PI 3-Kinase (Class I) HTRF® Assay

1 plate (384 wells)

Catalog # 33-016

I. STORAGE AND STABILITY 2

II. ASSAY OVERVIEW 2

III. ASSAY SYSTEM COMPONENTS 4

IV. ASSAY PROCEDURE 5

A. Preparation of Assay Solutions 5

B. Assay Protocol 6

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I. STORAGE AND STABILITY

Storage: All materials provided should be stored as indicated in Table 1.
Note DM A and DM B are light sensitive.

Freeze thawing of reagents should be avoided.

PI 3-Kinases supplied by Millipore should be stored as aliquots at -80ºC. A fresh aliquot should be used for each experiment. The enzyme aliquots should be thawed rapidly, vortexed gently and diluted immediately prior to use.

The PIP_2 substrate binds non-specifically to plastic. When using this reagent pipetting steps should be minimized and polypropylene tips and tubes should be used wherever possible. Consistent procedures for the preparation of both PIP_2 and PI 3-Kinases are strongly recommended for all experiments.

Thaw the PIP_2 substrate, STOP A, STOP B, DM A, and DM B solutions by placing into a water bath at room temperature. Ensure reagents are fully thawed and mixed thoroughly before use. If necessary, centrifuge briefly to collect residual liquid that may have collected in the lids.

4x Reaction Buffer and DM C should be warmed to room temperature prior to use. These may be stored at 4°C after initial thawing.

Stability: Components stable for 6 months from date of shipment if stored and handled correctly. We recommend that all enzymes to be used with this kit are stored as aliquots and a fresh aliquot used for each experiment.

