



Echelon Biosciences Inc.
 675 Arapeen Drive, Suite 302
 Salt Lake City, UT 84108
 Telephone 866-588-0455
 Fax 801-588-0497
 echelon@echelon-inc.com
 www.echelon-inc.com

Technical Data Sheet

For research use only
 Not intended or approved for
 diagnostic or therapeutic use.

Protocol for PIP Strip™ membrane-type Products

For use with product numbers: P-6001, P-6100, P-M600, P-6002, P-6003, and S-6000

Procedure (optimized for Echelon PIP2 Grip™, G-4501)

1. Block: cover the membrane with PBS+ 1% nonfat-dry milk and gently agitate for one hour at room temperature (RT).

{Note: during all incubation and wash steps, make sure the membrane stays wet and never dries. We also recommend gentle agitation during all incubation and wash steps. For some proteins, we have observed that using 3% fatty-acid free bovine serum albumin, BSA (Sigma # A-7030) or 0.1% ovalbumin (Sigma # A-5253) in TBS or PBS results in lowered background and increased specificity for PIP binding.}

2. Add Protein of interest: discard blocking solution and incubate the membrane with 1.0 µg/mL PIP2 Grip™ protein in PBS+1% nonfat-dry milk for 1 hr at RT or at 4 °C overnight.

{Note: 0.5 ug/mL is given as a starting concentration; and 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: discard the protein solution and wash the membrane with PBS-T three times with gentle agitation for ten minutes each.

4. Anti-GST antibody: discard wash solution and incubate strip for 1 hr at RT with anti-GST monoclonal antibody (Sigma # G-1160) diluted 1:2,000 to 1:15,000 in blocking solution.

{Note: The primary and secondary antibodies listed are used routinely at Echelon. Other similar antibodies are likely to work effectively in protein-lipid overlay assays. We recommend including "no primary antibody" and "no secondary antibody" control experiments.}

5. Wash: as in step 3

6. Anti-mouse HRP antibody: discard wash solution and incubate strip for 1 hr at RT with anti-mouse IgG-HRP (Sigma # A-9917) diluted 1:2,000 to 1:15,000 in blocking solution.

7. Wash: as in step 3

8. Detect: the bound protein using method of choice e.g., Chemiluminescent or ECL™ detection from Amersham or similar, Precipitating TMB from Clinical science products or similar, or through infrared detection using the Li-Cor system.

{Note: Please see the Echelon Troubleshooting Guide for additional information and recommendations. We **do not** recommend stripping and reprobng the membrane strips or arrays using Western/protein blot protocols. The stability of the individual lipid spots following such treatment has not been confirmed.}

Suggested Buffers for Optimization

TBST Washing Solution	PBST Washing Solution	TBS or PBS + 3% BSA Blocking Solution	TBS or PBS + 1% milk Blocking Solution	TBS or PBS +0.1% ovalbumin Blocking Solution
10 mM Tris 150 mM NaCl pH 8.0. For TBS-T Add 0.1% (v/v) Tween-20	Dissolve PBS tablet (Sigma # P4417) in 200 mL H ₂ O. For PBS-T Add 0.1% (v/v) Tween-20	Add 3 g fatty acid free BSA to 100 mL TBS or PBS	Add 1 g non-fat dry milk to 100 mL TBS or PBS	Add 0.1 g ovalbumin to 100 mL TBS or PBS

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-Echelon membrane-type products. For example, Yu et al. write 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 <http://www.echelon-inc.com>; or by phone, toll-free 866-588-0455, with any questions or to provide feedback and suggestions.

References

1. Yu, J. W., Mendrola, J. M., Audhya, A., Singh, S., Keleti, D., DeWald, D. B., Murray, D., Emr, S. D., and Lemmon, M. A., Genome-wide analysis of membrane targeting by *S. cerevisiae* pleckstrin homology domains, *Mol Cell*, 13, 677 (2004).
2. Ferguson, C. G., James, R. D., Bigman, C. S., Shepard, D. A., Abdiche, Y., Katsamba, P. S., Myszka, D. G., and Prestwich, G. D., Phosphoinositide-containing polymerized liposomes: stable membrane-mimetic vesicles for protein-lipid binding analysis, *Bioconjug Chem*, 16, 1475 (2005).

