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FAQ - PIP Mass Assays

For use with product numbers: K-2500s, K-3300, K-3800, K-4000E, K-4500

Phosphoinositides (PIP_n), phosphorylated forms of Phosphatidylinositol (PI), are an important class of lipids involved in cell signaling and membrane trafficking events. PIP_n is formed when the inositol ring of PI is phosphorylated, by a variety of kinases, on the 3, 4, and 5 hydroxyl groups in seven different combinations. All seven variations have been found in animals and six (with the exception of PI(3,4,5)P₃) are found in plant cells. These lipids have been found to be critical for the regulation of many aspects of membrane homeostasis and signaling with relevance to human physiology and disease. The ability to study the relative levels of PIP_n is crucial to understanding their functions.

Echelon currently carries 5 PIP_n Mass ELISA kits that can measure the relative levels of 5 different PIP_n. Each PIP_n Mass ELISA employs a lipid extraction protocol designed for the extraction of phosphoinositide (PIP_n) from millions of cells. These PIP_n extractions are then analyzed using a competitive ELISA. This document addresses frequently asked questions regarding the use of these assays. Please also refer to the specific products Technical Data Sheets (TDS). For questions e-mail echelon at echelon@echelon-inc.com, or call technical support at 801-588-0455.

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PIP _n	PIP Mass ELISA Kits	Catalog	PIP Mass Strip Kits	Catalog
PI(3)P	PI(3)P Mass ELISA	K-3300	°PI(3)P Mass Strip Kit	°K-3600
PI(4)P	PI(4)P Mass ELISA	K-4000E	°PI(4)P Mass Strip Kit	°K-4000
PI(3,4)P ₂	PI(3,4)P ₂ Mass ELISA	K-3800	°PI(3,4)P ₂ Mass Strip Kit	°K-3400
PI(4,5)P ₂	PI(4,5)P ₂ Mass ELISA	K-4500	°PI(4,5)P ₂ Mass Strip Kit	°K-2900
PI(3,4,5)P ₃	PIP ₃ Mass ELISA	K-2500S	°PIP ₃ Mass Strip Kit	°K-2400

Disclaimer

The PIP_n Mass Assays are used to quantify the total amount of a specific lipid that has been extracted from cells. However, since the lipids are substrates for enzymes in multiple pathways the data obtained may not correlate with what has been observed with isolated enzyme reactions or visualized with immunohistochemistry.

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Cells

The PIP_n extraction protocols have been verified using the cells lines: NIH-3T3, HL-60, MDA-MB-231, and MDA-MB-468. Adherent Cell Lines, NIH-3T3 mouse fibroblasts, MDA-MB-231 human breast cancer, and MDA-MB-468 PTEN deficient human breast cancer were used to validate the assays. Table 2 lists the published cell lines and tissue samples that have been used in various assays.

