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Updated Protocol

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-1000)

1. Block: cover the membrane with PBS-T+ 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-T or PBS-T 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-T+1% nonfat-dry milk for 1 hr at RT or at 4 ºC 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 chemiluminescent method of choice e.g., ECL™ detection from Amersham or similar.

9. **Image:** expose strip to film or use a scientific imager capable of chemiluminescent detection

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

Possible Blocking and Washing Solutions

TBS-T	**PBS-T**	**TBS-T or PBS-T +3%BSA**	**TBS-T or PBS-T +1% milk**	**TBS-T or PBS-T +0.1% ovalbumin**
10 mM Tris 150 mM NaCl pH 8.0 Add 0.1% (v/v) Tween-20	Dissolve PBS tablet (Sigma # P4417) in 200 mL H_2O Add 0.1% (v/v) Tween-20	Add 3 g fatty acid free BSA to 100 mL TBS-T or PBS-T	Add 1 g non-fat dry milk to 100 mL TBS-T or PBS-T	Add 0.1 g ovalbumin to 100 mL TBS-T or PBS-T

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

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Detailed protocol and comments

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PIP Strips™, PIP Array™, PIP Microstrips™, and Sphingostrips™

Troubleshooting Guide (Q&A)

Product Number: P-6001, P-6100, P-M600, S-6000

High Background: **Resulting in overall membrane background.**

Cross reactivity between blocking agent and primary antibody:
Can be eliminated with the addition of detergent (Tween-20) to the washing buffer or incubation buffers. If background persists, changing the blocking agent is recommended.

To few wash steps:
Washing the membrane between incubation steps is essential in reduction of background signal caused from non-specific binding of the primary and/or secondary antibodies to the membrane. Increasing the number and/or the length of the each wash step can help to reduce the background. Note: we suggest many short washing steps over a few long ones.

Concentrations of either primary and/or secondary antibody are too high:
High primary and/or secondary antibody concentrations increase non-specific binding to the membrane. Determining the ideal concentrations of primary and/or secondary antibodies can reduce background signal caused from non-specific binding.

Incubation times of either primary and/or secondary antibodies steps are too long:
The longer the incubation time of the primary and/or secondary antibodies, the greater the non-specific binding. If long incubation times are necessary to increase protein binding, you might try raising the incubation temperature (eg. to 37 °C) instead.

Membrane allowed to dry between or during incubation steps:
Care should be taken, between and/or during incubation steps, to keep the membrane from drying out.

Little or No Signal: **Resulting in little or no signal over membrane background.**

Detergent is too harsh:
SDS, Nonidet, P-40, and Triton X-100 disrupt binding between proteins and between proteins and lipids. Tween-20 is the most commonly used and recommended detergent for washing and incubation solutions. Removal of detergent from incubation and wash steps can increase protein binding to its target, but may also cause increased non-specific binding to the membrane. We suggest removing the detergent from incubation steps before removing it from wash steps.

Inhibition of Secondary antibody HRP conjugate:
HRP labeled antibodies should not be used in the presence of sodium azide or hemoglobin. Your HRP conjugated antibody can be spotted directly onto the membrane before blocking as a positive control.

Lipid target is not recognized by lipid recognition protein:
Refer to binding specificity section.

Incubation time of film with membrane:
The amount of time that the film is exposed to the membrane can increase or decrease the amount of signal that the film is able to detect. Try longer incubation times with the film if no signal is detected.

Substrate has lost activity:
Test your chemiluminescent kit for activity. Your secondary antibody can be spotted directly onto the membrane before blocking as a control to validate your HRP conjugated antibody, as well as, the chemiluminecent substrate you are using.

Binding specificity: **Non-existent or different than what was expected.**

Detergent is too harsh:
SDS, Nonidet, P-40, and Triton X-100 disrupt binding between proteins. Tween-20 is the most commonly used and recommended detergent for washing and incubation solutions. Removal of the detergent from incubation steps has been known to change binding specificities of some protein. We do not suggest removal of detergent from wash buffers.

Blocking Buffer:
For some proteins, we have observed that using 0.1% ovalbumin (Sigma # A-5253) in TBS-T or 4% non-fat dry milk in TBS-T is a satisfactory replacement for TBS-T +3% BSA blocking solution. Use of alternative blocking solutions can result in lowered background, increased specificity, and changes in PIP binding patterns. If you use BSA, use the fatty acid free variety.

Primary binding protein has lost activity:
A positive control GST-tagged lipid recognition protein (G-1100) is available to validate the assay performance in your lab. This control can help identify if the protein you are using has lost activity towards its specified lipid.

Incubation time of film with membrane:
The amount of time that the film is exposed to the membrane can increase or decrease how specific your protein appears. This length of time is determined by how strongly the primary and/or secondary antibody binds and, therefore, may need to be optimized for your specific protein.

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Technical Data Sheet

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

Product Name: **SphingoStrips™**

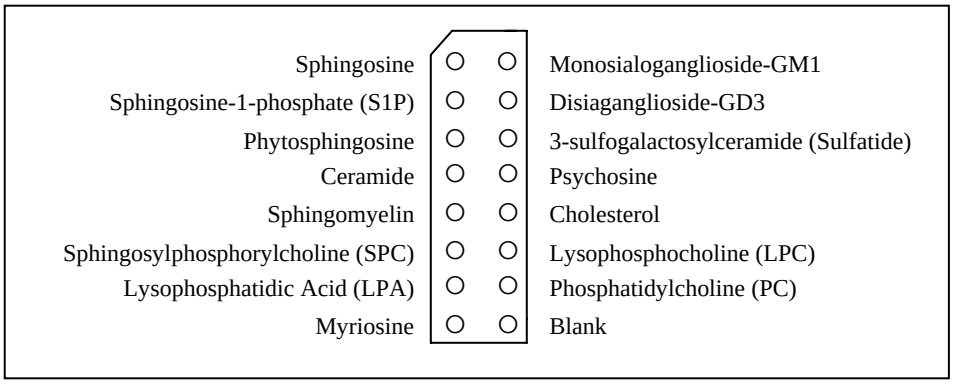
Product Number: S-6000

General Description:
SpingoStrips™ are 2 x 6 cm hydrophobic membranes that have been spotted with 15 different biologically active lipids at 100 pmol per spot. These membranes can be used to determine lipid-protein interactions through a simple protein-lipid overlay experiment. This allows researchers a convenient way to determine if their protein of interest interacts with one or more of the bound lipids.

Storage:
Store at 2-8 °C. Product is moisture and light sensitive.

Format:
The membrane has a diagonal cut on its top left corner and Ponceau S* staining to assist in orientation of the strip. See template below for location of lipids.

Membrane Template:



Suggested Usage:
See support protocol "basic Protocol for PIP strips™, PIP Array™, PIP Microstrips™, and SphingoStrips™" on our website www.echelon-inc.com

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*Final concentration of 0.1% (v/v) Ponceau S was added for accuracy during spotting.

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