Protocol

GOLDBIO®

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Western Blotting and Immunodetection Protocol

Introduction

Immunoblotting (also called Western Blotting) is used extensively in different protein studies aiming to characterize specific proteins, their interactions, and their modifications. This assay allows the detection of specific antigens by recognizing them with polyclonal or monoclonal antibodies. First, protein samples are solubilized, the antigen is separated by SDS-PAGE and transferred to a membrane, which is then probed with primary and secondary antibodies. Finally, antibody-antigen complexes are identified with chromogenic or luminescent substrates. This protocol includes all the steps following a 1 or 2 dimensional electrophoresis, including solution recipes, to allow for a clear immunoblot.

Materials

For Protein Blotting with Tank Transfer Systems

- Sample for analysis
- Protein molecular weight standards, compatible with colorimetric and fluorescent detection methods
- Transfer buffer
- Gloves
- Sponge
- 3mm filter paper
- Transfer membrane - nylon or nitrocellulose
- Electroblotting apparatus
- Indelible pen or soft pencil

For Immunoprobing with Directly Conjugated Secondary Antibody

- Transferred proteins from Protein Blotting
- Blocking buffer appropriate for membrane detection protocol (see recipe)
- Primary antibody specific for protein of interest
- Tris-Buffered Saline and Tween-20 (TTBS) (for nitrocellulose or PVDF) or Tris-buffered saline (TBS) (for nylon)
- Secondary antibody conjugate: species-specific anti-Ig conjugated to horseradish peroxidase or alkaline phosphatase
- Heat-sealable plastic bag
- Orbital shaker *or* rocking platform
- Powder-free gloves

- Plastic Box

For Chromogenic Substrate Visualization

- Membrane with transferred proteins and probed with antibody-enzyme complex.
- TBS
- Chromogenic visualization solution
- Additional reagents and equipment for gel photography

Staining Reagents

One of the following is needed (depending on application, see Protein Blotting, III.4):

- Coomassie blue (GoldBio Catalog # C-460)
- Ponceau S (GoldBio Catalog # P-330)
- India ink
- Naphthol blue
- Colloidal gold

Preparation of Transfer buffer

- Add 18.2 g Tris base (GoldBio Catalog # T-400) and 86.5 g glycine (GoldBio Catalog # G-630) to 4 liters of water.
- Add 1200 ml methanol and bring to 6 liters with water.

Note: The pH of the solution is ~8.2 to 8.4. For use with PVDF filters, decrease methanol concentration to 15%; for nylon filters, omit methanol altogether.

Preparation of blocking buffer

- For Colorimetric Detection –

  Nitrocellulose and PVDF: 0.1% (v/v) Tween-20 in Tris-buffered saline (TTBS). Store up to 1 week at 4°C.

  Neutral and positively charged nylon: Tris-buffered saline (TBS) containing 10% (w/v) nonfat dry milk, prepared just before use.
- For Luminescent Detection –

  Nitrocellulose, PVDF and neutral nylon: With constant mixing, add 0.2% casein to 65°C TTBS. Stir for 5 minutes, cool before use. Prepare just before use.

  Positively charged nylon: With constant mixing, add 6% (w/v) casein and 1% (v/v) polyvinyl pyrrolidone (PVP) to 65°C TTBS. Stir for 5 minutes. Cool before use, and prepare just before use.

Alkaline Phosphate substrate buffer

- 100mM Tris-HCl (GoldBio Catalog # T-095) at pH 9.5
- 100mM NaCl

- 5mM $MgCl_2$

Dioxetane phosphate substrate buffer

- 1mM $MgCl_2$
- 0.1M diethanolamine
- 0.02% sodium azide (optional)
- Adjust to pH 10 with HCl and use upon preparation.

