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Technical Data Sheet

For research use only

Not intended or approved for diagnostic or therapeutic use.



Product Name: TRueFRET™ PI3-K Activity Assay
Time Resolved Universally Enhanced Fluorescence Resonance Energy Transfer (TRueFRET™) 384-well assay kit for the detection and quantification of PI3-Kinase Activity

Product Number: K-2700

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1 Storage and kit contents: Store kit components at 4 °C for up to one month.

DO NOT freeze PI(3,4,5)P₃ Detector. The detector is stable at 4 °C, for up to 6 months.

Kit Contents:

(Reagents for 400 points)

- 1 vial PI(3,4,5)P₃ Detector* (K-2701); 44 µL of 600 nM
- 1 vial lyophilized Probe (K-2702)
- 1 vial lyophilized PI(4,5)P₂ Substrate (K-2703)
- 1 vial PI(3,4,5)P₃ lyophilized Standard (K-2704)
- Black 384-well plate

Additional Materials Required:

- Detection Buffer
- Source of PI3-K Enzyme and Reaction Buffer
- Microplate Reader with time-resolved fluorescence capability and correct filter set

*Detector conjugate incorporates Lumiphore's luminescent Terbium complex (Tb-Lumi4™).

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2 Product Overview

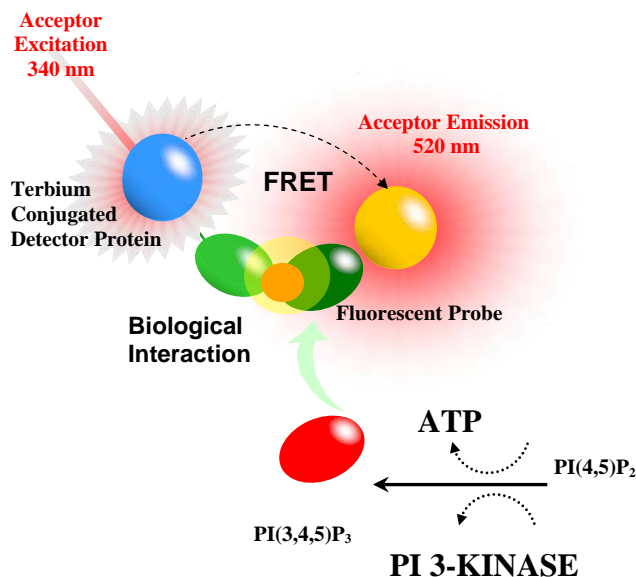


Fig. 1 Illustrates the principal of Echelon's TRueFRET™ PI3-K Activity Assay. PI(3,4,5)P₃ levels increase as PI3-K enzyme reactions progress. PI(3,4,5)P₃ competes with the probe for binding to the detector protein. The signal is inversely correlated with enzyme activity.

2.1 Assay Principal

The TRueFRET™ PI3-K Activity kit 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 (PI3-K) reactions progress, PI(3,4,5)P₃ levels increase and compete with the fluorescent probe for binding to the Detector (Fig. 1). Lower TRueFRET signal indicates higher PI3-K activity.

2.2 TR-FRET Principal

Echelon's TRueFRET™ assay is based on the principles of time-resolved fluorescence resonance energy transfer (TR-FRET). When the donor fluorophore is in close proximity to the acceptor fluorophore, excitation of the donor results in energy transfer to the acceptor. TR-FRET assays utilize lanthanide chelates as the donor species due to their long excited state lifetime of a millisecond or longer (compared to other fluorophores in the nanosecond range). This unique characteristic of lanthanide dyes is useful in avoiding problems with interference from compound autofluorescence or light scattering. TR-FRET assays are generally measured after a suitable delay of 50-100 microseconds after excitation by flash lamp.

2.3 TRueFRET™ Technology

Time-resolved universally enhanced fluorescent resonance energy transfer (TRueFRET™) utilizes highly luminescent Terbium (Tb³⁺) complexes with a quantum yield of 60 % making them the brightest lanthanide complexes available for commercial time-resolved applications in aqueous environments. This new macrocyclic Tb³⁺ complex (Tb-Lumi4™) has two peak emission lines at 490 nm and 545nm as seen in Fig 2.

2.4 Application

Quantification of PI3-K activity through the measurement of its end product, PI(3,4,5)P₃.

2.5 Specificity

The Detector has been tested for binding selectivity of PI(3,4,5)P₃, the enzymatic product of PI3-K, relative to the PI3-K substrate, PI(4,5)P₂. Detector binds the product with approximately 300-fold greater affinity than the substrate as determined by comparing competition EC₅₀ values.

