Instruction Manual For
PI3 Kinase Activity/Inhibitor Assay Kit

Catalog No.

Generic Manual for:
17-493
17-10013
17-10001
17-10002
17-10003
17-10004
17-10005

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Not for use in diagnostic procedures.

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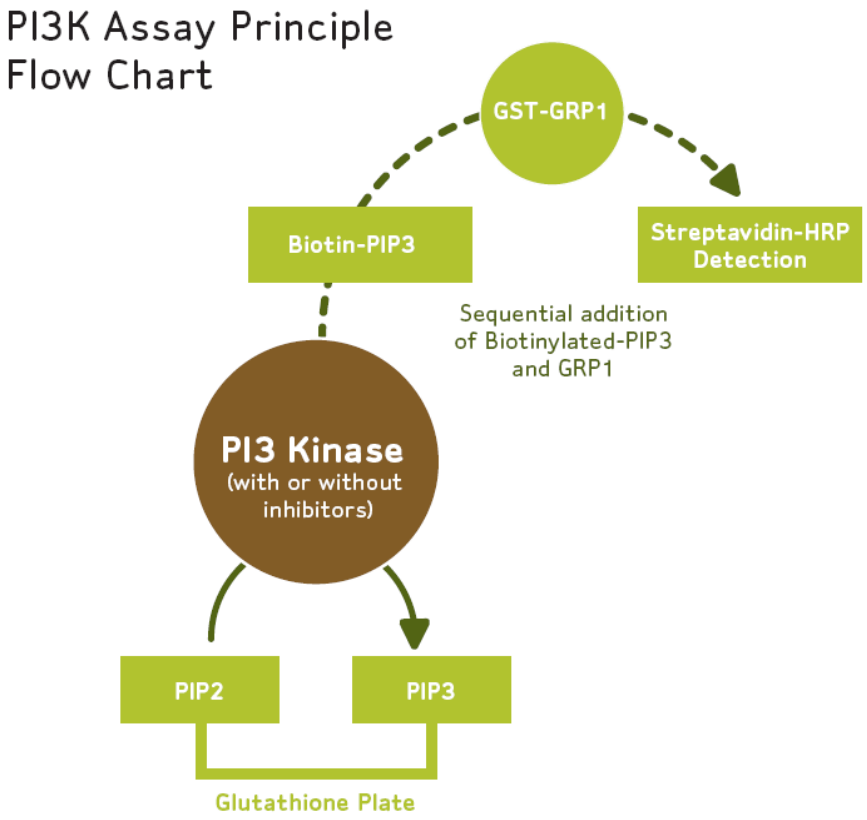
Introduction

Assay Principle

The PI3 Kinase Activity/Inhibitor Assay is a competitive assay used for the fast and sensitive quantitation of activity of the four class I PI3 kinases (p110 α, β, γ, δ). The PI3 Kinase Activity/Inhibitor Assay works on the principle that PI3 Kinase phosphorylates PI(3,4)P2 (PIP2) converting it to PI(3,4,5)P3 (PIP3). The PH domain of the protein GRP-1 binds PIP3 with high affinity and specificity. The kit includes this recombinant protein that is used as the capture protein. This protein binds to the glutathione plate and captures either the PIP3 generated as part of the kinase reaction or the biotinylated-PIP3 tracer included in the kit. The captured biotinylated-PIP3 is detected using streptavidin-HRP conjugate and a colorimetric read out (OD 450). The lower the signal, the higher the PI3 Kinase activity. Depending on the variable kits used, either 1, 2, or all 4 of the class I PI3 Kinases are included with the kit.

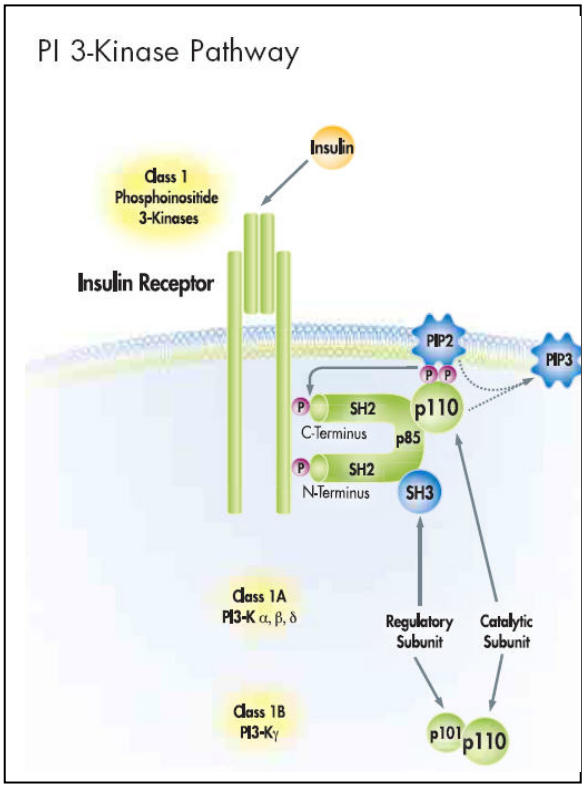
This assay is used for:

