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## Technical Data Sheet

### For research use only

Not intended or approved for  
diagnostic or therapeutic use.

## Class III PI3-Kinase Kit

### 96-well ELISA Assay for Detection of PI(3)P (Patent Protected)

**Product Number: K-3000**

### Storage

- Store kit part 1 of 2 at 4 °C.
- Store kit part 2 of 2 at -20 °C.

### Materials Provided

Part #	Description	Quantity
K-3001	Class III Detection Plate, 12 x 8-strip well plate	1
K-3002	PI Substrate, diC <sub>8</sub> , MW = 609 g/mol	30 µg
K-3003	PI(3)P Standard, diC <sub>8</sub> , MW = 733 g/mol	3 µg
K-3004	5x PI(3)P Detection Buffer (5x DB)	4 mL
K-EDTA	100 mM EDTA, pH 8	500 µL
K-TBST	10x TBS-T Buffer	20 mL
K-3305	PI(3)P Detector (store at -20 °C)	4 x 10 µg/vial
K-2306	HRP Conjugate	25 µL
K-TMB1	TMB Solution	12 mL
K-STOPt	1 N H <sub>2</sub> SO <sub>4</sub> Stop Solution	10 mL
Incubation Plate	96-well polypropylene U-bottom plate	1
Plate Sealers	Clear acetate sheet, 1 side adhesive	3

### Additional Materials Provided by User

- PI3-Kinase enzyme
- 5x PI3-Kinase reaction buffer (see note below). Store at -20 °C
- Absorbance plate reader capable of reading at 450 nm

**NOTE:** The composition of the 5x kinase reaction buffer is determined by the user. Two sample recipes are listed below for human Vps34 (PIK3C3). To offset any potential interference caused by the kinase reaction buffer to the detection of PI(3)P product, make sure to appropriately dilute controls and PI(3)P standards with Detection Buffer Blend as shown in assay protocol.

Recipe #1: 250 mM HEPES pH 7.5, 750 mM NaCl, 5 mM CHAPS, 5 mM DTT, 250 µM ATP, and 25 mM MnCl<sub>2</sub>.

Recipe #2: 50 mM Tris pH 8, 500 mM NaCl, 5 mM EDTA, 250 µM ATP, and 50 mM MnCl<sub>2</sub>.

## Background and Product Description

PI3-Kinases (PI3-K) are grouped into three classes according to their structural homology and their *in vivo* lipid substrate preference. Class III PI3-K uses phosphatidylinositol (PI) as substrate to generate PI(3)P product. While Class I PI3-Ks are well-recognized for their role in cell growth and division, the physiological roles of the Class II and III enzymes are still emerging.<sup>1</sup> There is only one Class III family member which was originally identified in yeast and appropriately named vacuolar protein-sorting defective 34 (Vps34) indicating its role in intracellular vesicular transport in this organism.<sup>2</sup> The human homolog, hVps34, consists of catalytic and regulatory subunits encoded by the *VPS34* and *PIK3R4* genes, respectively. This enzyme was recently shown to mediate the cellular response to nutrient deprivation by inhibiting the activities of the translational regulators mTOR and p70 S6 kinase.<sup>3,4</sup> Due to possible roles in human metabolic disorders, including Type II diabetes, activity in Class III PI3-K research and drug discovery efforts will certainly increase; and will ultimately lead to a better understanding of the cellular functions of each PI3-K family member.

Under proper conditions, all three classes of PI3-Kinases use phosphatidylinositol as substrate *in vitro* to produce PI(3)P. Detection of PI(3)P is thus useful for determining *in vitro* activity of all PI3-Ks (i.e. Class I, Class II, or Class III). Traditionally, experiments to measure Class III PI3-K activity have involved phosphorylation of phosphatidylinositol substrate using ATP-gamma <sup>32</sup>P, then extracting radioactive products, and separation using thin-layer chromatography. The assay method developed by Echelon Biosciences, Inc. allows the user to determine Class III PI3-K activity, using either recombinant or immunoprecipitated enzyme, by means of a standard 96-well ELISA format, eliminating the need for radioactivity, organic solvents, and thin layer chromatography.

