Instruction Manual For
PIP3 Quantification HTRF Assay

One plate of 96-well

Catalog No. 17-495

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Not for use in diagnostic procedures.

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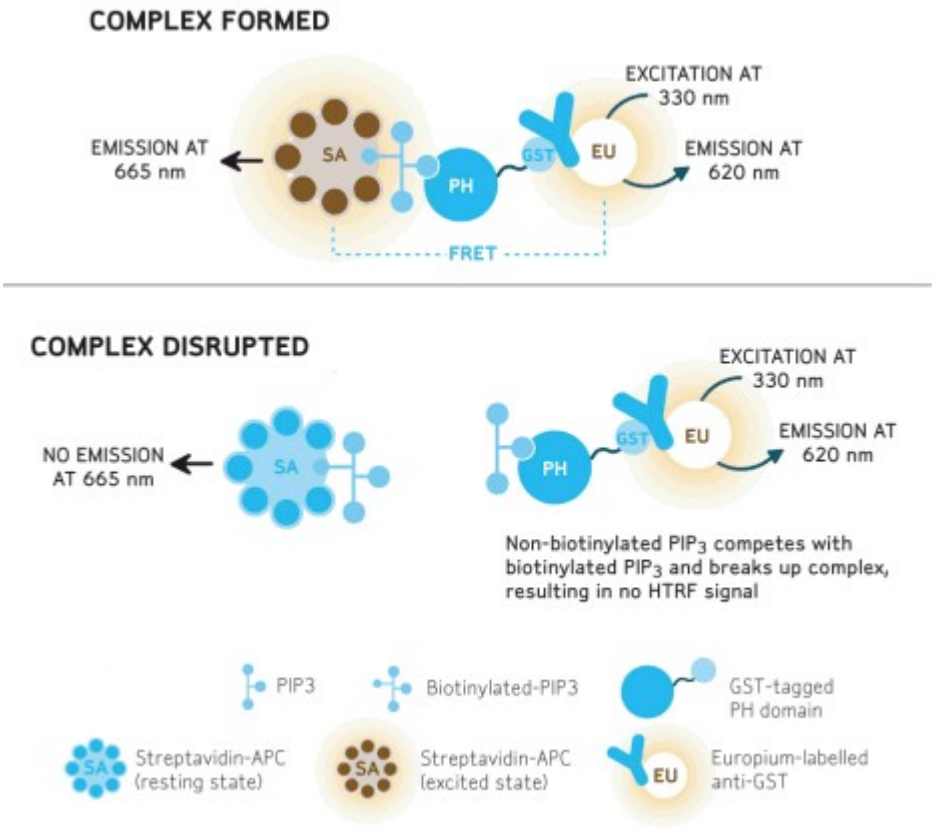
Introduction

Assay Principle

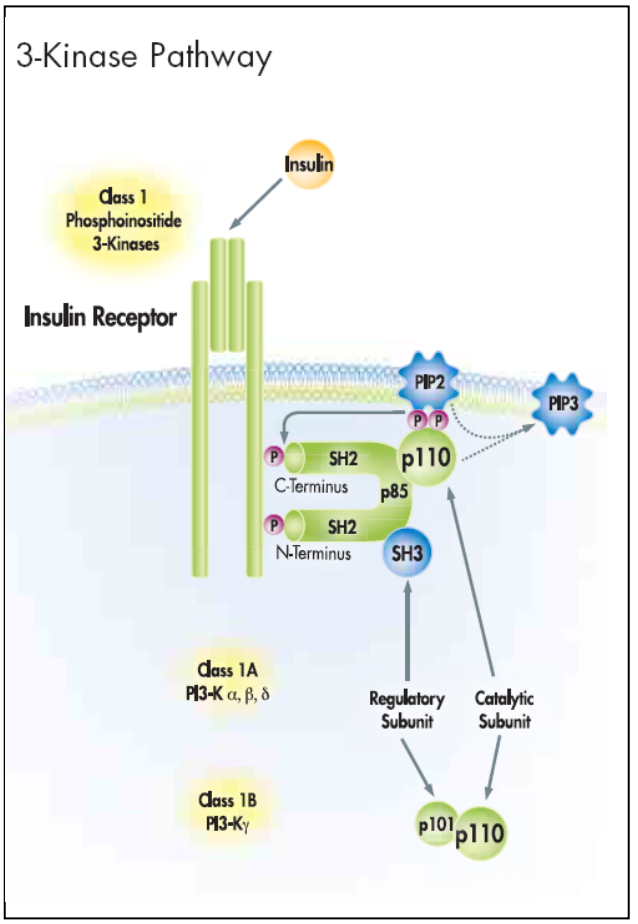
PI 3-Kinases α, β, γ and δ (h) catalyse the phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-trisphosphate (PIP3) in the presence of ATP and Mg2+. The PIP3 product is detected by displacement of biotin-PIP3 from an energy transfer complex consisting of Europium labelled anti-GST monoclonal antibody, a GSTtagged pleckstrin homology (PH) domain, biotinylated PIP3 and Streptavidin-Allophycocyanin (APC). Excitation of Europium in the complex results in an energy transfer to the APC and a fluorescent emission at 665 nm. The PIP3 product isolated from cell membranes displaces biotin-PIP3 from the complex resulting in a loss of energy transfer and thus a decrease in signal.

