

SDS-PAGE and Western Blotting

1. Equipment, Solutions and Reagents
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EQUIPMENT

- Electrophoresis apparatus
- Electroblotting apparatus
- Rocker platform
- X-ray film
- Acrylamide gel of appropriate percentage (preferably pre-cast)
- Blotting membrane (e.g. nitrocellulose or PVDF)

SOLUTIONS AND REAGENTS

- TBST (Tris-Buffered Saline/TWEEN[®]-20): 10 mM Tris-HCl, pH 8.0 (Cat. No. 648310), 150 mM NaCl (Cat. No. 567440), 0.05% TWEEN[®]-20 (Cat. No. 655205)
- TBST-milk: TBST containing 5% nonfat powdered milk for monoclonal antibodies or 10% nonfat powdered milk for polyclonal antibodies; store at 4°C for \leq 1 week
- Assay buffer (chemiluminescent detection): 100 mM Diethanolamine, pH 10.0, 1 mM MgCl₂ (Cat. No. 442615), and 0.2% NaN₃
- Substrate buffer (alkaline phosphatase colorimetric detection): 100 mM Tris-HCl, pH 9.5 (Cat. No. 648310), 100 mM NaCl (Cat. No. 567440), 5 mM MgCl₂ (Cat. No. 442615)
- Alkaline phosphatase substrate solution: for each ml of substrate solution combine 1 ml of substrate buffer with 4 μ l of NBT (*p*-nitroblue tetrazolium, Cat. No. 484235) and mix; add 4 μ l of BCIP (5-bromo-4-chloro-3-indolylphosphate, Cat. No.203788) and mix again; use within 30 minutes
- Stop solution: 20 mM Tris-HCl, pH 8.0 (Cat. No. 648310), 5 mM EDTA (Cat. No. 34103)
- Loading buffer: 200 mM Tris-HCl, pH 6.8 (Cat. No. 648310), 4% SDS (Cat. No. 428015 or 428016), 20% glycerol (Cat. No. 356350), bromophenol blue, and 200 mM DTT (Cleland's reagent, Cat. No. 233155) (**NOTE: add DTT only if reducing conditions are desired**)
- Running buffer: this will depend on the gel system used; follow recommendations of the manufacturer
- Transfer buffer: this will depend on the gel transfer system used, the type of membrane, *etc.*; follow the recommendations of the manufacturer
- Protein Molecular Weight Markers (Cat. No. MW03, 69149, or 69079)

PROCEDURE

This protocol is written for colorimetric detection using alkaline phosphatase conjugated secondary reagents. Alternatively, one can use a colorimetric Western blotting system or chemiluminescent system.

1. Prepare samples for SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) by mixing an appropriate volume of sample with an equal volume of loading buffer; heat to 95°C for 5 minutes.
2. Set up the acrylamide gel in the electrophoresis apparatus with running buffer.
3. Load appropriate molecular weight markers and samples into the wells and apply electric current to separate proteins.
4. Transfer the proteins from the polyacrylamide gel onto blotting membrane using the electroblotting apparatus.
5. Rinse the membrane in TBST.
6. Block the membrane in TBST-5% milk for 30 minutes at room temperature on a rocker platform using 1 - 2 ml/cm² of membrane.
7. Incubate the membrane for 1 hour on a rocker platform at room temperature with primary antibody diluted appropriately in TBST-milk. (*Amount of primary antibody can be minimized using a container just large enough to fit the blot.*)
8. Wash the membrane with 2 changes of TBST, 10 minutes per wash, on a rocker platform.
9. Incubate the membrane for 1 hour on a rocker platform at room temperature with secondary antibody (alkaline phosphatase conjugated for colorimetric detection or horseradish peroxidase conjugated for chemiluminescent detection) diluted in TBST-milk).
10. Wash the membrane with 3 changes of TBST, 10 minutes per wash, on a rocker platform.
11. Colorimetric Detection
 - A. Incubate the membrane in alkaline phosphatase substrate solution until the bands begin to appear.
 - B. When the bands are developed to the desired intensity, stop the reaction by washing the blot in stop solution.
 - C. Air-dry the blot at room temperature.
 - D. Photograph and/or mount the blot under a plastic sheet; fading of blots will occur with time and will be accelerated by exposure to direct light; re-wetting the blot will re-intensify the bands.

12. Chemiluminescent Detection

NOTES

- The sensitivity of Western blotting is dependent, in part, on the efficiency of transfer from the polyacrylamide gel onto the membrane; in general, thinner and lower percentage gels give greater transfer efficiency and thus higher Western blotting sensitivity; overnight transfer at a lower voltage may increase the transfer efficiency; for proteins <20 kDa, use a 0.2 µm, rather than a 0.45 µm, nitrocellulose filter.
- Nonfat powdered milk is an excellent blocking agent, but other proteins, such as casein, gelatin (Cat. No. 345808), BSA, and ovalbumin, may also be used; for phosphoserine, phosphotyrosine, and phosphothreonine it is recommended to use blocking agents other than milk, as it contains numerous phospho-proteins that lead to high background.
- Membranes should be rocked during all washing and incubation steps.
- Any wash step may be extended to overnight at 4°C, if desired.

- Blocking may be carried out overnight at 4°C, if desired.
- Strips of blots corresponding to individual gel lanes can be cut with a razor blade or a scalpel and probed individually with different antibodies in separate trays.
- The concentrations of primary and secondary antibodies should be titrated to determine the optimal concentration.
- If the substrate solution develops precipitates during storage at +4°C, warm it to room temperature and mix; a sonicating water bath may also be used; a small amount of precipitate in these solutions will not harm the performance of the product; keep substrate solution away from open flames and avoid contact with skin, eyes, and mouth.
- Improved performance may be obtained using subcellular fractions for Western blotting, i.e. nuclei for nuclear proteins or membranes for membrane receptors; concentration of antigens by immunoaffinity chromatography or immunoprecipitation aids in obtaining more intense bands on Western blots.
- After visualizing the immobilized proteins, it is possible to strip the primary antibody from the membrane; a blot may be stripped several times; expect some loss of sensitivity with each stripping:
 - Stripping buffer: 62.5 mM Tris-HCl, pH 6.7(Cat. No. 648310), 100 mM β -mercaptoethanol (Cat. No. 444203), 2% SDS (Cat. No. 428015 or 428016)
 - Incubate the blot at +50°C for 30 minutes in stripping buffer.
 - Wash the membrane with 2 changes of TBST.
 - Proceed with Western blotting procedure.

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