Echelon's Class III PI3K Activity Assay is a competitive ELISA in which the signal is inversely proportional to the amount of PI(3)P produced. After the PI3-K reactions are complete, reaction products are added to the PI(3)P-coated microplate, for competitive binding to a PI(3)P detector protein. The amount of PI(3)P detector protein bound to the plate is determined through colorimetric detection. This ELISA is sensitive as well as specific. As low as 0.5 pmol PI(3)P can be detected in a 100  $\mu$ L detection mixture, corresponding to a sensitivity of 5 nM. A typical PI(3)P standard competition curve generates an EC<sub>50</sub> value of below 100 nM (10 pmol) PI(3)P, with excellent assay robustness (*Z'* value of about 0.8).

## References

1. Engelman, J. A., Luo, J., and Cantley, L. C., The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism, *Nat Rev Genet*, 7, 606 (2006).
2. Odorizzi, G., Babst, M., and Emr, S. D., Phosphoinositide signaling and the regulation of membrane trafficking in yeast, *Trends Biochem Sci*, 25, 229 (2000).
3. Byfield, M. P., Murray, J. T., and Backer, J. M., hVps34 is a nutrient-regulated lipid kinase required for activation of p70 S6 kinase, *J Biol Chem*, 280, 33076 (2005).
4. Nobukuni, T., Joaquin, M., Rocco, M., Dann, S. G., Kim, S. Y., Gulati, P., Byfield, M. P., Backer, J. M., Natt, F., Bos, J. L., Zwartkruis, F. J., and Thomas, G., Amino acids mediate mTOR/raptor signaling through activation of class 3 phosphatidylinositol 3OH-kinase, *Proc Natl Acad Sci U S A*, 102, 14238 (2005).

## Related Products

1. PI(3)P Mass Strip Assay Kit, cat # K-3600
2. PI(3)P Mass ELISA, cat # K-3300
3. Anti-Vps34 antibody for Immunoprecipitation, cat # Z-R015
4. Anti-Vps34 antibody for Western Blot, cat # Z-R016

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## Assay Protocol

### Kinase Reaction

1. Isolate or prepare PI3-Kinase according to user's standard protocol.
2. Prepare 500  $\mu\text{M}$  of PI stock by adding 99  $\mu\text{L}$  of ddH<sub>2</sub>O to the vial of 30  $\mu\text{g}$  PI Substrate (K-3002). Pipet up and down repeatedly or vortex shortly to fully reconstitute the lipid. Spin down briefly and place vial on ice. One vial of PI substrate contains enough for 96 assay points at 500 pmol (304 ng) of diC<sub>8</sub> PI per point. Store remaining PI stock solution at -20 °C for up to 3 months.

One possible format for setting up PI3-K reactions in the incubation plate follows. For each PI3-K reaction, add the following to a single well of the 96-well incubation plate: 5  $\mu\text{L}$  of 5 x PI3-K reaction buffer, 4  $\mu\text{L}$  of 500  $\mu\text{M}$  PI substrate (2 nmol), enough ddH<sub>2</sub>O to bring the total volume (including PI3-Kinase) to 25  $\mu\text{L}$ , and finally add PI3-Kinase to start the reaction. Seal the plate with plate sealer.

If you are using immunoprecipitated enzyme bound on beads, add the components listed above directly to the beads in a micro tube and then add ddH<sub>2</sub>O to bring the final volume to 25  $\mu\text{L}$ .

Let the kinase reaction proceed without shaking for appropriate time (to be determined by user, typically 0.5-3 hours) at desired temperature: room temperature, 30 °C or 37 °C.

3. Quench the kinase reactions by adding 5  $\mu\text{L}$  100 mM EDTA (K-EDTA) to each well of 25  $\mu\text{L}$  reaction. Then dilute the quenched reaction with 130  $\mu\text{L}$  ddH<sub>2</sub>O. If enzyme is bound to beads or otherwise immobilized, the enzyme reaction can be stopped by simply pelleting the beads by centrifugation and transferring the reaction mixture to the incubation plate, adding 5  $\mu\text{L}$  of 100 mM EDTA, and bringing volume to 160  $\mu\text{L}$  with ddH<sub>2</sub>O.

