Echelon Biosciences Inc.

FlowPIPs: Multiplex Lipid Beads

Product #:	P-FB01	Size:	10 μg	50 μg	100 μg

Description: FlowPIPs are microparticles with attached lipids that can be used to determine and study lipid-protein interactions. Each pack of FlowPIPs contain two mixes of assay particles. Each bead mix, Mix A & Mix B, contains six separately coated beads that vary in both the coating lipid and the fluorescence intensity of the bead, allowing the user to determine if their protein of interest binds with one or more of the bound lipids in a multiplex cytometric format.

Sizes:

10 µg size in 20 µL slurry	Suitable for evaluating two lipid-protein interactions.
50 µg size in 100 µL slurry	Suitable for evaluating ten lipid-protein interactions.
100 µg size in 200 µL slurry	Suitable for evaluating twenty lipid-protein interactions.

Technical Notes:

- 2 pmol of total lipid, 0.33 pmol of each individual lipid, bound per 5 µg of each FlowPIP Bead Mix.
- Particle size 2.5 – 2.9 µm; Mean size 2.52 µm
- A flow cytometer capable of excitation with a Blue Laser, 488 nm, is required.
- Six fluorescence intensities in each FlowPIP Bead Mix are analyzed in the Yellow channel.
- Fluorescent conjugates can be used for detection in the Green, Red, and near IR channels.

Formulation: FlowPIPs are provided at 0.5 mg/mL in a 1x PBS, pH 7.4 buffer solution.

Storage: Store product at 2-8 °C. Product is temperature and light sensitive. Do not freeze.

QA / Product Testing: Each lot of FlowPIPs Multiplex Lipid Bead Mix is evaluated with 3 anti-PIP abs and 5 PIP Grips.

FlowPIPs Resources: Visit our website www.echelon-inc.com. At the bottom of this product's webpage is:

1. Certificate of Analysis: Providing lot-specific information.
2. Question and Answer Document (P-6000): support document providing suggestions for improving and troubleshooting lipid-protein binding interactions.

Fluorescence Intensity Peak #	Bead Mix A Coated w/	Bead Mix B Coated w/
6	PtdIns	PtdIns(3,4)P2
5	PtdIns(3)P	PtdIns(3,5)P2
4	PtdIns(4)P	PtdIns(4,5)P2
3	PtdIns(5)P	PtdIns(3,4,5)P3
2	PtdEthanolamine (PE)	Phosphatidic Acid (PA)
1	No Lipid / Blank	Phosphatidylserine (PS)
Peak # is in order of fluorescence signal strength		

Table 1: Multiplex Lipid Bead Mixes A and B each contain a mixture of beads with six discreet fluorescence intensities when excited with a 488 nm laser. Each separate fluorescence peak intensity bead is coated with a specific lipid, listed below.

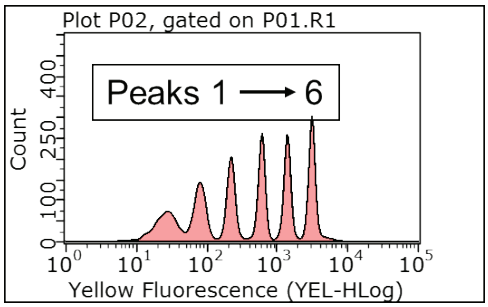

Figure 1. Demonstrates the separation of each bead's peak fluorescence signal when analyzed in the Yellow channel, with a flow cytometer. Each separately coated bead mix allows the user to analyze specific lipid-protein binding for 6 lipid

FlowPIPs: Multiplex Lipid Beads
Protocol for Detection of Lipid-Protein Interactions

For use with product number: P-FB01A and P-FB01B

Suggested Buffers:

1x PBS, pH 7.4: (Sigma Tablets / P-4417)
1. Add 600 mL deionized H_2O to 3 PBS tablets. Mix thoroughly.

FlowPIP Buffer 1: (1x PBS, pH 7.4, 1% BSA, 1% GS, 0.09% Sodium azide)
1. Combine 2.5 g BSA + 2.5 mL Goat Serum + 6.92 mL of 3.25% Sodium azide Solution.
2. Bring final volume to 250 mL with 1x PBS. Store at 4 °C. (Stable for 6 months.)