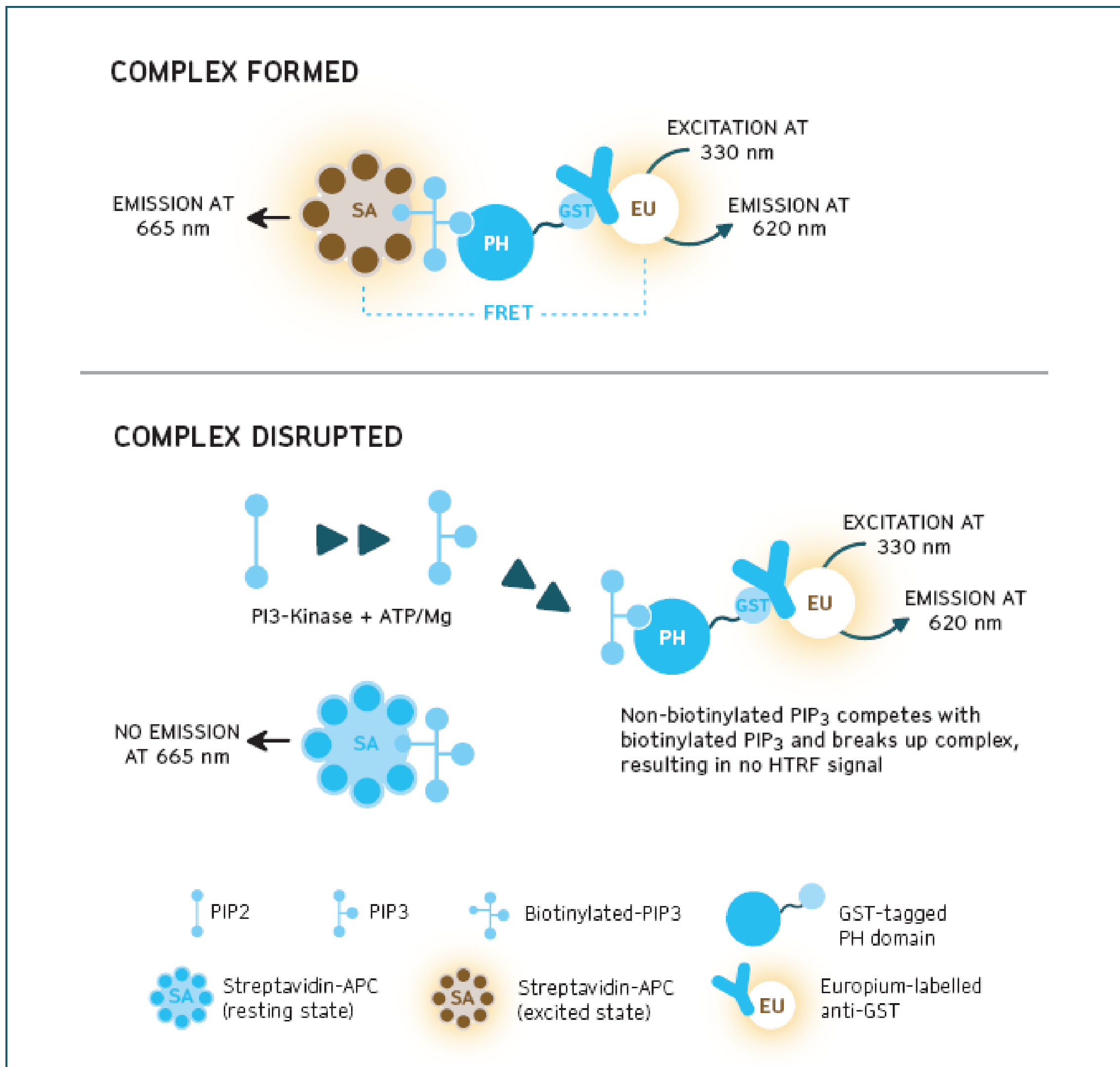
II. ASSAY OVERVIEW

All PI 3-Kinases catalyse the phosphorylation of the 3 position of phosphatidylinositols.

The Class I enzymes utilize phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate phosphatidylinositol 3,4,5-trisphosphate (PIP_3) in the presence of ATP and Mg^{2+}. $PI(3,4,5)P_3$ product is detected by displacement of a biotinylated ligand from an energy transfer complex consisting of a Europium-labeled anti-GST monoclonal antibody, a GST-tagged PH domain, biotinylated $PI(3,4,5)P_3$ 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 $PI(3,4,5)P_3$ product formed by Class I PI 3-Kinase activity displaces biotin-$PI(3,4,5)P_3$ from the complex resulting in a loss of energy transfer and thus a decrease in signal.
The Class I PI 3-Kinase HTRF Assay kit provides a method for assaying PI 3-Kinases in a homogenous 384 well format. The assay is suitable for screening of potential inhibitors of these enzymes.

Figure 1: Schematic of Assay System. In the absence of PI3-Kinase activity, the complex is free to form. Upon introduction of enzyme, substrate and ATP, a phosphorylated lipid species is generated which competes with the biotinylated ligand for sites on the PH domain. Excitation of the europium will generate a FRET signal when the complex is formed, but not when the complex is disrupted.



III. ASSAY SYSTEM COMPONENTS

A. Provided Reagents

Reagent	Part Number	Volume Supplied	Storage
4x Reaction Buffer	33-002	3000 µL	-80°C
PIP2 (1 mM Stock)	33-004	90 µL	-80°C
Stop A	33-006	1620 µL	-80°C
Stop B	33-008	540 µL	-80°C
DM A	33-010	106 µL	-80°C
DM B	33-012	106 µL	-80°C
DM C	33-014	3000 µL	-80°C

Please note that the volumes reported in the table above and on the vial tubes include an overfill to compensate for material potentially lost through aspiration. Customers should be assured that, even if they cannot dispense the total reported volume, there is sufficient material within the tube to conduct the 1 plate assay.

B. Materials required but not provided

Material	Recommended Supplier	Concentration
Black 384-well Plate	Corning Costar	-
ATP	Sigma, A7699	10 mM
DTT	Sigma, D5545	1 M
Water (18.2MΩ)	Millipore	