Add 40  $\mu\text{L}$  of 5x PI(3)P Detection Buffer (K-3004) to each 160  $\mu\text{L}$  reaction mixture to bring the final volume to 200  $\mu\text{L}$  ([PI] + [PI(3)P] = 10  $\mu\text{M}$  in Detection Buffer Blend, final EDTA concentration  $\geq$  2.5 mM). This will provide enough samples for triplicate assay points in following detection step.

**NOTE:** User must optimize the amount of PI3-Kinase in each reaction by doing an enzyme titration. We suggest 5 nM PI3-K enzyme as a starting point for optimization. Substrate [PI] titration is optional. We also suggest including one reaction without PI3-K as a "No Enzyme" control; and one reaction without PI as a "No Substrate" control.

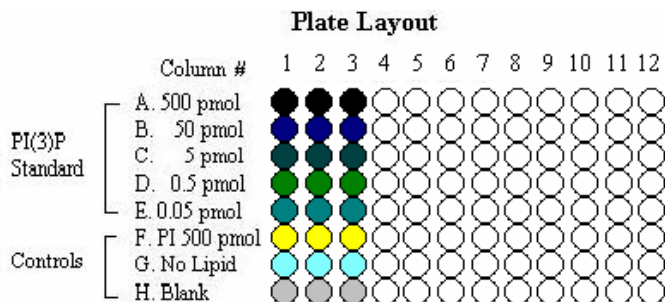
### PI(3)P Detection

1. Prepare 2 mL **Detection Buffer Blend (DBB)** on ice by adding 400  $\mu\text{L}$  5x Detection Buffer, 50  $\mu\text{L}$  100 mM EDTA, and 50  $\mu\text{L}$  5x PI3-K reaction buffer to 1.5 mL ddH<sub>2</sub>O.
2. Prepare PI(3)P Standard solutions in DBB.
  - 1) Add 410  $\mu\text{L}$  DBB to 3  $\mu\text{g}$  PI(3)P Standard (K-3003) for a concentration of 10  $\mu\text{M}$ . Pipet up and down repeatedly or vortex shortly to fully reconstitute the lipid. Spin down briefly and place vial on ice.
  - 2) Further dilute 10  $\mu\text{M}$  PI(3)P 10x serially in DBB into unused wells of the incubation plate for concentrations of 1  $\mu\text{M}$  through 1 nM, respectively. Refer to the table below to make enough for triplicate wells of standard curve at 50  $\mu\text{L}$ /well. Store unused portion of 10  $\mu\text{M}$  PI(3)P at -20 °C for up to 3 months.

[PI(3)P]	Dilution Factor (serial)	PI(3)P, $\mu\text{L}$ (Previous conc.)	DBB, $\mu\text{L}$	PI(3)P, pmol/50 $\mu\text{L}$
10 $\mu\text{M}$	1x	180	-	500
1 $\mu\text{M}$	10x	20	180	50
100 nM	10x	20	180	5
10 nM	10x	20	180	0.5
1 nM	10x	20	180	0.05

3. Set up the Detection plate as shown to the right.

- 1) Add 50  $\mu\text{L}$  of DBB to wells H1-H3 as blank control.
- 2) Add 50  $\mu\text{L}$  DBB to wells G1-G3 as no lipid control.
- 3) Add 50  $\mu\text{L}$  PI(3)P dilutions, as PI(3)P competition standards, to wells 1-3 of rows A through E as indicated in plate layout.
- 4) Add 50  $\mu\text{L}$  no enzyme reaction from incubation plate (10  $\mu\text{M}$  PI) to wells F1-F3 as substrate competition control.
- 5) Add 50  $\mu\text{L}$  of each PI3-K reaction from incubation plate to triplicate or other desired replicate wells, use non-shaded part of the detection plate.



4. Prepare 8 mL of PI(3)P Detection Buffer (**DB**) by diluting 1.6 mL of 5x PI(3)P Detection Buffer (K-3004) in 6.4 mL ddH<sub>2</sub>O. Add 50  $\mu\text{L}$  DB to blank control wells H1-H3.