1. Detection of the degree of inhibition of general and isoform-specific class I PI3 Kinase inhibitors.
2. Screening inhibitors or activators of class I PI3 Kinases.
3. Detecting the effects of other compounds on the class I isoforms of PI3 Kinases.
4. Detecting the activity of class I catalytic PI3 Kinases.



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) includes the α, β, and δ isoforms and class I(B) includes the γ isoform. 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

Kit components must be stored immediately at the temperatures specified

Store at 2-8ºC

Glutathione-Coated Plate, 96-well strip format: (Part No. CS203297) 1 plate.

20% Tween® 20: (Part No. 20-246) One vial containing 3 mL.

20X TBS Wash: (Part No. 20-190) One bottle containing 50 mL.

Substrate TMB: (Part No. 90348) One amber bottle containing 12 mL. Protect from light.

Stop Buffer: (Part No. 2007598) One bottle vial containing 12 mL.

SA-HRP Detection Reagent (Part No. CS203296) One vial containing 15 µL.

Store at -80ºC

GRP1-GST: (Part No.CS203301) 1 vial containing 10 µL.

Kinase Reaction Buffer, 5X: (Part No. CS203302) 1 vial containing 1 mL.

Wortmannin (Part No.CS203298) 1 vial containing 0.1 mg.

PIP2 (Part No. CS203300) 1 vial containing 30 µL.

Biotinylated-PIP3 (Part No. CS203299) 1 vial containing 200 µL. EDTA is included.

Included Recombinant Active Kinase(s)

- **PI3K, p110α** (Part No. CS203304) 1 vial containing 50 µL PI3K, p110α at 10 µg/mL.
- **PI3K, p110β** (Part No. CS203305) 1 vial containing 50 µL PI3K, p110β at 50 µg/mL.
- **PI3K, p110δ** (Part No. CS203293) 1 vial containing 50 µL PI3K, p110δ at 20 µg/mL.
- **PI3K, p120γ** (Part No. CS203318) 1 vial containing 50 µL PI3K, p120γ at 50 µg/mL.

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
7. DMSO

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. Centrifuge briefly at 4°C. Aliquot the enzyme to avoid repeated thawing and freezing. Immediately snap-freeze the vials in liquid nitrogen prior to re-storage at -80°C.
- 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.

Storage

Kit components arrive on dry ice, and must be stored immediately at the temperatures specified above. Kit components are stable for 3 months from date of shipment when stored as directed.

Preparation of Buffers/Solutions

1. 1X TBS: Prepare 200 mL of 1X TBS by adding 10 mL of 20X TBS (Catalog # 20-190) to 190 mL of Milli-Q or distilled water. Store at room temperature.

2. 1X TBST (Wash Buffer): Prepare 800 mL of 1X TBST by adding 2 mL of 20% Tween® 20 (v/v) (Catalog # 20-246) and 40 mL of 20X TBS (Catalog # 20-190) to 758 mL of Milli-Q or distilled water. Store at room temperature.

3. Wortmannin (Catalog #CS203298): Add 233 µL DMSO to the 0.1 mg vial to make a stock solution. Aliquot and store at -20°C. Further dilute with aqueous buffer just prior to use. The DMSO stock solutions are stable for 1-3 months at -20 °C.

4. Wortmannin working solution: Serial dilute the stock Wortmannin solution in distilled H_2O 1:100, followed by another serial dilution of 1:50. Leaving out the mention of 2X removes confusion with 5X in the table.

5. PIP2 Working Solution: Dilute the stock PIP2 1:20 to make the working solution in distilled H_2O, keep on ice.

6. PI3 Kinase Working Solution: Dilute the stock kinase 1:10 to make the working solution in distilled H_2O, keep on ice.

