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.