5. Apply PI(3)P Detector.

- 1) Reconstitute each vial of 10  $\mu\text{g}$  PI(3)P Detector (K-3305) with 1.5 mL DB. Each vial provides PI(3)P Detector for up to 30 assay points. Prepare freshly prior to use.
- 2) Add 50  $\mu\text{L}$  of reconstituted PI(3)P detector to all wells except blank control wells H1-H3.
- 3) Seal plate and incubate on plate shaker at room temperature for 1 hour.

**NOTE:** Never let the detection plate dry out after the assay has started. Always get the next solution ready before discarding the current one from wells in use.

Strip wells not in use should be removed from the plate, sealed in a plastic bag, and stored at 4 °C for later use.

6. Wash plate

- 1) Dilute 20 mL 10x TBS-T buffer (K-TBST) with 180 mL ddH<sub>2</sub>O to get 200 mL TBS-T buffer. TBS-T buffer is stable at room temperature for at least 1 year.
- 2) Discard solution from Detection Plate and wash plate twice briefly with TBS-T 300  $\mu\text{L}$ /well.

7. Apply HRP Conjugate

Dilute HRP Conjugate (K-2306) 1:500 with TBS-T. Prepare enough for current assay only. For entire plate, dilute 22  $\mu\text{L}$  HRP Conjugate with 11 mL TBS-T and mix well. Discard TBS-T wash from plate, and add 100  $\mu\text{L}$  diluted HRP Conjugate to each well. Seal plate and incubate for 1 hour at room temperature on plate shaker.

8. Discard solution from Detection Plate and wash plate 3 times with TBS-T 300  $\mu\text{L}$ /well.

9. Discard TBS-T wash completely from plate. Add TMB Solution (K-TMB1) 100  $\mu\text{L}$  to each well. Seal plate with a new sealer. Allow color to develop for 3-20 minutes in dark (or cover plate with aluminum foil). Periodically check for blue color development and DO NOT overdevelop. Stop color development by adding 50  $\mu\text{L}$  of 1 N H<sub>2</sub>SO<sub>4</sub> stop solution (K-STOPT) to each well when the color is still clear or very light blue in 500 pmol PI(3)P standard wells and has turned ocean blue in No Lipid control wells. Blue color will change to yellow color upon addition of stop solution. Eliminate any big air bubbles present in wells before reading the plate.

**Note:** Use caution when using acid stop solution.

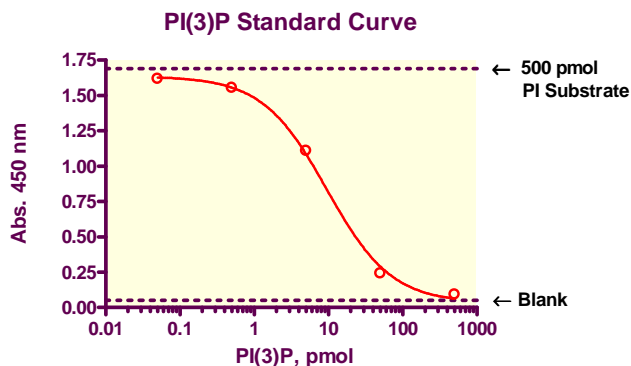
10. Read absorbance at 450 nm on a plate reader.

**Caution:** Be aware of the dynamic range of your plate reader instrument. Make sure the highest absorbance reading is within this range.

## Results

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 absorbance values vs. Log PI(3)P in pmol to generate a standard curve using one site competition, non-linear regression analysis as shown to the right. Determine the PI(3)P value in pmol for each enzymatic reaction assay point (50  $\mu\text{L}$  out of 200  $\mu\text{L}$  final reaction mixture) by interpolation from the standard curve. Calculate [PI(3)P] in the original 25  $\mu\text{L}$  PI3-K reaction as follows:



$$[\text{PI}(3)\text{P}] = \text{_____ pmol} \times (200 \mu\text{L}/50 \mu\text{L})/25 \mu\text{L} = \text{_____ } \mu\text{M}$$

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