Assay Principle Flow Chart



PI3 Kinase Background

Phosphoinositol-3-kinases (PI3 Kinase, PI3K) are a family of lipid kinases that mediate many intracellular signaling responses in both physiological and pathophysiological states. PI3 Kinase is a heteroduplex with three classes (I, II, & III) of catalytic domain. The class I PI3 Kinases are made up of a p85 regulatory protein and a p110 catalytic domain. There are 4 isoforms of the class I p110 catalytic domains. Class I(A) include α, β, δ and class I(B) include γ. They are activated downstream via receptor tyrosine kinases or G-protein coupled receptors. Once activated, PI3K generates phosphatidylinositol 3,4,5-trisphosphate (PIP3) from PIP2. It was later found that PTEN, a lipid phosphatase, acts opposite to PI3K by dephosphorylating PIP3, converting it to PIP2, thus de-activating the effects of PI3K. Increased levels of PIP3 in the cell leads to the activation of many key signaling regulators including Akt and PDK as well as calcium release. One of the primary roles of PI3K is its regulation of cell growth and cell metabolism. Both of these play a critical role in cancer as some believe that a tumor's shift to glycolysis is due to the activation of PI3K. PI3 kinases have been linked with numerous disease states, including cancer, diabetes, allergic response, hypertension, cardiac contractility, atherosclerosis, sepsis and autoimmune/ inflammatory disorders.



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Kit Components: Received in two boxes (17-495-1 and 17-495-2)

Store at Room Temperature

Black 96 well plate: (Part No.CS203342) 1 black 96 well plate.

Store at -20º C

Detection Buffer : (Part No. CS203350) One vial containing 14 mL.

Lipid Recovery Buffer: (Part No. CS203348) One bottle containing 14 mL

1M DTT: (Part No. 90499) 1 vial containing 100 µL of 1M DTT.

Store at -80º C

Biotin-PIP3 : (Part No. CS203346) 1 vial containing 210 μL.

Detection Component 1: (Part No. CS203344) 1 vial containing 21 μL.

Detection Component 2: (Part No.CS203330) 1 vial containing 21 μL.

PIP3 Standard (Part No. CS203356) 1 vial containing 80μL of 1mM PIP3.

Materials Not Supplied

1. Multi-channel or repeating pipettes
2. Plate shaker at room temperature.
3. Pipettors & tips capable of accurately measuring 1-1000 µL
4. Graphing software for plotting data or graph paper for manual plotting of data
5. Multi-channel pipettor reservoirs
6. Nuclease free water/ deionized water

Precautions

- The PI3 Kinase Activity Assay kit is designed for research use only and not recommended for internal use in humans or animals. All chemicals should be considered potentially hazardous and principles of good laboratory practice should be followed.
- The instructions provided have been designed to optimize the kit's performance. Deviation from the instructions may result in suboptimal performance of the kit and the failure to produce accurate data.

