NEUROMICS

Standard Western Blot Protocol-Cell Lysates

This protocol is intended to provide a set of initial conditions for analysis of cell lysates samples by Western blot. Further optimization may be required for individual samples or analytes. Follow manufacturer's protocols for specific reagents when applicable.

Preparation of Cell Lysates for Western blots:

Prepare total cell lysates by solubilizing cells in an appropriate sample buffer, such as 2X SDS sample buffer (20 mM dithiothreitol, 6% SDS, 0.25 M Tris, pH 6.8, 10% glycerol, 10 mM NaF and bromophenyl blue), at approximately $2x10^6$-$1x10^7$ cells per mL. The extracts are heated in a boiling water bath for 5 minutes and then sonicated with 3-4 bursts of 5-10 seconds each.

Immunoblotting:

1. Prepare the following solutions:

Blotting Buffer	Blocking Solution	Antibody Solution
25 mM Tris, pH 7.4 0.15 M NaCl 0.1% Tween® 20	2-5% nonfat dry milk in Blotting Buffer Adjust pH to 7.4	1-5% nonfat dry milk in Blotting Buffer Adjust pH to 7.4

2. Transfer the electrophoresed proteins to a PVDF membrane and incubate for 1 hour at room temperature in Blocking Solution.
3. Incubate the membrane overnight at 4°C in Antibody Solution containing primary antibody.
4. Wash the membrane at room temperature for 30-60 minutes with 5 or more changes of Blotting Buffer.
5. Incubate the membrane for 1 hour at room temperature in Antibody Solution containing appropriate dilution of HRP-conjugated secondary antibody.
6. Wash the membrane for 30-60 minutes with 5 or more changes of Blotting Buffer.
7. Detect with Chemiluminescent Detection Substrate.
8. Expose to film and develop image.

Optimization of Immunoblotting for Cell Lysates:

A number of parameters may be modified to optimize an antibody for detection of endogenous protein levels. The objective in optimizing blotting condition is to maximize signal strength and minimize non-specific bands and background noise. The variables with the most significant impact are listed below. Optimization may be done as an initial checkerboard screen where multiple conditions are applied in a single experiment or sequentially, changing one set of parameters at a time and optimizing conditions over several blots.

Recommended starting conditions:

Antibody concentration. 0.1-0.5 microgram/mL. Adjust antibody concentration from 0.05 to 2.0 microgram/mL to obtain desired signal strength and low background.

Sample concentration. 10-20 microliter of cell lysates at $1x10^7$ cells per mL. (This is typically equivalent to 15-30 microgram of total protein). Adjust up or down to obtain desired signal strength and low background.

Blocking buffer. Start with 5% nonfat dry milk for block, and 2% nonfat dry milk for primary and secondary antibody dilution. Adjust concentration of milk up or down to obtain desired signal strength and low background. If the intensity of the target band is still too low, but background is not a problem, 1% BSA can be used as the blocking component.

NaCl concentration. Recommended concentration is 0.15M NaCl. Increasing the salt concentration in all buffers to 0.5M NaCl will reduce background. *Note: high salt can also reduce signal strength of the target protein.*

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