Echelon Biosciences Inc.

Protocol for Lipid Strip and Lipid Array Products

For use with product numbers: P-6001, P-6100, P-6002, P-6003, S-6000, S-6001, P-6111, and P-P100

Positive Controls

Description	Catalog Number	Concentration	Block Solution	Secondary Antibody*
PIP Grip Proteins				(volume to add to 5 mL of Block Solution)
Multi-PIP Grip (LL5α)	G-9901	0.5 µg/mL	PBS-T 3% BSA	K-SEC2, 75 µL
Multi-PIP Grip (LL5α)	G-9901	2.0 µg/mL	TBS-T 3% BSA	K-SEC2, 75 µL
PI(3)P Grip (p40PX)	G-0302	1.0 µg/mL	PBS-T 3% BSA	K-SEC2, 75 µL
PI(4)P Grip (SidC_3C)	G-0402	0.5 µg/mL	PBS-T 3% BSA	K-SEC2, 75 µL
PI(4,5)P2 Grip (PLCδ1)	G-4501	0.5 µg/mL	PBS-T 3% BSA or PBS 1% Milk	K-SEC2, 75 µL
PI(3,4,5)P3 Grip (Grp1)	G-3901	0.25 µg/mL	PBS-T 3% BSA or PBS 1% Milk	K-SEC2, 75 µL
Antibodies				
Anti-PI(4)P IgM	Z-P004	1.0 µg/mL	PBS-T 3% BSA	Jackson Immunoresearch #715-035-140
Anti-PI(3,4)P2 IgG	Z-P034b	2.0 µg/mL	PBS-T 3% BSA	K-SEC1, 100 µL
Anti-PI(3,4,5)P3 IgG	Z-P345b	4.0 µg/mL	PBS-T 3% BSA	K-SEC1, 100 µL

*K-SEC1 and K-SEC2 can be purchased from EBI upon request or an appropriate secondary antibody can be substituted.

This is an example protocol that has been optimized for use with EBI's PI(4,5)P2 Grip (Cat # G-4501). Please modify as needed for your protein of interest or for your preferred control protein from the 'Positive Control' table above. Please read the entire protocol and assay notes before beginning.

Optimized procedure for PI(4,5)P2 Grip™ (GST-PLC-d1-PH), Catalog # G-4501

1. Add controls (optional): Spot 1 µL of PI(4,5)P2 Grip and 1 µL of secondary antibody directly onto open areas of the dry membrane. Allow the spots to dry completely before proceeding to step 2.This is a control for the HRP conjugate and detection reagent (see Assay Note 5).
2. Block: Cover the membrane with 5 to 10 mL of blocking buffer PBS-T + 3% BSA and gently agitate for one hour at room temperature (RT) or overnight at 4°C.
3. Add Protein of interest: Discard blocking buffer and add 0.5 µg/mL PI(4,5)P2 Grip™ protein in 5 mL PBS-T + 3% BSA, or enough to cover the membrane. Incubate for 1 hr at RT with gentle agitation.
4. Wash: Discard the protein solution and wash with >5 mL PBS-T three times with gentle agitation for five to ten minutes each.
5. Anti-GST-HRP antibody: Discard wash solution and add 75 µL anti-GST-HRP antibody (K-SEC2) to 5 mL PBS-T + 3% BSA blocking solution and incubate for 1 hr at RT with gentle agitation.
6. Wash: As in step 3
7. Detect: Discard wash solution and detect the bound protein by detection methods of choice e.g., Echelon's K-TMBP, TMB Precipitating or similar, Chemiluminescent or ECL detection from KPL or similar.
   For K-TMBP, after discarding the final wash, add 1 to 2 mL TMB Precipitating (catalog K-TMBP) per membrane with gentle agitation. Protein-lipid interaction will develop within 2-20 minutes with the spots turning blue. Stop the reaction by adding an ample amount of dH2O (≥ 15mL). Avoid overdevelopment to avoid high background. Record an image of the membrane.