Technical Notes

- For maximum recovery of product, centrifuge original vial after fast thawing prior to removing the cap. Rapidly thaw the vial under cold water and immediately place on ice or keep at room temperature following the protocols. Aliquot the -80°C components to avoid repeated thawing and freezing. Immediately snap-freeze the vials in liquid nitrogen prior to re-storage at -80°C.
- Do not use reagents beyond the expiration date of the kit.
- Do not mix or interchange reagents from various kit lots.
- Manual Plate Washing: Vigorous washing and complete removal of all liquid by aspiration at the end of each washing step is very important to obtain low background values. Gentle agitation during the wash steps or a 2-3 minute soak may reduce background values.

Preparation of Buffers/Solutions

1. **Complete Detection Buffer:** Add 2 μL of 1M DTT per mL Detection Buffer (CS203350) to make the ***complete detection buffer***, keep on ice. Use to prepare the Detection Mixture in Step 1 of the assay protocol.

2. **Complete Lipid Recovery Buffer:** Add 2 μL of 1M DTT per mL Lipid Recovery Buffer (CS203348) to make the ***complete lipid recovery buffer***, keep on ice. Use for PIP3 standard curve preparation and lipid sample reconstitution.

Storage

Kit components arrive on dry ice, and must immediately be stored at the temperatures specified below.
Kit components are stable for 4 months from date of shipment when stored as directed.

PI3 Kinase TR-FRET Assay Kit Protocol

1. Setup the Detection Mixture.

Table 1. Detection Mixture Setup (per reaction):

Reagents	Volume (μL)
Biotin-PIP3	2.1
Detection Component 1	0.21
Detection Component 2	0.21
Complete Detection Buffer	22.48
Final Volume	25

Incubate at RT for 30 minutes, protect from light.

2. Prepare the PIP3 Standard Curve during the last 10 minutes of the Detection Mixture incubation period.. The kit provided vial (CS203356) will serve as the top point at 500μM. Make 5-fold serial dilutions using *complete lipid recovery buffer*. i.e. Pipette 20 μL of 1st point, add to 80 μL buffer, vortex to mix well, this is the 2nd point. Repeat the procedure for the following points. There will be a total of (11) points plus a buffer blank. The curve ranges from [0-500μM].

   Add 25 μL/ well in duplicate per point.

3. Lipid samples from acid extraction will be reconstituted with *complete lipid recovery buffer*. Sonicate for 10-15 seconds at mild power in a cooled micro bath sonicator. (Appendix I.)

   Add 25 μL /well in duplicate.

4. Add 25 μL/well of the detection mixture from step 1, cover with a plate sealer and incubate for 4 hours at RT with gentle agitation. Protect from light.

   Note: *4 hours is recommended, the standard curve is stable up to 12 hours.*

5. Measure TR-FRET ratio on an appropriate reader according to the following parameters (these are guideline parameters based on Molecular Devices –Analyst. Please refer to parameters recommended in the instrument instruction manual):

Excitation	330 nm
Emission	660-50 nm and 620-35nm
Counting Delay	50 μsec
Integration time	400 μsec
Z Height	0.5mm

HTRF Ratios are calculated as follows:

$$HTRF\ Ratio = \left(\frac{Emission\ at\ 660nm}{Emission\ at\ 620nm} \right) \times 10000$$

6. Data is interpreted by GraphPad PRISM nonlinear fit, Sigmoidal dose-response (variable slope).

Typical Assay Results

Z' factor was calculated to be 0.6 from three separate trials using 24 replicates for each of no B-PIP3 detection mixture as background signal and B-PIP3 detection mixture as positive signal.

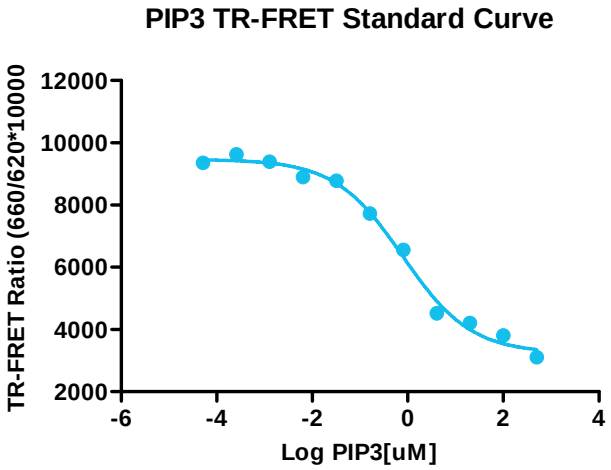

Figure 1. A typical PIP3 Standard curve following the assay protocol outlined in the manual above. PIP3 ranges from 0-500μM final in the 50μL assay volume/well. The curve was plotted with GraphPad Prism using Sigmoidal dose-response (variable slope).