2.6 PI3-Kinase Background

Class I Phosphoinositide 3-kinases (PI3-K), are ubiquitously expressed lipid kinases which phosphorylate PI(4,5)P₂ at the 3'-hydroxyl of the inositol ring producing PtdIns(3,4,5)P₃. PtdIns(3,4,5)P₃ has shown to be an

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important lipid second messenger with key roles in fundamental cellular responses such as proliferation and survival. Due to these interesting biological roles, PI3-K and PtdIns(3,4,5)P₃ have become attractive targets for research and drug development in many diseases including inflammation and cancer.

3 Instrument Settings

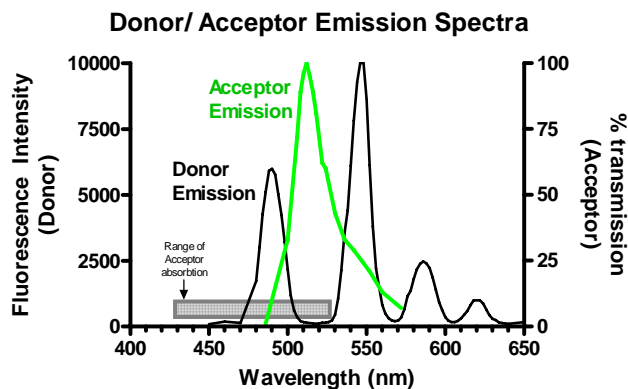


Fig. 2: Emission spectra of donor and acceptor fluorophore.

3.1 Donor Excitation Filter

The Tb³⁺ donor fluorophore can be excited using a 340 nm excitation filter with a 30 nm bandpass or larger.

3.2 Donor Emission Filter

It is not necessary to record a donor emission; the acceptor emission does not need to be referenced. If the acceptor emission vs. donor emission ratio is needed to correct for well to well volume differences or quenching from colored compounds, a filter centered at 490 nm with a 8-10 nm bandpass can be used.

3.3 Acceptor Emission Filter

In order to measure the acceptor fluorophore emission, without interference from the Tb³⁺ donor, it is important to use a filter centered at 520 nm with a bandpass no larger than 20 nm. A larger filter band pass will pass light associated with the Tb³⁺ donor peaks, giving higher background.

3.4 Other Instrument Settings

Instrument settings to run TRUEFRET™ assays are similar to other TR-FRET technologies. As a starting point for optimization, use the guidelines provided by the instrument manufacturer. A delay time of 100 μs followed by a 400 μs integration time and 100 flashes per well are typical settings for TR-FRET assays. Please set these parameters as advised by your instrument manufacturer.

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4 Suggested Protocol

4.1 Preparation of working solutions

Enzyme Buffer: Dependent upon your enzyme source. Refer to manufacturer's recommendations and/or literature for your specific enzyme.

Detection Buffer: 150 mM NaCl, 50 mM Hepes pH 7.4, 0.1 % Tween 20

PI(4,5)P₂ Substrate: Reconstitute PI(4,5)P₂ Substrate by adding 440 µL dH₂O for 12 µM stock solution. Dilute 12 µM stock solution 1:5 in Detection Buffer or Enzyme Buffer (dependant upon your source of enzyme) for 2.4 µM working stock solution.

Note: Store at -20 °C once reconstituted.

PI(3,4,5)P₃ Standard: Reconstitute PI(3,4,5)P₃ Standard by adding 44 µL dH₂O for 12 µM stock solution. Dilute 12 µM stock solution 1:10 in Detection Buffer or Enzyme Buffer (dependant upon your source of enzyme) for 1.2 µM working stock. Then dilute 1.2 µM working stock 1:2 in Buffer 7 times for a total of 8- PI(3,4,5)P₃ Standards at working stock concentrations of 12 µM to 0.009 µM (see Table, section 4.1; preparation of working solutions).

Note: Store at -20 °C once reconstituted.

Working Stock Concentrations (10 µL/well)	Final Concentrations (30 µL/well)
1.2 µM PI(3,4,5)P ₃ Standard	0.4 µM PI(3,4,5)P ₃ Standard
0.6 µM PI(3,4,5)P ₃ Standard	0.2 µM PI(3,4,5)P ₃ Standard
0.3 µM PI(3,4,5)P ₃ Standard	0.1 µM PI(3,4,5)P ₃ Standard
0.15 µM PI(3,4,5)P ₃ Standard	0.05 µM PI(3,4,5)P ₃ Standard
0.075 µM PI(3,4,5)P ₃ Standard	0.025 µM PI(3,4,5)P ₃ Standard
0.0375 µM PI(3,4,5)P ₃ Standard	0.0125 µM PI(3,4,5)P ₃ Standard
0.01875 µM PI(3,4,5)P ₃ Standard	0.00625 µM PI(3,4,5)P ₃ Standard
0.009375 µM PI(3,4,5)P ₃ Standard	0.003125 µM PI(3,4,5)P ₃ Standard

PI(3,4,5)P₃ Detector: Dilute 600 nM stock solution 1:100 in Detection Buffer for 6 nM working stock.