Suggested Buffers for Optimization

Wash Solutions		Block Solutions		
TBS TBS tablet (Amresco K859) in 100 mL dH2O For **TBS-T** Add 0.1% (v/v) Tween-20	**PBS** Dissolve PBS Tablet (Sigma P4417) in 200 mL H2O For **PBS-T** Add 0.1% (v/v) Tween-20. Adjust pH to 7.2	**TBS or PBS + 3% BSA** Add 3 g fatty acid free BSA (Seracare 1900-0016) to 100 mL TBS or PBS For **PBS-T + 3% BSA** Add 3 g BSA to 100 mL PBS-T	**TBS or PBS + 1% milk** Add 1 g non-fat dry milk to 100 mL TBS or PBS	**TBS or PBS +0.1% ovalbumin** Add 0.1 g ovalbumin (Sigma A5253) to 100 mL TBS or PBS

Assay Notes

1. During all incubation and wash steps, make sure the membrane stays wet and never dries. We recommend gentle agitation during all incubation and wash steps. We have found that protein-lipid binding can be affected by the blocking buffer, we recommend trying different buffers for each protein of interest, see table above for suggested buffers.
2. 1-2 µg/mL is recommended as a starting concentration for a new protein of interest. The end user must optimize protein concentration for each protein of interest. If high background is experienced or a protein interacts with multiple lipids instead of showing the expected specificity, we recommend decreasing the amount of protein used or running the protein in a different blocking buffer. We do not recommend incubating the protein overnight at 4°C. This may degrade the protein and cause a decrease in activity.

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3. We do not recommend using cell lysate, only purified protein or antibodies.
4. The primary and secondary antibodies listed are used routinely at Echelon. Other similar antibodies are likely to work effectively in protein-lipid overlay assays.
5. We recommend including control spots. Test the secondary HRP antibody and detection reagent with the detector by spotting 1 µL of detector proteins and secondary detector on the strip before running the protein lipid overlay assay. The spots will show up strongly with the detectors, indicating that both are working.
6. Chemiluminescent or ECL detection may produce variable results depending on the kind of ECL used, exposure times, volume, film, and development analysis software. ECL is prone to loosing activity close to and after the expiration date labeled on the bottle. Avoid using ultrasensitive ECL reagents due to possible high background.
7. Please see EBI's FAQ for Lipid Strip and Lipid Array Products for additional information and recommendations.
8. We do not recommend stripping and re-probing the membrane strips or arrays using Western/protein blot protocols. The stability of the individual lipid spots following such treatment has not been confirmed.
9. The blue blank spot contains traces of Xylene Cyanol FF which shows fluorescence around 615 nm and may be visible when using fluorescence based imgaing such as Licor.

Statement, Notes, and Additional Help

The binding pattern obtained with Echelon Strip products can be different compared to binding interactions determined by other methods and non-EBI membrane-type products. For example, Yu et al. writes that compared to surface plasmon resonance analysis, lipid overlay experiments are sensitive but that "caution must be exercised in interpreting its results"(1). Further, results at Echelon indicate that the binding pattern of certain PH-domain containing proteins is altered by the use of different protein concentrations and different blocking and washing buffers. Therefore we provide the preceding protocol as a guide, and strongly encourage researchers to consult the scientific literature and conduct optimization experiments in order to establish the most favorable procedures for their protein of interest. A few references are provided below for your convenience. In addition to protein-lipid overlay experiments, Echelon recommends researchers use alternative methods to fully characterize the lipid binding preference of a particular protein. In addition to membrane-type products, Echelon has a number of innovative products useful for determining protein-lipid interactions. These products include stabilized liposomes (PolyPIPosomes™ e.g. Y-P039, ref(2)), PIP Beads™ (e.g. P-B00S), and PIP-Plates™ (e.g. H-6300). Please contact our technical service representatives by email at echelon@echelon-inc.com; or by phone, toll-free 866-588-0455, with any questions or to provide feedback and suggestions.

References

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