7. Biotinylated-PIP3 Working Solution: Dilute the stock Biotinylated-PIP3 1:18 to make a working solution in 1XTBS. Sufficient EDTA is included to stop the kinase reaction.

8. GRP1 Working Solution: Dilute the stock GRP1 1:1000 to make a working solution in 1XTBS.

9. Compounds (not provided in kit): Prepare a concentrated stock with 100% DMSO, then further dilute in distilled water to have DMSO no more than 2% final when preincubated with the kinase in Step 2 of the protocol below.

10. SA-HRP Working Solution: Dilute the stock SA-HRP detection reagent 1:2000 in 1X TBST.

PI3 Kinase Activity Assay Kit Protocol

1. Take out as many strips as needed to perform the assay. It is recommended to set-up duplicate wells (see table #1 below). Store the remaining strips with desiccant at 4°C.
   Note: *Strips can be better held in place by taping down the ends of the strips onto the plate frame.*

2. Setup the PI3 Kinase reaction in the Glutathione-coated strips/plate for inhibitor reaction:

   a. Preincubate the kinase and inhibitor for 10 minutes prior to adding PIP2 substrate.

   b. Add 5 μL/well of 5X Kinase reaction buffer.

   c. Add 5 μL/well of PIP2 substrate.

   d. Add distilled H_2O to each well based on Table 1 below to make up to a final 25 μL/well.

3. Incubate at RT for 1 hour.

Table 1. Setup the Kinase/Inhibitor Reactions:

Wells / Reagents	Buffer Control (μL)	Positive Control (μL)	Inhibitor Wells (μL)	B-PIP3 only (μL)
5X Reaction Buffer	5	5	5	5
PIP2(50 μM)	5	5	5	5
Kinase	-	5	5	-
Wortmannin or customer compound	-	-	5	-
Distilled H_2O	15	10	5	15
Final Volume (μL)	25	25	25	25

4. Add 25 μL/well of Biotinylated-PIP3/EDTA working solution **excluding** the buffer control wells.

5. Add 25 μL/well 1XTBS to the buffer control wells.

6. Add 50 μL/well of GRP1 working solution to all wells.

7. Incubate at RT for 1 hour.

8. Wash the wells 4 times with 200 μL/well 1XTBST.

9. Add 50 μL/well SA-HRP working solution, incubate at RT for 1 hour.

   Note: Allow the Substrate TMB (Part No. 90348) and Stop Solution (Part No. 2007598) to warm up at RT.

10. Wash the wells 3 times with 200 μL of 1X TBST per well, then 2 times with 200 μL of 1X TBS per well.

11. Add 100 μL of the Substrate TMB (Catalog 90348) per well, develop in the dark for 5-20 minutes. Monitor the appearance of the blue color to avoid over-development.

12. Stop the reaction by adding 100 μL of the Stop Solution (Part No. 2007598) per well. Read at 450nm.

Calculation of Relative Percentage to Biotinylated-PIP3:

Take the direct absorbance read signal at 450nm, set the positive Biotinylated-PIP3 wells (average of duplicate or triplicate wells) as 100, all the other signals are divided by the Biotinylated-PIP3 average, then times 100 to show the relative percentage to the positive signal.

$$\text{Relative \% to B-PIP3} = \frac{\text{A450* of samples include buffer, kinase \& inhibitors}}{\text{A450* of Biotinylated-PIP3 average}} \times 100$$

*A450 means the absorbance signal at 450nm (direct read signal of the instrument at end user's preference).