Cell Line or Tissue and Reference Number	PIP _n	PIP _n Mass Assay Catalog Number
NIH-3T3 ¹ , MDA-MB-231 ¹⁸ , MDA-MB-468, HL-60 ¹ , Yeast strain BY4742 ³ , Human platelets ³ , Guinea pig brain and kidney ⁴	All	PIP _n Extraction Protocol
HL-60 ¹ and NIH-3T3 ¹ , Mouse and Human neutrophils ²	PIP ₃ and PI(3,4)P ₂	PIP _n Extraction Protocol
A431 ⁶ HeLa ²⁴	PI(3)P	°K-3600
human T-cells ⁴⁰	PI(3,4)P ₂	°K-3400
Rat brain ⁵ , Lobster Olfactory receptor neurons (ORNs) ⁴⁵	PI(4,5)P ₂	°K-2900
Rodent Olfactory Ciliary Membranes ¹²	PIP ₃	°K-2400
<i>Plasmodium falciparum</i> ²⁹ , <i>Arabidopsis</i> ³⁹ , Mouse Embryonic Fibroblasts (MEF) ⁴³	PI(3)P	K-3300
Mouse Neutrophils ²⁵ , Rat basophilic leukemia (RBL-2H3) ⁴⁴	PI(3,4)P ₂	K-3800
Hippocampal neurons from the fetuses of female rats ³¹ , mouse cardiac myocytes ³²	PI(4)P	°K-4000
Mouse Dorsal Root Ganglion (DRG) Neurons ²⁷ , HEK ²⁸ , Hippocampal neurons from the fetuses of female rats ³¹ , rat liver tissue ³⁴ , HCC827 and MDA-MB-453 ³⁷ , <i>Arabidopsis</i> ³⁹ , Mouse brain tissue ⁴¹ , Neuroblastoma ⁴⁶ , MCF-10A ⁴⁷ , Mouse Spleen CD4+ T cells ⁴⁸ , HER2 ⁵¹	PI(4,5)P ₂	K-4500
<i>Dictyostelium</i> ^{10,11} , Rat endothelial progenitor cells ¹³ , Jurkat and H9 ²⁰ , c-Myc-OX40 T cells ²⁶ , Sprague-Dawley rats ^{30,49} , Hippocampal neurons from the fetuses of female rats ³¹ , HeLa ³³ , rat liver tissue ³⁴ , human ovarian cell lines CLDN3 and CLDN4 ³⁵ , Mouse T-cells ³⁶ , HCC827 and MDA-MB-453 ³⁷ , 3T3-L1 adipocytes ³⁸ , Mouse brain tissue ⁴¹ , Mouse osteoclasts ⁵⁰ , HER2 ⁵¹	PIP ₃	K-2500s

Counting Cells

Normalize cells by counting or running a cellular protein assay.

- Adherent cells can be counted before stimulation or before the cell extraction procedure. After counting, return cells to a flask for 1 to 3 hours for re-attachment.
- Non-adherent cells can be counted before stimulation or before the cell extraction procedure. After stimulation, spin cells down, decant the media and add ice cold 0.5 M TCA and proceed with the cell extraction protocol.
- Count or perform protein assay on a separate flask with the same cell dilution as the flasks to be used for cell extraction.
- Counting cells, either by haemocytometer or protein assay, is not recommended after the 0.5 M TCA is added.
- Do not trypsinize cells before adding 0.5 M TCA; it can alter stimulation time and phosphoinositide levels.
- Please refer to the product specific TDS for optimal cell number.

Extraction Protocol

The cell extraction protocol has been verified internally at Echelon using the cells lines: NIH-3T3, HL-60, MDA-MB-231, and MDA-MB-468. Please also see Table 2 for published cell lines and tissues.

Solutions for Extraction

- 0.5 M TCA:** For 50 mL, dissolve 4.08 g TCA (Trichloroacetic Acid) in dH₂O. Bring volume to 50 mL.
 - 0.5 M TCA should be cold before use.
 - Store at room temperature. Prolonged storage at 4°C will cause TCA to precipitate.
 - TCA is used to precipitate proteins, DNA and RNA and attenuates cellular production and/or degradation of PIP_n
- 5% TCA with 1 mM EDTA:** For 50 mL, dissolve 2.5 g TCA in dH₂O, add 100 µL 0.5 M EDTA, and bring volume to 50 mL with dH₂O.
- MeOH:CHCl₃ (2:1):** For 60 mL, add 40 mL MeOH to 20 mL CHCl₃
 - CHCl₃ will dissolve plastic equipment. Do not use plastic pipettes or containers. Measure out Chloroform with glass pipette or glass

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cylinder and store solution in a glass bottle. Chloroform melts plastic, which can cause the precipitation in this solution.