Troubleshooting Guide

Problem	Potential Cause	Experimental Suggestions
No signal or weak signal in all wells	Missing components or key steps	Check to make sure all components were added in the appropriate steps and amounts.
	Standard or Biotinylated-PIP3 or Capture protein is no longer active or has reduced activity	Make sure all components are stored at the recommended temperature and minimize the freeze/thaw cycle as manual recommended. Make aliquots when first thaw the components if planning more than one assay.
	Plate reader or settings are not optimal	Verify the measurement, read time, and filter on the plate reader.
	Incorrect storage temperatures Incorrect assay temperature	Items are to be stored at the appropriate storage temperatures. Performance can be negatively affected if reagents are not stored and used in the appropriate time period.
No detectable lipid level in samples	Low PIP3 level or out of the standard curve range or not a good lipid extraction	Check the attached lipid extraction protocol to make sure a good sample preparation, adjust the dilution if out of detectable range.

Appendix I. Lipid Extraction Protocol

Extraction of PI(3,4,5)P3 from cells

The procedure as verified with about 1 x 10^6 cells (6-well culture plate at 80% confluency). Larger or smaller amounts of cells require proportional adjustments of volumes. The amount of cells necessary for PI(3,4,5)P3 quantification needs to be determined for each cell type.

Solutions for Extraction:

1. 0.5 M TCA: For 50 mL, dissolve 4.08 g TCA (Trichloroacetic Acid) in dH_2O and bring volume to 50 mL.
2. 5% TCA with 1 mM EDTA: For 50 mL, dissolve 2.5 g TCA in dH_2O, add 100 µL 0.5 M EDTA, and bring volume to 50 mL with dH_2O.
3. Methanol:Chloroform (2:1): For 60 mL, add 40 mL Methanol to 20 mL Chloroform
4. Methanol:Chloroform:12 N HCl (80:40:1): For 60 mL, combine 40 mL Methanol, 20mL Chloroform, and 0.5 mL 12 N HCl
5. 0.1 N HCl: For 50 mL, add 0.42 mL 12 N HCl to 50 mL dH_2O

Procedure:

1. Collect cells: Aspirate media from cells. Add 1 mL cold 0.5 M TCA. Incubate on ice for 5 min. Scrape cells and transfer into 1.5 mL tube. Centrifuge at 13,000 rpm for 5 min at 4°C. Discard supernatant.
2. Wash pellet: Add 1 mL 5% TCA/1 mM EDTA to cell pellet. Vortex and centrifuge at 13,000 rpm for 5 min at 4°C. Discard supernatant.
3. Extract neutral lipids: Add 1 mL Methanol: Chloroform (2:1) to pellet. Vortex 3-4 times over a 10 min period at room temp. Centrifuge at 13,000 rpm for 5 min at 4°C. Discard supernatant. **Repeat once.**
4. Extract Acidic Lipids : Add 0.5 mL Chloroform: Methanol: 12 N HCl (40:80:1) to pellet. Incubate 15 min at room temperature. Vortex occasionally during incubation period. Add 180µL Chloroform following with 320µL 0.1 N HCl, vortex. Centrifuge at 13,000 rpm for 5 min at 4°C. Transfer organic (lower) phase to 1.5mL tube, add 30µL of 0.1M ammonium hydroxide in methanol. The neutralized organic phase was then dry in a vacuum dryer.

   Use Auto setting, medium dry rate. It takes about 1hr for 1ml organic phase.

Reconstitute Lipid:

Reconstitute the dried lipid samples in 60µL **Lipid Recovery Buffer (part # CS203348 in kit 17-494/17-495)**, vortex samples for 30 seconds, then sonicate using a cooled bath sonicator for 10-15 seconds with mild power.

***** Sonication condition needs to be optimized due to different sonicator at end user's preference. A mild power will be good.

Note: It's strongly recommended to reconstitute lipid sample before storage. The lipid solution can be stored at -20°C. OR a small volume of Chloroform or Methanol can be added to the dried lipid, store at -20°C, re-dry before detection.

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