Probe: Reconstitute probe by adding 44 µL of Detection Buffer to Probe vial for 3 µM stock solution. Then dilute 3 µM stock solution 1:100 in Detection Buffer for 30 nM working stock solution.

Note: For best results, we recommend reconstituting probe immediately before use. Store at -20 °C once reconstituted.

4.2 Protocol

The entire procedure, including enzymatic reactions, can be performed directly in the black 384-well plate provided in the kit. It is suggested that all points be performed in triplicate. Enzymatic reactions can also be performed directly in the detection plate or in vials and then transferred.

4.2.1 Enzyme Reaction

To Black 384-well plate add:

- 1- 5 µL/well working stocks of PI(4,5)P₂ Substrate in all enzymatic reaction wells and Substrate Only control wells.
- 2- 5 µL/well PI3-K Enzyme at 2 times the desired concentrations diluted in enzyme buffer (determined by enzyme type and source).
Note: If testing compounds it is recommended to pre-incubate the compound with enzyme 15-30 minutes prior to adding substrate.
- 3- Incubate enzyme reactions for desired time.

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Note: Do NOT stop PI3-K enzyme reactions with EDTA (refer to section 7.1.3: EDTA)

4.2.2 Detection Reaction

- 1- 10 µL/well working stock PI(3,4,5)P₃ Standard, Enzyme Reactions and Enzyme Reaction Controls or, Detection Buffer to “NC” and “Background” wells (refer to Reference Guide below).
- 2- 10 µL/well working stock of PI(3,4,5)P₃ Detector to all wells.
- 3- 10 µL/well working stock of probe to all wells except Background control wells. Add Detection Buffer to Background control wells.
- 4- Shake plate 5-30 min at room temperature, protected from the light, on an orbital plate shaker before reading on microplate reader.

Note: Assay signal decays slowly over time without affecting assay stability. For example, after 2 hours the positive control signal has decreased by less than 20%

Quick reference guide for section 4.2.2; Detection Reaction.

Volume of working stocks to add/well (Total well volume = 30 µL)	Step 1			Step 2	Step 3	
	Enzyme Reactions and Enzyme Reaction Controls*	Standards	Enzyme Buffer** or Detection Buffer	Detector Protein working stock solution	Probe working stock solution	Detection Buffer
Standards	-----	10 µL	-----	10 µL	10 µL	-----
Enzyme Reactions	10 µL	-----	-----	10 µL	10 µL	-----
Substrate Only Control	5 µL	-----	5 µL	10 µL	10 µL	-----
Enzyme Only Control	5 µL	-----	5 µL	10 µL	10 µL	-----
No Competitor Control	-----	-----	10 µL	10 µL	10 µL	-----
Background Control	-----	-----	10 µL	10 µL	-----	10 µL

*The enzyme reaction controls included in this guide are suggested and may or may not include all necessary controls. For example: Inhibitors and appropriate vehicle control should be added in step1, with the corresponding adjustments to enzyme, substrate, and ATP concentrations while maintaining volume; 10 µL

** Buffer selected for control(s) is dependent upon what you used for enzyme reactions.

5 Optimization

TRUEFRET™ results vary depending on instrument and filter sets used. For best results, optimization of Probe and Detector pair concentrations on your instrument is suggested before running your samples. For a quick start the assay can be run using the protocol and reagent dilutions suggested in section 4.

5.1 Optimization: Detector

The suggested concentration for the Detector protein is 1-2 nM (final concentration in 30 µL volume) depending on filters used and instrument. Increasing Detector concentration should increase acceptor emission signal without significantly affecting sensitivity for the competing PI(3,4,5)P₃. Enough Detector is provided for 400 points at 2 nM. Increasing Detector concentration over 2nM will decrease the amount of reactions available in the kit.

5.2 Optimization: Probe

The suggested concentration for the Probe is 5-10 nM (final concentration in 30 µL well volume) depending on the instrument and filters used. Increased concentrations of the Probe will increase the overall signal of the

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assay. However, increasing the probe may decrease the sensitivity for the competing PI(3,4,5)P₃. The Probe concentration at 5nM should give an EC₅₀ value for PI(3,4,5)P₃ of ~ 10-100 nM. If acceptor emission is low we suggest increasing Detector concentrations before changing Probe concentrations. Increasing the Probe concentrations over 10 nM will decrease the amount of reactions available in kit.

