Western Blotting (Immunoblotting)

Research Use Only

Introduction

The immunoblotting technique provides information about the presence, relative molecular weight, and/or quantity of an antigen by combining protein separation via gel electrophoresis with specific recognition of antigens by antibodies. Immunoblotting is useful when the antigen of interest is insoluble or readily degraded. Since most gel electrophoresis procedures result in denaturation of the antigen, only polyclonal and monoclonal antibodies that recognize the denatured form of an antigen can be utilized in immunoblotting. To study proteins that are expressed at very low levels, it is recommended that immunoprecipitation be followed by immunoblotting for more sensitive detection.

Useful websites

Westernblotting.org (http://www.westernblotting.org/)
This website is a western blot methods resource with access to blotting protocols, troubleshooting, research articles, etc.

Protocol Online (http://www.protocol-online.org/prot/Molecular_Biology/Protein/Western_Blotting/index.html)
Protocol Online is a database of research protocols contributed by researchers worldwide.

Molecular Station (http://www.molecularstation.com/protocol-links/detail/link-3413.html)
Molecular Station is a website that was started by a group of graduate students and scientists to provide a site of compiled resources for researchers in molecular biology and science in general.

Western Blotting

Materials

- Nitrocellulose or PVDF membrane (0.22 or 0.45µm)
- Primary antibody
- Secondary antibody conjugated to horseradish peroxidase (HRP) - If using mouse, rabbit, goat, or sheep IgG primary antibody
- Chemiluminscent-HRP Substrate System
- X-Ray film

Buffers

- Blocking Buffer
- TBST-T
- Antibody Binding Buffer (if necessary)
- Sample Loading Buffer

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Instruments

- Gel electrophoresis system
- Protein transfer apparatus
- Rocking platform
- Film processor

Experimental Procedure

1. Prepare samples/controls and resolve proteins via SDS-PAGE.
   Note: Typically samples are mixed with Sample Loading buffer that contains SDS, glycerol, and dye. Please consider whether reducing agents and/or protease or phosphatase inhibitors are needed. In addition, it is best to optimize the denaturation step as some proteins should be heated at 60°C rather than the typical 100°C.
2. Transfer proteins from the gel onto a nitrocellulose or PVDF membrane. For best results, follow the instructions provided by the manufacturer of the transfer system.
3. **Optional:** To determine whether the proteins have been transferred to the membrane, stain with a 0.1% Ponceau S solution. Protein bands can be visualized after staining for 5 minutes. To remove the Ponceau S stain, rinse with distilled water or TBS-T until most of the dye is removed. Residual dye will not affect subsequent steps.
4. Place the membrane into blocking buffer (enough to cover the membrane) and incubate for 2 hours at room temperature or overnight at 4^0C on a rocking platform.
5. Remove the blocking buffer and rinse the blot with TBS-T.
6. Prepare the primary antibody in Antibody Binding Buffer or Blocking Buffer as recommended by the supplier. If recommended concentration is unknown, use a standard concentration of 1-2 µg/mL. If using hybridoma tissue culture supernatant or serum for immunoblotting, preliminary experiments should be performed to evaluate whether dilution is needed for best results.
7. Incubate the blot with primary antibody for at least 2 hours or overnight at 4°C on a rocking platform.
   Note: Shorter times should be determined empirically for optimal results.
8. After the overnight incubation with primary antibody, wash the blot at least three to five times for a minimum of 5 to 10 minutes in TBS-T.
   Prepare the secondary antibody as recommended by the supplier in the Antibody Binding Buffer or Blocking Buffer.
   Note: Please avoid the presence of sodium azide in this step as it is deleterious to the HRP enzyme.
9. Incubate the blot with secondary antibody for one hour at room temperature on a rocking platform.
10. Wash the blot at least three to five times for at least 10 minutes in TBS-T.
11. Incubate the blot with the Chemiluminescent-HRP substrate according to the manufacturer's instructions.
12. Expose the blot to X-ray film for the appropriate time period that yields desired results. For best results, expose for ten seconds, one minute, five minutes and 20 minutes.

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Buffer Recipes

2X SDS Reducing Sample Loading Buffer (containing 50 mM DTT)

- 950 mL of 2X SDS sample buffer
- 50 mL of 1M DTT

Note: Use within 1 hour and discard remainder.

TBS-Tween (TBS-T):

- 25 mM Tris-HCl, pH 8.0
- 125 mM NaCl
- 0.1% Tween 20

Blocking Buffer:

- 5% nonfat dry milk in TBS-T

Antibody Binding Buffer:

- 1% nonfat dry milk in TBS-T

References

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