- b. MeOH: CHCl₃ (2:1) should be prepared in a glass bottle. This solution is not stable long term and should be used within a month of preparation. It is safe to use plastic ware to transfer this solution.
4. MeOH: CHCl₃: 12 N HCl (80:40:1): For 60 mL, combine 40 mL MeOH, 20 mL CHCl₃, and 0.5 mL 12 N HCl.
 - a. CHCl₃ will dissolve plastic equipment. Do not use plastic pipettes or containers. Measure out Chloroform with glass pipette or glass cylinder and store solution in a glass bottle. Chloroform melts plastic, which can cause the precipitation in this solution.
 - b. Use 12 N concentrated 36% - 38% HCl. Do not use diluted acid.
 - c. The MeOH: CHCl₃: 12 N HCl (80:40:1) solution should be clear. The solution will become cloudy or precipitate when diluted or impure reagents are added together and/or less than 12 N HCl is used.
5. 0.1 N HCl: For 50 mL, add 0.42 mL 12 N HCl to 50 mL dH₂O.

PIPn Extraction Protocol

Volumes are not included in this protocol. Please refer to the specific products TDS for suggested cell number and volumes needed for extraction.

1. Collect Cells:
 - a. For adherent cells, remove medium by gentle aspiration and immediately add ice cold 0.5 M TCA. Incubate cells on ice for 5 minutes. Scrape the cells from flask. Use additional 0.5 M TCA if needed and transfer to a centrifuge tube. Centrifuge at 3,000 RPM (approximately 900-1,000 RCF) for 7 minutes at 4°C. Discard the supernatant. The remaining steps are performed at room temperature.
 - b. For non-adherent cells, collect cells into centrifuge tube, spin the cells down, decant media, add ice cold 0.5 M TCA and vortex. Incubate cells on ice for 5 minutes. Centrifuge at 3,000 RPM (approximately 900-1,000 RCF) for 7 minutes at 4°C. Discard the supernatant. The remaining steps are performed at room temperature.
 - c. Tissue samples should be ground in a mortar and pestle under liquid nitrogen and homogenized before the addition of 0.5 M TCA⁴.
2. Wash Pellet: Add 5% TCA/1 mM EDTA to the pellet. Vortex for 30 seconds. Centrifuge at 3000 RPM for 5 minutes. Discard the supernatant. Repeat wash one more time.

Note: It is important to remove as much of the supernatant as possible after final wash. Otherwise, too much water will be present to allow a single phase to form on subsequent extraction steps.
3. Extract neutral lipids: Add MeOH: CHCl₃ (2:1) and vortex for 10 minutes at room temperature. Centrifuge at 3000 RPM for 5 minutes, discard the supernatant. Repeat neutral lipid extraction one more time. Depending on the number of cells used, a small white pellet should be visible after this step.
4. Extract acidic lipids: Add MeOH: CHCl₃: 12 N HCl (80:40:1) and vortex for 25 minutes at room temperature. Centrifuge at 3,000 RPM for 5 minutes. Transfer supernatant to a new tube. Discard pellet.
5. Phase split: To supernatant from step 4, add CHCl₃ and then the 0.1 N HCl. Vortex for 30 seconds. Centrifuge at 3,000 RPM for 5 minutes to separate organic and aqueous phases. Disregard any excess cellular debris that may appear between the two layers. Collect the organic (lower) phase, with a positive displacement pipette, into a new vial and dry in a vacuum dryer (45-60 minutes). Dried lipid can be stored at -20°C for up to 12 months. The dried lipid should not be visible. If there is a visible substance at the end of this step, it is most likely cell debris that was not eliminated in the extraction.