5.3 Optimization: Substrate/ Standards

There is enough PI(4,5)P₂ substrate provided for 400 points at 400 nM (final concentration). There is enough PI(3,4,5)P₃ standard provided, at the suggested concentrations, for 7 full curves (see Table, section 4.1; preparation of working solutions).

6 Data Assessment

Enzyme activity can be estimated by comparing the values from the wells containing enzymatic reaction products to the values in the standard curve. Plot the values obtained vs. amount of PI(3,4,5)P₃ per standard to generate a standard curve. Determine the activity by comparing the values obtained from your samples to the standard curve. We find a sigmoidal dose-response curve generally provides the best fit of the standard curve data; and non-linear regression analysis can be used to determine inhibitor values.

7 Troubleshooting:

7.1 Low or No Signal: Resulting in little or no signal over Background Control

7.1.1 Effects of ATP

ATP starts to compete the TRUEFRET™ signal at 1-10 μM final concentrations. If you are using ATP at these concentrations this may affect the TR-FRET signal and the resulting “top” and “bottom” of an assay window. This can be corrected by adding DTT. For Example, in a 5 μL PI3-K reaction volume the effects of 100 mM ATP can be eliminated by the addition of 5 mM DTT in the same volume.

7.1.2 Probe has been compromised

The probe is sensitive to light and, once in solution, to temperature. After reconstitution, the 3 μM stock solution of Probe should be stored at -20 °C and protected from light.

7.1.3 EDTA was used

Metal chelators, such as EDTA, will destabilize the lanthanide dye resulting in low signal.

7.2 Poor Sensitivity: Resulting in a poor standard curve or no enzymatic activity

7.2.1 Too much Probe

Using too much Probe will result in decreased sensitivity to PI(3,4,5)P₃ competition. Decreasing the probe to 5-10 nM final concentration should correct this problem.

8 References:

Hennessy, Brayan T., Smith, Debra L., Ram, Prahlad T., Lu, Yiling, and Mills, Gordon B. Exploiting the PI3K/AKT pathway for cancer drug discovery. *Nature Reviews Drug Discovery* **2005**, 4, 988-1004.

Petoud, S., Cohen, S. M., Bunzli, J. C., and Raymond, K. N. Stable lanthanide luminescence agents highly emissive in aqueous solution: multidentate 2-hydroxyisophthalamide complexes of Sm(3+), Eu(3+), Tb(3+), Dy(3+). *J. Am. Chem. Soc.* **2003**, 125, 13324-13325.

Shaw, Reuben T, and Cantely, Lewis C. Ras, PI(3)K, and mTOR signaling controls tumor cell growth. *Nature* **2006**, 441 (7092) 424-430.

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9 Other Related Products:

	Product	General Description	Detection Mode	Cat. No.
PI3-Kinase Activity Assays*	PI3-K FP	Detects PI3-K activity in purified and immuno-precipitated samples through detection of PI(3,4,5)P ₃ (Enzyme product of PI3-Kinase activity)	Flourescence Polarization	K-1100
	PI-3K Alpha	Detects PI3-K activity in purified and immuno-precipitated samples through detection of PI(3,4,5)P ₃ (Enzyme product of PI3-Kinase activity)	Alphascreen	K-1300
	PI3-K ELISA	Detects PI3-K activity in purified and immuno-precipitated samples through detection of PI(3,4,5)P ₃ (Enzyme product of PI3-Kinase activity)	Absorbance 450 nm	K-1000
	PI3-K ELISA (384 well)	Detects PI3-K activity in purified and immuno-precipitated samples through detection of PI(3,4,5)P ₃ (Enzyme product of PI3-Kinase activity)	Absorbance 450 nm	K-2600
Possible PI3-Kinase Substrates	Non-labeled Substrate	Synthesized and provided as a lyophilized powder. Available in long chain (di-C ₁₆) or short chain (di-C ₈) PtdIns(4,5)P ₂ ; or inositol head group.		P-4516 P-4508 Q-0145
	Labeled Substrate	Synthesized and provided as a lyophilized powder. Substrates available with flourescent tag or biotinylated label.		H-45TM H-45FL H-45BT C-45F6a C-45M6 C-45M6a C-45M16a C-45B6a

Check Echelon's website (www.echelon-inc.com) for other phosphatase/kinase activity assays, purified enzymes, and substrates.

*US Pat. 7,067,269,B2

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