TTBS and TBS

For TTBS:

- 50mM Tris-HCl
- 150mM NaCl
- 0.05% Tween 20

For TBS:

- 50mM Tris-Cl at pH 7.5
- 150mM NaCl

Visualization Solution

For NBT stock

- 50 mg/ml nitroblue tetrazolium (NBT) (GoldBio Catalog # NBT) in 70% N,N-dimethylformamide (DMF). Store < 1 year at 4°C.

For BCIP stock

- 50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (GoldBio Catalog # B-500) in 100% DMF. Store < 1 year at 4°C.
- Mix 33 µl NBT stock and 5 ml alkaline phosphate substrate buffer. Add 17 µl BCIP stock and mix. Stable for 1 hour at room temperature.

Substrate Specific Visualization Solutions

The visualization solution used depends on the system undergoing Immunoblotting. Reference Table 2 to evaluate which one is right for your procedure.

For 4CN:

Mix 20 ml ice-cold methanol with 60 mg 4-chloro-1-naphthol (4CN) (GoldBio Catalog # C-273). Separately mix 60 µl of 30% H_2O_2 with 100 ml TBS at room temperature. Rapidly mix the two solutions and use immediately.

For DAB/$NiCl_2$:

- 5 ml 100mM Tris-HCl at pH 7.5
- 100 µl 3,3'–diaminobenzidine (DAB) (GoldBio Catalog # D-320) stock (40 mg/ml in H_2O, stored in 100 µl aliquots at -20°C)
- 25 µl $NiCl_2$ stock (80 mg/ml in H_2O, stored in 100 µl aliquots at -20°C)
- 15 µl 3% H_2O_2

- Mix prior to use.

CAUTION: HANDLE DAB *CAREFULLY*; WEARING GLOVES AND MASK, AS IT IS A CARCINOGEN.

For Luminol

- 0.5 ml 10x luminol (GoldBio Catalog # L-145) stock (40 mg luminol in 10 ml DMSO)
- 0.5 ml 10x *p*-iodophenol stock
- 2.5 ml 100mM Tris-Cl at pH 7.5
- 25 µl 3% H_2O_2
- H_2O to 5 ml
- Prepared immediately prior to use.

For Ponceau S

- Dissolve 0.5 g Ponceau S (GoldBio Catalog # P-330) in 1 ml glacial acetic acid. Bring to 100 ml with water. Prepare immediately prior to use.

Method

Electrophorese the protein sample

1. Prepare antigenic samples and separate proteins using small or standard sized one or two-dimensional gels. Include pre-stained or biotinylated protein molecular weight standards in one or more gel lanes.

Note: The protein makers will transfer to the membrane and conveniently indicate membrane orientation and sizes of proteins after immunostaining.

Assemble the immunoblot sandwich

1. When electrophoresis is complete, disassemble gel sandwich and remove stacking gel. Equilibrate the gel 30 minutes at room temperature in transfer buffer.

Note: Use gloves when manipulating any apparatuses to avoid hand oil blocking transfer.

Note: Match the appropriate transfer buffer to the membrane.

Note: Gel equilibration is required to prevent a change in the size of the gel during transfer.

Note: Any shift in gel dimension will result in a blurred transfer pattern.

2. Assemble transfer sandwich in a tray large enough to hold the plastic transfer cassette. Fill with transfer buffer so that cassette is covered.

Note: The transfer cassette should be assembled under buffer to minimize trapping of air bubbles.

3. On the bottom half of the plastic transfer cassette, place a sponge, followed by a sheet of filter paper cut to the same size as the gel, then prewet with transfer buffer.

4. Place the gel on top of filter paper; the side of the gel touching the paper arbitrarily becomes the cathode side of the gel (i.e., ultimately toward the negative electrode when positioned in the tank). Remove any air bubbles between gel and filter paper by gently rolling a test tube or glass rod over the surface of the gel.

    Note: Any bubbles between the filter paper, gel and membrane will block current flow and prevent protein transfer. This problem is indicated on the membrane by sharply defined white areas devoid of transferred protein.