Other Things to Consider: PIPn Extraction Protocol

1. The amount of cells necessary for PIPn quantification needs to be determined for each cell and PIPn. For a starting point please refer to the products specific TDS. If larger or smaller amounts of cells are required, over what is specified in the products TDS, the extraction volumes will need to be proportionately adjusted.
2. For a PIPn extraction positive control, add 20 pmol diC₁₆ PIPn to step 4, of the PIPn extraction protocol, without cells. Dilute diC₁₆ PIPn control lipid in CHCl₃: MeOH: H₂O (1: 2: 0.8) solution at 0.2 μM. See Table 3 for appropriate diC₁₆ PIPn controls.
3. If air-displacement pipets are being used to dispense volatile solvents or to recover the lower organic phase, then they should first be well-primed with the organic solvent so the air inside of the pipet becomes saturated with the vapor; otherwise the first few samples will be short-measured.
4. Once the extraction solutions are prepared, plastic tips and vials can be used for the remaining steps of the extraction.
5. Once step 1 of the extraction protocol is complete, the cell pellet does not need to be on ice.
6. If you are running a large number of cell extractions, we suggest staggering the flasks during step 1 and 2 to limit the amount of time the PIPn is in the TCA solutions. The cell pellet, after the final wash in step 2, can be incubated on ice or at -20°C for up to 2 hours. Thaw cell pellets before continuing with the cell extraction protocol or they will not break apart while vortexing. PIPn may not be stable in TCA.
7. The approximate end volume from the PIPn extraction takes about 1 hour to dry in a speed vacuum dryer. If you do not have a speed vacuum dryer a nitrogen stream or lyophilizer can be utilized.
8. The dried lipid should not be visible. If there is a visible substance it is most likely cell debris that was not eliminated during the extraction. Proceed with detection regardless if a pellet can be seen or not. If the pellet is visible, it may look colorless, white, yellow, brown, or black. Do not attempt to dissolve visible cell debris in the detection assay buffer. This black to yellow substance should also be avoided when pipetting the lipid extraction samples into the PIPn Mass ELISA wells.
9. Due to the variable nature of cells and the difficulty of the PIPn extraction, we recommend running samples in multiples; multiple dilutions, extractions, and times per condition per data point.

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ELISA Detection

Once PIP_n has been extracted from cellular samples, it is brought up into an aqueous buffer before being incubated with a PIP_n detector protein. The PIP_n detector /extraction sample are then transferred to the PIP_n-coated plate for competitive binding. A peroxidase-linked secondary detection reagent and colorimetric substrate is used to detect the PIP_n detector protein that has bound the plate. Since this is a competitive ELISA, the colorimetric signal is inversely proportional to the amount of PIP_n extracted from cells. The quantity of PIP_n may vary for each cell type, stimulant, and number of cells used.

ELISA Selectivity

The antibodies and binding proteins utilized in each assay have been tested for selectivity of its preferred PIP_n over the other synthetic diC16 PIP_n (see Table 3 for the catalog numbers of the lipids tested) and have been found selective for the PIP_n they were designed to measure. We developed the kit's selectivity based on the IC₅₀ values obtained for each PI/PIP_n while also considering the relative levels of PIP_n found in cells. Please note, that the isolated synthetic diC16 PIP_n (shown in Table 3), may not fully represent the complex mixture of lipids and lipid chain lengths present in a biological extraction.

Table 3: Selectivity			PI(3)P	PI(4)P	PI(3,4)P ₂	PI(4,5)P ₂	PI(3,4,5)P ₃
			K-3300	K-4000E	K-3800	K-4500	K-2500s
PI/PIP _n	Echelon Catalog Number	MW (g/mol)	IC ₅₀ ratio (IC ₅₀ of interfering lipid / IC ₅₀ of standard curve)				
PI	P-0016	833.01	272.4	*	*	6,889.0	*
PI(3)P	P-3016	956.96	1	63.7	*	743.8	*
PI(4)P	P-4016	956.96	526.4	1	*	1215.2	*
PI(5)P	P-5016	956.96	102.3	**	*	96.2	*
PI(3,4)P ₂	P-3416	1,080.9	79.2	72.5	1	594.2	3,141.2
PI(3,5)P ₂	P-3516	1,080.9	48.6	**	*	273.4	17,910.0
PI(4,5)P ₂	P-4516	1,080.9	94.3	107.9	62.7	1	*
PI(3,4,5)P ₃	P-3916	1,204.84	31.7	152.2	23.7	51.1	1

The relative levels of PI/PIP_n are dependent upon the cell source, drug treatment, and its disease. For instance, PI(4)P and PI(4,5)P₂ constitutes about 2-5% of total PtdIns in mammalian cells^{53,54}, with PI(4)P at ~ 50% the amount of PI(4,5)P₂, yet, in yeast and plants cells PI(4)P is found at levels higher than PI(4,5)P₂^{55,56}. As another example, PI(3)P is found at 5-10% of PI(4)P in mammalian cells⁵⁷ but in

yeast PI(3)P is found at the same levels of PI(4)P⁵⁸. PI(3,5)P₂ levels in yeast are ~50% of PI(4,5)P₂^{59,60} but it is significantly lower in mammalian cells (1% of PI(4,5)P₂)^{61,62}. Additionally, PI(3)P₃, PI(5)P₂, PI(3,5)P₂ and PI(3,4,5)P₃ are all minor lipids in mammalian cells (1-5% of PI(4,5)P₂), that can substantially increase upon activation.