Early descriptions and papers of lipids immobilized on membranes for determining protein binding

3. Stevenson, J. M., Perera, I. Y., and Boss, W. F., A phosphatidylinositol 4-kinase pleckstrin homology domain that binds phosphatidylinositol 4-monophosphate, *J Biol Chem*, 273, 22761 (1998).
4. Dowler, S., Currie, R. A., Campbell, D. G., Deak, M., Kular, G., Downes, C. P., and Alessi, D. R., Identification of pleckstrin-homology-domain-containing proteins with novel phosphoinositide-binding specificities, *Biochem J*, 351, 19 (2000).

Lipid-binding domain reviews

5. Cho, W., and Stahelin, R. V., Membrane-protein interactions in cell signaling and membrane trafficking, *Annu Rev Biophys Biomol Struct*, 34, 119 (2005).
6. Ellson, C. D., Andrews, S., Stephens, L. R., and Hawkins, P. T., The PX domain: a new phosphoinositide-binding module, *J Cell Sci*, 115, 1099 (2002).

Detailed protocol and comments

7. Dowler, S., Kular, G., and Alessi, D. R., Protein lipid overlay assay, *Sci STKE*, 2002, L6. (2002).

PH, GRAM, and GLUE domains

8. Varnai, P., Lin, X., Lee, S. B., Tuymetova, G., Bondeva, T., Spat, A., Rhee, S. G., Hajnoczky, G., and Balla, T., Inositol lipid binding and membrane localization of isolated pleckstrin homology (PH) domains. Studies on the PH domains of phospholipase C delta 1 and p130, *J Biol Chem*, 277, 27412 (2002).

9. Berger, P., Schaffitzel, C., Berger, I., Ban, N., and Suter, U., Membrane association of myotubularin-related protein 2 is mediated by a pleckstrin homology-GRAM domain and a coiled-coil dimerization module, *Proc Natl Acad Sci U S A*, 100, 12177 (2003).
10. Slagsvold, T., Aasland, R., Hirano, S., Bache, K. G., Raiborg, C., Trambaiolo, D., Wakatsuki, S., and Stenmark, H., Eap45 in Mammalian ESCRT-II Binds Ubiquitin via a Phosphoinositide-interacting GLUE Domain, *J. Biol. Chem.*, 280, 19600 (2005).

PX domains

11. Yu, J. W., and Lemmon, M. A., All phox homology (PX) domains from *Saccharomyces cerevisiae* specifically recognize phosphatidylinositol 3-phosphate, *J Biol Chem*, 276, 44179 (2001).
12. Cheever, M. L., Sato, T. K., de Beer, T., Kutateladze, T. G., Emr, S. D., and Overduin, M., Phox domain interaction with PtdIns(3)P targets the Vam7 t-SNARE to vacuole membranes, *Nat Cell Biol*, 3, 613 (2001).
13. Du, G., Altshuler, Y. M., Vitale, N., Huang, P., Chasserot-Golaz, S., Morris, A. J., Bader, M. F., and Frohman, M. A., Regulation of phospholipase D1 subcellular cycling through coordination of multiple membrane association motifs, *J Cell Biol*, 162, 305 (2003).

FYVE domains

14. Misra, S., Miller, G. J., and Hurley, J. H., Recognizing phosphatidylinositol 3-phosphate, *Cell*, 107, 559 (2001).
15. Sbrissa, D., Ikonomov, O. C., and Shisheva, A., Phosphatidylinositol 3-phosphate-interacting domains in PIKfyve. Binding specificity and role in PIKfyve. Endomembrane localization, *J Biol Chem*, 277, 6073 (2002).
16. Simonsen, A., Birkeland, H. C. G., Gillooly, D. J., Mizushima, N., Kuma, A., Yoshimori, T., Slagsvold, T., Brech, A., and Stenmark, H., Alf, a novel FYVE-domain-containing protein associated with protein granules and autophagic membranes, *J. Cell Sci.*, 117, 4239 (2004).

Tubby domain

17. Santagata, S., Boggon, T. J., Baird, C. L., Gomez, C. A., Zhao, J., Shan, W. S., Myszka, D. G., and Shapiro, L., G-protein signaling through tubby proteins, *Science*, 292, 2041 (2001).