Detection of Proteins on Blot Membranes

Staining of blot transfer membranes permits visualization of proteins and allows the extent of transfer to be monitored. In the protocols described in this unit, proteins are stained after electroblotting from one-dimensional or two-dimensional polyacrylamide gels to blot membranes such as polyvinylidene difluoride (PVDF), nitrocellulose, or nylon membranes (UNIT 10.7). PVDF is the preferred, more universal membrane and is emphasized here; however, most stains work similarly on nitrocellulose, and many can be used on alternative blotting membranes.

The first six Basic Protocols describe the use of six general protein stains—amido black, Coomassie blue, Ponceau S, colloidal gold, colloidal silver, and India ink. In addition, the fluorescent stains fluorescamine and IAEDANS, which covalently react with bound proteins, are described in Basic Protocol 7 and the Alternate Protocol. Table 10.8.1 lists approximate detection limits for each nonfluorescent stain as well as membrane compatibilities. The dimensional stability of blotted membranes facilitates direct, precise comparisons of staining patterns using different detection methods. For example, protein staining can be directly compared with immunoreactivity by staining one portion of a blot with a general protein stain while subjecting another portion containing duplicate samples to immunoblotting. When it is desirable to cut replicate lanes from the blot prior to any staining, prestained standards provide very useful visual reference points.

**NOTE:** High-purity water (from a Milli-Q purification system or equivalent) should be used throughout the protocols, and all plastic and glass boxes must be thoroughly cleaned before use to avoid staining artifacts. Membranes should be handled only by the edges with forceps. All steps should be performed at room temperature (unless otherwise described) and with gentle agitation. Use of an orbital shaker is recommended for steps that take longer than 1 min. If a PVDF membrane is allowed to dry after transfer, wet for 5 sec in 100% methanol and rinse with water before staining. Volumes of stain, destain, and wash solutions should be sufficient to cover the membrane and allow it to float freely. Unless noted otherwise, solutions may be stored for several months at room temperature.

### Table 10.8.1 Staining Sensitivities and Membrane Compatibilities for Nonfluorescent Stains

<table>
<thead>
<tr>
<th>Stain</th>
<th>Minimum amount detected</th>
<th>Membrane type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PVDF</td>
</tr>
<tr>
<td>Amido black</td>
<td>50 ng</td>
<td>+</td>
</tr>
<tr>
<td>Coomassie blue</td>
<td>50 ng</td>
<td>+</td>
</tr>
<tr>
<td>Ponceau S</td>
<td>200 ng</td>
<td>+</td>
</tr>
<tr>
<td>Colloidal gold</td>
<td>2 ng</td>
<td>+</td>
</tr>
<tr>
<td>Colloidal silver</td>
<td>5 ng</td>
<td>+</td>
</tr>
<tr>
<td>India ink</td>
<td>5 ng</td>
<td>+</td>
</tr>
</tbody>
</table>

*a* Sensitivity of fluorescent stains is very dependent on protein amino acid composition.

*b* Minimum amount detected based on amount of protein loaded onto gel. The actual amount on the blot will be slightly lower because of losses during electrotransfer. Values are based on use of a full-sized gel (11 cm × 16 cm × 1.5 mm). Sensitivity will be ~2 to 5 times higher when minigels (8 cm × 10 cm × 1.0 mm) are used because the protein bands are concentrated on a smaller area of membrane.

*c* + indicates stains well; − indicates membrane not compatible.
AMIDO BLACK STAINING

Amido black is used to stain proteins on blot transfer membranes. Transferred proteins (>50 ng/band) appear as dark blue bands on a light blue background. Amido black has a sensitivity similar to that of Coomassie blue, but it stains faster. It is the preferred stain for protein sequencing and in situ cleavage of proteins for determining internal sequences because the mild staining and destaining conditions minimize the likelihood that any protein will be extracted during the procedures.

**Materials**

- Protein sample electroblotted to PVDF, nitrocellulose, or nylon membrane
- Amido black 10B stain: 0.1% (w/v) amido black (naphthol blue black 10B, Sigma) in 10% (v/v) acetic acid
- 5% (v/v) acetic acid
- Plastic boxes

1. Place blot transfer membrane in a plastic box and wash with water three times for 5 min each.
2. Stain membrane with amido black 10B stain for 1 min.
   
