathways with aberrant signaling resulting in multiple pathologies. Thus pharmaceutical and biotech institutions are interested in HTS of small molecule inhibitors for these pathways. The competitive TRueFRETTM PI3-K activity assay was developed as a sensitive and robust screening tool. And performance was similar for both directly-conjugated and generic (i.e. streptavidin) Lumi4TM-Tb assay configurations. TRueFRETTM reagents were found to be brighter than a competitor's terbium-based products, potentially reducing reagents and cost by at least 10 fold while also offering dramatically lower detection limits. Together these results demonstrate advantages of Lumi4TM-Tb-based TRueFRETTM assays and reagents as versatile tools for lipid kinase and phosphatase drug discovery applications.