FlowPIP Buffer 2: (1x PBS, pH 7.4, 1% BSA, 1% GS, 0.05% Tween 20, 0.09% Soidum azide)
1. Combine 2.5 g BSA + 2.5 mL Goat Serum + 6.92 mL of 3.25% Sodium azide Solution + 125 µL Tween 20.
2. Bring final volume to 250 mL with 1x PBS. Store at 4 °C. (Stable for 6 months.)

Suggested Procedure: (Example of protocol optimized for Echelon's PI(4,5)P_2 Grip™, G-4501)

1. Prepare FlowPIPs: Gently vortex each set of FlowPIP beads, FlowPIP Mix A (P-FB01A) and FlowPIP Mix B (P-FB01B), to ensure they are evenly distributed. Pipette 10 µL of each FlowPIP Bead Mix into separate 0.5 mL Microcentrifuge tubes. Each tube contains 5 µg of each type of FlowPIP Multiplex Lipid Bead which is sufficient to evaluate the protein of interest.
2. Add Protein of Interest: Add 100 µL of 0.1 µg/mL PI(4,5)P_2 Grip™ protein in FlowPIP Buffer 1 to each separate FlowPIP Bead Mix A & B. Incubate for 1 hr. at RT or at 4 °C overnight with gentle agitation.

   {Note: 0.1 ug/mL is given as a starting concentration for the PI(4,5)P_2 Grip™ protein; the same protein can show different binding patterns at different concentrations. The end user must optimize the protein concentration for each protein of interest. If high background is experienced or proteins interact with multiple lipids instead of showing the expected specificity, we recommend decreasing the amount of protein used.}
3. Wash: Add 250 µL FlowPIP Buffer 2 to each of the FlowPIP Bead bead mixes (Prepared in step. 2). Vortex briefly for 10 seconds (setting 5/6). Centrifuge @ 4,000 x g for 10 minutes. Carefully remove the remaining supernatant and avoid removing the pellet. Repeat 2X for a total of 3 washes.

   {Note: If the pellet is not visible; carefully remove the supernatant leaving ~50 µL supernatant. This volume will contain the bound FlowPIps.}
4. Add 2° Anti-GST antibody: Carefully remove the remaining supernatant and avoid removing the pellet (from the last wash step). Add 100 µL of Alexa Fluor 488 αGST IgG *(Molecular Probes / Cat.# A-11131)*, diluted 1:1,000 in FlowPIP Buffer 1, at a final concentration of 2 µg/mL. Incubate for 1 hr. at RT or at 4 °C overnight with gentle agitation.

   {Note: The secondary antibody listed is used routinely at Echelon. Other similar antibodies are likely to work effectively in this assay. We recommend including "no primary antibody" and "no secondary antibody" control experiments.}
5. Wash: as in step 3.
6. Detection: Add 250 µL 1x PBS after the final wash step. Further dilute the bead mixture to the ideal concentration for analysis on your specific flow cytometer. The Guava easyCyte 8HT flow cytometer recommends reading ≤ 500 cells / µL for accurate results. Analyze forward vs. side scatter (fsc vs. ssc) and gate the region that clearly contains the population of beads. Adjust the gain settings (see suggestions below) prior to collecting data on the lipid-protein binding, perform compensation for fluorophore bleed-over correction, and analyze lipid-protein binding.

Figure 2. Demonstrates the specific lipid – protein binding pattern of the PI(4,5)P_2 Grip™ : PLCδ, *(Echelon Biosciences, Inc. / Cat.# G-4501)* with FlowPIP Mixes A and B. Alexa Fluor 488 αGST IgG *(Molecular Probes / Cat.# A-11131)* was utilized as the 2° Detection Ab. Analysis was performed utilizing a Guava easyCyte 8HT flow cytometer (*Millipore*.)

Suggested Gain Settings:

fsc	ssc	grn	yel	red	nir	red2	nir2	thr
7.66	6.94	8.35	24.70	8.00	8.00	8.00	8.00	fsc