TYPICAL ASSAY RESULTS

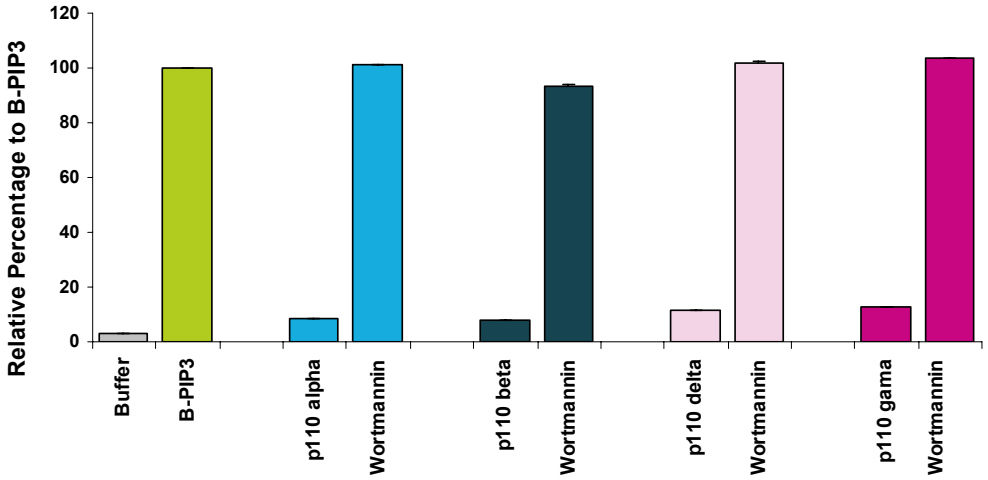

Figure 1. PI3 Kinase Activity Assay Kit Performance using the general class I PI3K inhibitor Wortmannin
Results of the assay following the protocol outlined in the manual above. 100 nM Wortmannin was applied to show the inhibitor effects on the PI3 Kinase reactions. The Biotinylated-PIP3 was set as 100%, the kinase reactions with/without inhibitors were referenced to the Biotinylated-PIP3 signal to have the relative percentage, which reflected the inhibitor effects. The buffer background, which contains no Biotinylated-PIP3, is included to show the signal over noise ratio.

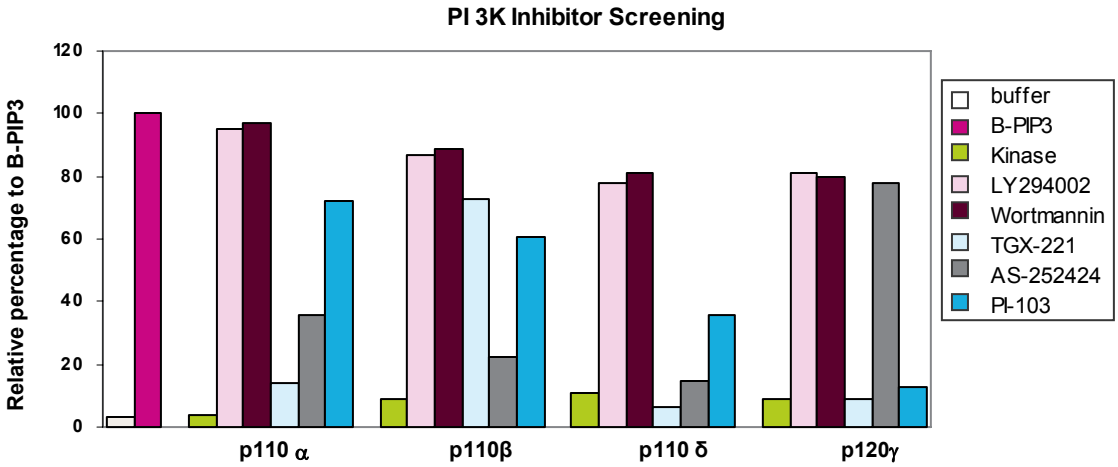

Figure 2. PI3 Kinase Activity/Inhibitor Assay Kit Performance using either isoform-specific or general class I inhibitors. Following the protocol outlined in the manual above. The buffer background, which does not contain Biotinylated-PIP3, is included to show the signal over noise ratio. The Biotinylated-PIP3 was set as 100%, the kinases reactions with/without inhibitors were referenced to the Biotinylated-PIP3 signal to have the relative percentage, which reflected the inhibitor effects. The isoform specific inhibitors used were as follows: PI-103 (p110α) (100 nM), TGX-221 (p110β) (50 nM), AS-252424 (p110γ) (200 nM), both Wortmannin (100 nM) and LY294002 (100 μM) are general class I PI3 Kinase inhibitors. Inhibitors were applied to show the isoform specific inhibitor effects on the various PI3 Kinase reactions.

