Western Blot

BETHYL LABORATORIES, INC

Western Blot Protocol
Reagents Needed:

20X Running Buffer

Tricine (free base)	71.7 g
Tris (free base)	72.6 g
SDS	10.0 g
Sodium Bisulfite	2.5 g

Adjust to 500 ml with ultra pure water.
Store at 4°C. For 1X Running Buffer, add 10 ml of 20X Running Buffer to 190 ml of distilled water.

10X Transfer Buffer

Tris (free base)	15.2 g
Glycine	72.1 g
SDS	5.0 g

Ultra pure water to 500 ml
10X Transfer Buffer is available from PAGEgels (Cat# CB82500)
Store at 4°C.

1X Transfer Buffer

10X Transfer Buffer	50 ml
Methanol	100 ml
Distilled water	350 ml

Make fresh for each use.

5% non-fat dry milk in TBST

Carnation non-fat dry milk	50 g
TBST	1 liter

TBST (Tris Buffered Saline with Tween 20, pH8.0)

Tris	6.1 g
NaC l	8.68 g
Tween-20	500 mcl

Adjust the volume to 1 liter with distilled water. Adjust pH to 8.0 with HCL.
Store at 4-25°C.

Electrophoresis in SDS-Polyacrylamide Gel and Transfer to Nitrocellulose:
Many of the reagents for these procedures are commercially available. Sources that are preferred by Bethyl Laboratories, Inc. are:
Polyacrylamide gels (4-12% Tricine), running buffer and transfer buffer from PAGEgel (San Diego, CA).
SeeBlue2 and HiMark molecular weight markers - Invitrogen (Carlsbad, CA).
Nitrocellulose membranes - Invitrogen (Carlsbad,CA).

1. Cut open the package that contains the gel cassette and drain away the buffer.
2. Rinse the wells with distilled water.
3. Rinse the wells with fresh 1x running buffer.
4. Place the gels on the buffer core so that the shorter plates face inward. If only using one gel, use a buffer dam to seal the other side.
5. Fill the inner core with fresh 1X running buffer. If there are no leaks, fill the outer core with running buffer. Load samples.
6. Run the gels at 150V until the dye front reaches the bottom of the gel (approximately 60 minutes).
7. Soak nitrocellulose membrane and blotting paper in 1X transfer buffer for at least 5 minutes prior to opening gel cassette.
8. Open gel cassettes and place the gel on the nitrocellulose membrane sandwiched between two pieces of blotting paper.
9. Place in transfer apparatus and fill with fresh 1X transfer buffer.
10. Run transfer apparatus for 60-75 minutes on 35V.

Western Blotting:

1. Remove the membrane from the transfer apparatus and place in 20 ml of 5% non-fat dry milk in TBST for one hour, with gentle shaking.
2. Dilute the primary antibody in 15 ml of 5% non-fat dry milk in TBST. For best results, the optimal dilution of antibody should be empirically defined.
3. Incubate the membrane in diluted primary antibody for two hours to overnight with gentle shaking at room temperature.
4. Wash the membrane three times, 10 minutes each time in TBST.
5. Dilute the secondary HRP conjugated antibody (Bethyl anti-Rabbit IgG-HRP Cat. # A120-101P or Bethyl anti-Goat IgG-HRP Cat. # A50-100P) in 15 ml of reconstituted 5% non-fat dry milk in TBST. For best results, the optimal concentration of the secondary HRP conjugated antibody should be empirically defined.
6. Incubate the membrane in diluted anti-Rabbit IgG-HRP Conjugate
7. or anti-Goat IgG-HRP Conjugate for 60 minutes.
8. Wash as directed in step 4.
9. Develop blots with substrate solution and place in plastic membrane protector.
10. Expose membrane to film or CCD camera.

Western Blot Protocol using Biotin conjugated Antibody

Follow general Western Blot Protocol up to gel transfer step and proceed as follows:

1. Remove the membrane from the transfer apparatus and place in 20 ml of 1% non-fat dry milk in TBST for one hour at room temperature, with gentle shaking.
2. Wash the membrane three (3) times for 5 minutes each in TBST.
3. Dilute the primary biotin-conjugated antibody in 15 ml of 1% non-fat dry milk in TBST. For best results, the optimal dilution of antibody should be empirically defined.
4. Incubate the membrane in diluted primary antibody for two hours to overnight with gentle shaking at room temperature.
5. Wash the membrane three (3) times for 10 minutes each in TBST.
6. Dilute streptavidin-HRP conjugate (Thermo Scientific, Rockford, IL, Prod# 21130) in 15 ml of 1% non-fat dry milk in TBST. Typical dilutions are in the range of 1:5000 to 1:15,000 of a 1 mg/ml stock. For best results, the optimal concentration of streptavidin-HRP should be empirically defined.
7. Incubate the membrane in diluted streptavidin-HRP at room temperature for 60 minutes.
8. Wash as directed in step 5.
9. Develop blots with substrate solution (e.g. Super Signal West Dura, Thermo Scientific Cat# 34075) and place in plastic membrane protector.
10. Expose membrane to film or CCD camera.

Western Blot FAQs

What protocol was used to perform the blot shown in the data sheet of the Bethyl antibodies?
All of Bethyl's antibodies are tested using the reagents from the ReliaBLOT® kit. A ReliaBLOT protocol and western blot protocol can be found in the Protocols Section of the Bethyl website.

If I don't use the ReliaBLOT® kit, what blocking buffer should I use?
The recipe for ReliaBLOT® blocking buffer is proprietary. An acceptable substitute would be 5% milk (Carnation, non-fat, dry)/TBS/0.5% tween. A protocol for Western Blotting can be found in the Protocols Section of the Bethyl website.

Can you give me the exact sequence of the peptide used as the immunogen to generate the antibody?
The sequences of the immunogens are proprietary. All of the data sheets on our polyclonal antibodies provide a range of amino acids in the protein to which the peptide maps.

Why is my band/signal not migrating at the same size that you show in your data sheet?
1. The migration patterns of molecular weight markers can differ between vendors.
2. The type of gel and buffer system used can have effects on protein mobility.

Why do some proteins not migrate at the predicted molecular weight?
1. Proteins that are highly basic or acidic (charged) may not migrate at their theoretical molecular weight.
2. Sometimes proteins will retain some secondary structure (re-folding) which can alter mobility.
3. Post-translational modifications will alter protein mobility.