   *Longer staining times only increase background and hence decrease sensitivity.*
3. Destain the membrane with 5% acetic acid twice for 1 min each.
4. Rinse the membrane with water twice for 10 min each, then air dry.

COOMASSIE BLUE R-250 STAINING

Coomassie blue R-250 can be used with most types of blot membranes except nitrocellulose (high concentrations of organic solvents can dissolve nitrocellulose membranes). Coomassie blue has a similar sensitivity to amido black. Coomassie blue–stained proteins (>50 ng/band) appear as dark blue bands against a light blue background. The sequence of washing with water, staining, and destaining is similar to that for amido black staining (Basic Protocol 1), but the staining step is lengthened to 5 min.

**Materials**

- Blot transfer membrane *(UNIT 10.7)*
- Coomassie blue stain: 0.025% (w/v) Coomassie brilliant blue R-250 (Bio-Rad) in 40% methanol/7% acetic acid (v/v)
- 50% methanol/7% acetic acid (v/v)
- Plastic box

1. Place blot transfer membrane in a clear plastic box. Wash with water three times for 5 min each.
2. Stain membrane with Coomassie blue stain for 5 min.
   
   *If proteins on PVDF membranes are to be subjected to N-terminal sequencing, acetic acid should be omitted from the staining and destaining solutions to minimize protein extraction.*
3. Destain membrane with 50% methanol/7% acetic acid for 5 to 10 min.
4. Rinse with water several times, then air dry.
PONCEAU S STAINING

Ponceau S is the least sensitive general protein stain described here. Transferred proteins (>200 ng/band) appear as red bands on a pink background. Major advantages of Ponceau S staining are that it is simple, rapid, and reversible. If desired, essentially all of the stain can be removed by extended destaining as described in steps 4 to 6. This can be particularly advantageous if the blot is to be reused after initial protein staining for a second detection method such as immunoblotting.

Materials

- Blot transfer membrane (UNIT 10.7)
- Ponceau S stain: 0.5% (w/v) Ponceau S (Sigma) in 1% (v/v) acetic acid
- 200 μM NaOH/20% (v/v) acetonitrile
- Plastic box

1. Place blot transfer membrane in a plastic box and wash with water three times for 5 min each.
2. Stain membrane with Ponceau S stain for 30 sec to 1 min.
3. Destain membrane with several changes of water for 30 sec to 1 min each, then air dry. If the stain is to be extracted from protein bands (steps 4 to 6) prior to employing a second detection method, omit drying.

   Do not overdestain because the protein bands will be difficult to detect. Stop destaining when the background has a very slight pink tinge.

4. Make a permanent record of the staining pattern by photocopying or photographing the blot. Alternatively, mark the positions of the bands of interest directly on the blot by overlaying the wet blot with plastic wrap or a plastic bag and using a pencil or pen to outline the bands. Press with moderate pressure to make a permanent indentation on the membrane.
5. Extract Ponceau S stain from the protein bands with 200 μM NaOH/20% acetonitrile for 1 min.
6. Wash membrane with water three times for 5 min each, then air dry.

COLLOIDAL GOLD STAINING

Colloidal gold is a highly sensitive stain. AuroDye (Amersham) is a ready-to-use commercial source of colloidal gold stain in a low-pH buffer. AuroDye should be used without dilution. Transferred proteins (>2 ng/band) will appear as red bands on a pink background. A higher signal may be obtained with alkali treatment of the membrane prior to staining by washing the membrane with 1% KOH followed by several rinses with phosphate-buffered saline (PBS). Glass rather than plastic boxes must be used to hold the membranes and solutions. Glass is easier to clean than plastic and is less likely to give artifacts. Ideally, a set of glass trays should be dedicated to the procedure.

Materials

- Blot transfer membrane (UNIT 10.7)
- Tween 20 solution: 0.3% (v/v) Tween 20 in PBS (APPENDIX 2E; prepare solution fresh weekly and store at 4°C)
- AuroDye colloidal gold reagent (Amersham; store at 4°C)
- Glass box
1. Place blot transfer membrane in a glass box. Wash with water three times for 5 min each.
2. Incubate membrane with Tween 20 solution for 30 min at 37°C with gentle agitation.
3. Wash membrane with Tween 20 solution three times for 5 min each at room temperature.
4. Rinse membrane several times with water.
5. Stain membrane with AuroDye for 2 to 6 hr (until desired color formation).
   *Use only enough stain to cover the membrane completely.*
6. Rinse membrane thoroughly with water, then air dry.