    Note: Proteins are usually negatively charged in transfer buffer and move toward the positive anode. However, some proteins may be positively charged. An additional membrane placed on the cathode side of the gel will bind these proteins.

5. Prepare the transfer membrane. Cut membrane to same size as gel plus 1 to 2 mm on each edge. Place into distilled water slowly, with one edge at a 45° angle so that the water wicks up into the membrane, thereby wetting the entire surface. Equilibrate for 10-15 minutes in transfer buffer.

    Note: If the membrane is placed into the water too quickly, air will be trapped and will appear as white blotches in the membrane. Protein will not transfer onto these areas.

    Note: This wetting procedure works for nitrocellulose and nylon membranes only. PVDF membranes are hydrophobic and will not wet simply from being placed into distilled water or transfer buffer. For these membranes, first immerse 1 to 2 sec on 100% methanol, then equilibrate 10 to 15 minutes with transfer buffer. Do not let membrane dry out at any time. If this occurs, wet once again with methanol and transfer buffer as described above.

6. Moisten surface of gel with transfer buffer. Place prewetted membrane directly on the top side of the gel (i.e. anode side). To avoid bubbles, start by placing one corner of the membrane on the gel and slowly lower the rest of the membrane onto the gel. Remove all air bubbles as in step 4.

    Note: Poor contact between the gel and membrane will cause a swirled pattern of transferred proteins on the membrane. Some proteins will transfer as soon as the gel is placed on the membrane; repositioning the gel or membrane can result in a smeared or double image on the developed blot.

Note: The use of 0.2 µm membranes may improve retention of smaller-molecular-weight proteins.

7. Wet another piece of filter paper, place on anode side of the membrane and remove all air bubbles. Place another sponge on top of this filter paper.

8. Complete assembly by locking the top half of the transfer cassette into place.

Note: It is important to orient the sandwich so that the membrane faces the anode (positively charged) side of the tank.

Transfer proteins from gel to membrane

1. Fill the tank with transfer buffer and place transfer cassette containing sandwich into electroblotting apparatus in correct orientation. Connect leads of power supply to corresponding anode and cathode sides of electroblotting apparatus.

Note: Transfer buffer should cover the electrode panels but should not touch the base of the banana plug.

2. Electrophoretically transfer proteins from gel to membrane for 30 minutes to 1 hour at 100 V with cooling (ice) or overnight at 14 V (constant voltage) in a cold room.

Note: Transfer time is dependent on the thickness and the percent acrylamide of the gel, as well as the size of the protein being transferred. In general, proteins are transferred within 1 to 6 hours, but high-molecular-weight molecules may take longer. Overnight transfer at low voltage is reliable and convenient. Cooling (at 10° to 20°C) is required for transfers > 1 hour at high power. Heat exchanger cooling cores using a circulating water bath are placed into the transfer unit for cooling.

3. Turn off the power supply and disassemble the apparatus. Remove the membrane from the blotting apparatus and note the orientation by cutting a corner or marking with a soft pencil or pen.

Note: Ensure ahead of time that the ink remains on the membrane by testing.

Note: Membranes can be dried and stored in a resealable plastic bags at 4°C for 1 year or longer at this point. Prior to further processing, dried PVDF membranes must be placed into a small amount of 100% methanol to wet the membrane, then in distilled water to remove the methanol.

4. Stain the gel for total protein with Coomassie blue to verify transfer efficiency. If desired, visualize transferred proteins on nitrocellulose or PVDF membranes by staining reversibly with Ponceau S or irreversibly with Coomassie blue, India ink, naphthol blue or colloidal gold.

    Note: These staining procedures are incompatible with nylon membranes.