As described above, the cell source and treatment of cells can affect the PIP_n profile. Due to this, you may experience interference from a secondary PIP_n. This is most often observed with PI(4,5)P₂ because of its high level in cells compared to the other PIP_n. PI(4,5)P₂ cross reactivity is of greatest concern in the PI(3,4)P₂ assay (K-3800). If a sample is highly competitive in an assay and you think a secondary PIP_n may be interfering, the sample should be titrated until it competes in the lower detection range of the assay. The sample should show dilution linearity.

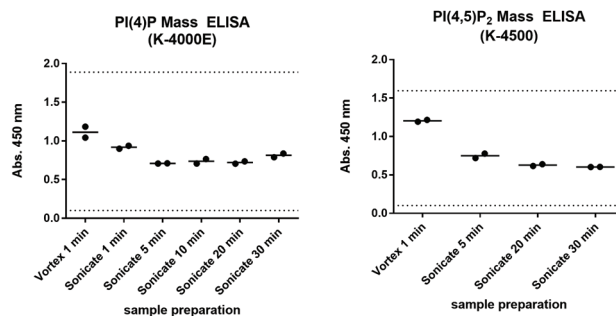
Preparing the PIP_n Extraction Sample for Detection in the ELISA Assay

Ensure the dried PIP_n extractions are at room temperature. The room temperature PIP_n extractions are then resuspended in assay buffer according to the product specific TDS before being vortexed for 1 min or sonicated for 5-10 min (or both) to solubilize the lipids.

When only vortexing is utilized to dissolve the PIP_n extraction sample, lower amounts of PIP_n are measured (see figure 1) and greater day to day variation is observed. The vortexed sample may not show dilution linearity. When the same sample is sonicated, however, we observe greater amounts of measurable PIP_n (see figure 1), consistent day to day variation (inter assay CV = 4.90%) and dilution linearity (2.89% at 1:3 dilution).

It can be difficult, however, to reproduce conditions of sonication; due to variation in the number of vials between batches, temperature of the water bath, and sonicator tuning. The suggested sonication time of 5-10 min was developed using

Figure 1: vortexing vs. sonication of extraction samples



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a water bath sonicator. The results observed with your water bath sonicator may be different. It is suggested that your sonicator is tested with extraction samples for day to day variation and time dependent consistency. If you are running other lipid mass assays you may want to consider dissolving your lipids the same manner. How you dissolve the lipid will affect how it goes into solution and can cause inconsistencies in your data if it is not held constant.