C. Enzymes available from Millipore

Cat #	Description	Species
14-790	PI3 Kinase (p110α/p65α)	Human
14-602	PI3 Kinase (p110α/p85α)	Human
14-782	PI3 Kinase (p110α(E542K)/p85α)	Human
14-783	PI3 Kinase (p110α(E545K)/p85α)	Human
14-792	PI3 Kinase (p110α(H1047R)/p85α)	Human
14-786	PI3 Kinase (p110α/p65α)	Mouse
14-785	PI3 Kinase (p110α/p85α)	Mouse
14-791	PI3 Kinase (p110α(E542K)/p85α)	Mouse
14-781	PI3 Kinase (p110α(E545K)/p85α)	Mouse
14-787	PI3 Kinase (p110α(H1047R)/p85α)	Mouse
14-603	PI3 Kinase (p110β/p85α)	Human
14-794	PI3 Kinase (p110β/p85α)	Mouse
14-788	PI3 Kinase (p110β/p85β)	Mouse
14-558	PI3 Kinase (p120γ)	Human
14-604	PI3 Kinase (p110δ/p85α)	Human
14-789	PI3 Kinase (p110δ/p85α)	Mouse

IV. ASSAY PROCEDURE

A. Preparation of Assay Solutions

Detection Component Preparation

1. **Stop Solution:**

   Prepare the Stop Solution by combining Stop A and Stop B in a ratio of 3:1 respectively (e.g. to prepare Stop solution for one well combine 1.25 µl Stop B and 3.75 µl Stop A). This solution is stable at room temperature for up to 6 hours.

2. **Detection Mix:**

   Prepare Detection Mix by combining DM C, DM B and DM A in a ratio of 18:1:1 respectively (e.g. to prepare Detection Mix for one well combine 4.5 µL DM C, 0.25 µL DM B and µL 0.25 DM A, NB follow given order of addition). This solution is stable at room temperature for up to 6 hours.

Reaction Component Preparation

1. **1x Reaction Buffer**

   Dilute 4x Reaction Buffer ($MgCl_2$ concentration, 40 mM) 4-fold with water and add DTT to a concentration of 5 mM. Prepare fresh for each assay.

   For 1mL 1 x Reaction Buffer add 0.25 mL 4x Reaction Buffer to 0.745 mL distilled water and 5 µL 1 M DTT and mix. This solution is used to make up the ATP solution and the 1x Reaction Buffer / PI solution.

2. **ATP Working Solution**

   Dilute the 10 mM ATP Stock Solution in 1x Reaction Buffer to 4x the required final reaction concentration (e.g. to prepare 1 mL 100 µM ATP final reaction concentration prepare a 400 µM Working Solution by adding µL 10 mM ATP to 960 µL 1x Reaction Buffer).

3. **Lipid Working Solution**

   Dilute the 1 mM PIP_2 stock in 1x Reaction Buffer to 1.38 x the final assay concentrations of 10 µM. The working concentration will be 13.8 µM.

   For 1 mL of Lipid Working Solution add 13.8 µL 1 mM PIP_2 to 986.2 µL 1x Reaction buffer and mix. Ensure you have enough solution to make up the Plus Kinase solution and some remaining for the Minus Kinase solution.

4. **PI 3-Kinase Working Solution**

   Dilute the PI 3-Kinase to the required concentration in Lipid Working Solution. The required enzyme concentration will need to be determined by titration prior to inhibitor studies.

5. **Minus Enzyme / PIP_2 Control**

   The remaining 1x Reaction Buffer / Lipid Working Solution is used for the Minus enzyme / PI control.

6. **Inhibitors**

   Prepare inhibitors in 100% DMSO at 40x the required final assay concentration and use 0.5 µL per assay.

B. Assay Protocol

For each enzyme assay set up a plus enzyme and minus enzyme control.

1. Add 0.5 µL 100% DMSO to minus enzyme controls wells and plus enzyme control wells. Add 0.5 µL inhibitor to test wells.

2. Add 14.5 µL Lipid Working Solution to Minus enzyme control wells. Add 14.5 µL PI 3-Kinase/ Lipid Working Solution to Plus enzyme control wells and inhibitor test wells.

3. Add 5 µL ATP Working Solution to all wells.

4. Incubate reaction for 30 minutes at room temperature.

5. Add 5 µL Stop Solution ensuring that good mixing is achieved with the well contents.

6. Add 5 µL Detection Mix again ensuring that the well contents are mixed. The Stop Solution and Detection Mix should never be mixed.

7. It is recommended that the plate is sealed to minimize reduction in reaction volume. Incubate for 2 hrs before reading.

8. Measure HTRF ratio on an appropriate microplate reader. Refer to your instrument manufacturer for guidance on measurement parameters recommended for HTRF.

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