    Note: If membrane shows significant staining on the backside, either the gel was heavily overloaded or the membrane has poor protein-binding capacity. In either case, protein-binding sites on the side facing the gel are saturated, allowing protein to migrate to the other side of the membrane. Nitrocellulose, in particular, will show diminished binding capacity with age or poor storage conditions (e.g., high temperature or humidity). In addition, some proteins simply do not bind well to a particular matrix. By using several membrane sheets in place of one, the protein can be detected as it passes through each consecutive sheet. This will give an indication of how efficiently the membrane binds to a particular protein.

5. Proceed with immunoprobing and visual detection of proteins.

Immunoprobing with Directly-Conjugated Secondary Antibody

In this procedure, the immobilized proteins on the membrane are probed with specific antibodies to identify any antigens present.

1. Place the membrane in heat-sealable plastic bag with 5 ml blocking buffer and seal the bag. Incubate for 30 minutes to 1 hour at room temperature with agitation on an orbital shaker or rocking platform.

    Note: Usually 5 ml buffer is sufficient for 2-3 membranes (14 x 14 cm size). If the membrane is to be stripped and reprobed, the blocking buffer must contain casein or nonfat dry milk.

    Note: Plastic incubation trays are often used in place of heat-sealable bags, and can be especially useful when processing large numbers of strips in different primary antibody solutions.

2. Calculate the amount of primary antibody to use. Dilute primary antibody in blocking buffer.

    Note: Primary antibody dilution is determined empirically, but is typically 1/100 to 1/1000 for a polyclonal antibody, 1/10 to 1/100 for hybridoma supernatants and ≥ 1/1000 for murine ascites fluid. Ten to one-hundred-fold higher dilutions can be used with AP or luminescence-based detection systems. To determine the appropriate antibody concentration, a dilution series is easily performed with membrane strips. Separate antigens on a preparative gel (i.e. a single large sample well) and immunoblot the entire gel. Cut 2- to 4-mm strips by hand or with a membrane cutter and incubate individual strips in a set of serial dilutions of primary antibody. The correct dilution should give low background and high specificity.

Note: Browse GoldBio antibody collection for a large variety of primary antibodies for your research. For all GoldBio primary antibodies, use our convenient Antibody Dilution Tool to calculate the volume you need.

Note: Both primary and secondary antibody solutions can be used at least twice, but long-term storage (i.e., > 2 days at 4°C) is not recommended.

3. Open bag and pour out blocking buffer. Replace with diluted primary antibody and incubate 30 minutes to 1 hour at room temperature with constant agitation.

Note: Usually 5 ml diluted primary antibody solution is sufficient for 2-3 membranes (14 x 14 cm size). Incubation time may vary depending on conjugate used.

Note: When using plastic trays, the primary and secondary antibody solution volume should be increased to 25 to 50 ml. For membrane strips, incubation trays with individual slots are recommended. Typically, 0.5 to 1 ml solution per slot is needed.

4. Remove the membrane from plastic bag with gloved hand. Place in plastic box and wash four times by agitating with 200 ml TTBS (nitrocellulose or PVDF) or TBS (nylon) 10 to 15 minutes each time.

5. Dilute secondary antibody conjugate in blocking buffer.

Note: Commercially available enzyme-conjugated secondary antibody is usually diluted 1/200 to 1/2000 prior to use (Harlow and Lane, 1999).

6. Place the membrane in new heat-sealable plastic bag, add diluted secondary antibody conjugate, and incubate 30 minutes to 1 hour at room temperature with constant agitation.

Note: When using plastic incubation trays, see step 3 annotation for proper antibody volumes.

7. Remove the membrane from bag and wash as in step 4. Develop according to appropriate visualization protocol.

Visualization of Bound Antigen with Chromogenic Substrates

Table 2 provides a list of Chromogenic and Luminescent Visualization Systems for your specific system under examination.

1. If the final membrane wash was performed in TTBS, wash the membrane 15 minutes at room temperature in 50 ml TBS.

Note: The Tween-20 in TTBS interferes with 4CN development.

2. Place the membrane into chromogenic visualization solution.

Note: Bands should appear in 10 to 30 minutes.