Other Things to Consider: ELISA Detection

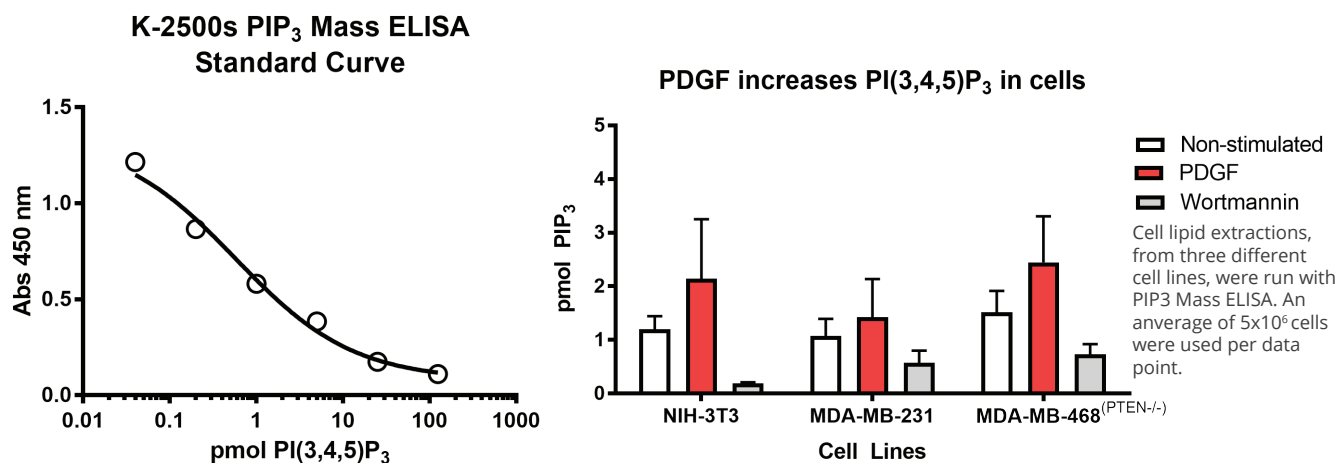
1. It is very important for the PIPn Standard and the extraction samples to be at room temperature before they are solubilized. Not doing so will affect how they behave in the assays.
2. We have not extensively tested the use of water bath sonication with ice, except to note that less than expected PIPn was measured as compared to same sample without ice.
3. Any yellow to black precipitate in the PIPn extraction sample is not PIPn and should be avoided when pipetting the cell samples into the PIPn Mass ELISA wells.
4. Cell dilutions higher than six may not work well on the ELISA. We recommend reducing the number of cells used before further diluting the cell extraction samples. Dilute cell extraction samples as needed.

Quantification: PIPn Mass ELISA

To quantify the relative PIPn levels in the PIPn extractions, plot the absorbance (O.D) values obtained vs. PIPn standard to generate a PIPn standard curve. Cellular PIPn levels can then be estimated by comparing the O.D. values of the PIPn extraction samples to the O.D. values on the standard curve.

PIP3 Mass ELISA Kit (catalog K-2500s) Example

Figure 2: PI(3,4,5)P₃ levels were calculated using Graphpad Prism software and nonlinear regression Sigmoidal Dose Response (variable slope) analysis. Breast cancer cell lines MDA-MB-231 and MDA-MB-468 (PTEN^{-/-}) were extracted from 12-well plates and run on the PIP3 Mass ELISA Kit (K-2500s). The cells were serum starved for 5 hours before they were stimulated with 10 ng/mL PDGF for 5 min or 300 nM Wortmannin for 3 hours respectively.



For examples of other kits, contact echelon@echelon-inc.com to see a poster presented at AACR in 2009 for products K-2500s, K-3300, K-3800, K-4000, and K-4500.

Sample normalization with PI(4,5)P₂

PI(4,5)P₂ is the most abundant PIPn in mammalian cells and therefore can be used to normalize samples by using the PI(4,5)P₂ measurement as an indicator of total phosphoinositide. This method has been used most recently in a publication by Costa, et al in 2015⁶³ for the measurement of PI(3,4,5)P₃. This publication justified the use of this PIPn over the others due to its high abundance⁶³ and PI3K's lack of influence over its levels⁶⁴. To use this control each extraction sample will need to be split into two vials at the end of the extraction, before the sample is dried. For example: if 1.5 mL of the lower (organic) layer is collected then 1.45 mL is transferred to one vial for PI(3,4,5)P₃ detection in the PIP3 Mass ELISA (K-2500s) and 0.05 mL is transferred to another vial for PI(4,5)P₂ detection in the PI(4,5)P₂ mass ELISA (K-4500). These are both dried and then brought up according to the protocol of the specific PIPn Mass ELISA kit being used. Once the ELISA's are run and the results quantified the pmol values are then entered into this formula:

$$\text{PI(3,4,5)P}_3/\text{PI(4,5)P}_2 = \% \text{ total PI(4,5)P}_2$$

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