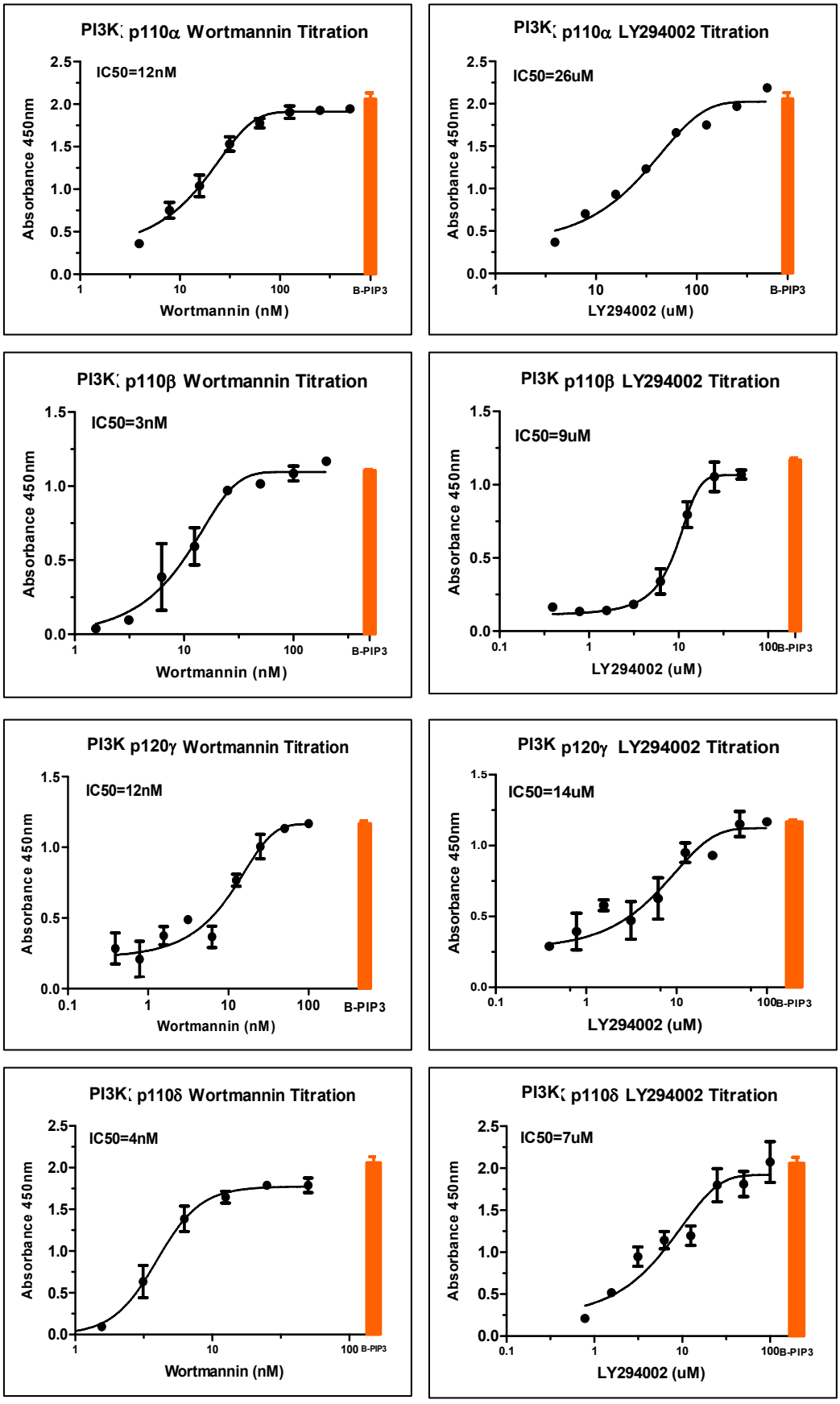

Figure 3.

PI3 Kinase Activity Assay Kit Titration studies using Wortmannin and LY294002. Following the protocol outlined in the manual above.

Both Wortmannin and LY294002 were titered to each of the PI3K isoforms for the IC50

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.
	Detection conjugate or substrate is no longer active or has reduced activity	Test directly by adding HRP-conjugated reagent with substrate.
	Plate reader or settings are not optimal	Verify the measurement, read time, and filter on the plate reader.
	Incorrect storage temperatures	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.
	Incorrect assay temperature	Colorimetric Substrate needs to be warmed to room temperature prior to use.
High background negative control wells	Added Biotinylated-PIP3 solution by mistake.	Leave out the Biotinylated-PIP3, add 1XTBS instead.
	Inadequate washing	Make sure to follow the recommended protocol for washing. Ensure all wells are filled with wash buffer.
No Signal or weak signal in inhibitor wells.	Missing components or wrong concentration.	Check to make sure the inhibitors were added and at the right concentrations.
No signal or weak signal in Biotinylated-PIP3 positive wells	Missing Biotinylated-PIP3.	Confirm if the Biotinylated-PIP3 was added.
	Possible reduced activity of GRP1.	Check if the GRP1 vial is stored properly as recommended and avoid repeated freeze/thaw.
	Low SA-HRP detection reagent.	Check if the detection reagent was prepared correctly.

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