3. Terminate the reaction by washing the membrane in distilled water. Air dry and photograph for a permanent record.

Table 1. Protein Standard and Applications

Protein Standard	Application
Unstained	Molecular weight calibration and transfer efficiency; can be visualized with total protein stains.
Tagged	Molecular weight calibration and transfer efficiency; visualized during immunodetection steps; a variety of potential tags, including biotinylated and antibody-specific amino acid sequence engineered into standard proteins.
Prestained	Excellent for checking transfer efficiency and visually inspecting the blot; typically does not produce as sharp a band as other standards, making precise molecular weight calculations difficult.

Table 2. Chromogenic and Luminescent Visualization Systems

System	Reagent	Reaction/Detection	Comments
Chromogenic Substrates			
HRP-Based	4CN (GoldBio Catalog # C-273)	Oxidized products form purple precipitate	Not very sensitive (inhibited by Tween 20); fades rapidly upon light exposure.
	$DAB/NiCl_2$	Forms dark brown precipitate	More sensitive than 4CN but potentially carcinogenic; resulting membrane easily scanned.
	TMB	Forms dark purple stain	More stable and less toxic than $DAB/NiCl_2$; can be used with all membrane types.
AP-based	BCIP/NBT (GoldBio	BCIP hydrolysis produces indigo precipitate after	More sensitive and reliable than other AP-precipitating

	Catalog # B-500 and NBT)	oxidation with NBT; reduced NBT precipitates; dark blue-gray stain results	substrates; note that phosphate inhibits AP sensitivity.

Luminescent Substrates

HRP-based	Luminol/H_2O_2/ *p*-iodophenol (GoldBio Catalog # L-830 or L-145)	Oxidized luminol substrate gives off blue light; *p*-iodophenol increases light output	Very convenient, sensitive system; reaction detected in seconds to 1 hour.
AP-based	Substituted 1,2-dioxetane-phosphates (e.g., AMPPD, CSPD, Lumigen)	Dephosphorylated substrate gives off light	Protocol described gives reasonable sensitivity on all membrane types. Consult instructions of reagent.
	AquaSpark™ Broad Range Phosphatase Substrate (GoldBio Catalog # AQ-105)	AquaSpark™ Broad Range Phosphatase Substrate gives off light in the presence of phosphatase activity.	Shows higher signal intensity than other luminogenic alkaline phosphatase substrates, single-reagent formula, works under various pH conditions, and is water soluble.
	AquaSpark™ Alkaline Phosphate Substrate (GoldBio Catalog # AQ-100)	AquaSpark™ Alkaline Phosphatase Substrate gives off light in the presence of alkaline phosphatase activity.	Higher sensitivity and efficiency than currently existing probes. Work as single agent. Suitable for Western Blotting or nucleic acid blotting procedures.

Associated Products

- Coomassie blue (GoldBio Catalog # C-460)
- Ponceau S (GoldBio Catalog # P-330)
- Tris base (GoldBio Catalog # T-400)
- Tris-HCl (GoldBio Catalog # T-095)
- Nitroblue tetrazolium (NBT) (GoldBio Catalog # NBT)
- 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (GoldBio Catalog # B-500)

- 4-chloro-1-naphthol (4CN) (GoldBio Catalog # C-273).
- 3,3'–diaminobenzidine (DAB) (GoldBio Catalog # D-320)
- Luminol (GoldBio Catalog # L-830) or Luminol, sodium salt (GoldBio Catalog # L-145)
- Glycine (GoldBio Catalog # G-630)
- AquaSpark™ Broad Range Phosphatase Substrate (GoldBio Catalog #AQ-105)
- AquaSpark™ Alkaline Phosphatase Substrate (GoldBio Catalog # AQ-100)

References

Gallagher, S. (2011). Immunoblotting and immunodetection. *Current protocols in cell biology*, 6-2.