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**COLLOIDAL SILVER STAINING**

Colloidal silver is a more economical stain than colloidal gold. The staining procedure is rapid, although the ferrous sulfate solution must be prepared immediately before use. Transferred proteins (>5 ng/band) appear as black bands on a light brown background for nitrocellulose and on a dark background for PVDF.

**Materials**

- Blot transfer membrane *(UNIT 10.7)*
- 40% (w/v) sodium citrate (store at 4°C for several months)
- 20% (w/v) ferrous sulfate (FeSO₄·7H₂O), prepared fresh
- 20% (w/v) silver nitrate (store at 4°C for several months)
- Glass box

1. Place blot transfer membrane in a glass box and wash with water three times for 5 min each.
2. Add 5 ml of 40% sodium citrate and 4 ml of 20% ferrous sulfate to 90 ml water. Stir vigorously and slowly add 1 ml of 20% silver nitrate over ~1 to 2 min to form a suspension.
3. Immediately use the suspension to stain the membrane for ~5 min.
   *The staining suspension should be used within 30 min.*
4. Rinse membrane with water, then air dry.

### BASIC PROTOCOL 6

**INDIA INK STAINING**

India ink is used to stain electroblotted proteins on blot transfer membranes. Transferred proteins (>5 ng/band) appear as black bands on a gray background. Sensitivity may be enhanced by brief alkali treatment of the membrane with 1% KOH followed by several rinses with PBS.

**Materials**

- Blot transfer membrane *(UNIT 10.7)*
- Tween 20 solution: 0.3% (v/v) Tween 20 in PBS *(APPENDIX 2E; prepare solution fresh weekly and store at 4°C)*
- India ink solution: 0.1% (v/v) India ink (Pelikan 17 black) in Tween 20 solution (store 1 month at room temperature)
- Plastic box

1. Place blot transfer membrane in a plastic box. Wash with water three times for 5 min each.
2. Wash membrane with Tween 20 solution four times for 10 min each.
3. Stain membrane with India ink solution for 2 hr or overnight.
4. Rinse with water until an acceptable background is obtained, then air dry.

**FLUORESCAMINE LABELING**

Fluorescamine, or 4-phenylspiro[furan-2(3H),1'-phthalan]-3,3'-dione, is used to introduce a fluorescent label on electrophobtotted proteins via reaction with free amines. Transferred proteins are visualized on blot transfer membranes with UV light. This stain can be very sensitive and can be used in conjunction with a second detection method such as immunoblotting (also see Basic Protocol 3). However, the protein is irreversibly modified because fluorescamine reacts with available amino groups (i.e., lysines and the protein N terminus if it was not previously blocked).

**Materials**

- Blot transfer membrane *(UNIT 10.7)*
- Sodium bicarbonate solution: 100 mM sodium bicarbonate in 0.3% (v/v) Tween 20, pH 9.0 (prepare fresh weekly and store at 4°C)
- Fluorescamine stain: 0.25 mg/ml fluorescamine (Sigma) in sodium bicarbonate solution (prepare fresh daily)
- Plastic box

1. Place blot transfer membrane in a plastic box. Wash with water three times for 5 min each.
2. Wash membrane with sodium bicarbonate solution twice for 10 min each.
3. Label protein bands with fluorescamine stain for 15 min. Use enough staining solution to cover the membrane completely.
4. Wash membrane with bicarbonate solution three times for 5 min each.
5. Rinse membrane several times with water.
6. Visualize transferred proteins with UV light.

**IAEDANS LABELING**

\(N\)-iodoacetyl-\(N\)'-(5-sulfo-1-naphthyl)ethylenediamine (IAEDANS or 1,5-I-AEDANS) is used for fluorescent labeling of electrophobtotted proteins on blot transfer membranes. Because IAEDANS reacts with free cysteines, disulfides in the sample must first be reduced with dithiothreitol (DTT). Transferred protein bands are visualized under UV light.

**Additional Materials** *(also see Basic Protocol 7)*

- DTT solution: 200 mM dithiothreitol (DTT) in 100 mM Tris·Cl, pH 8.6 *(APPENDIX 2E; prepare immediately before use)*
- 100 mM Tris·Cl, pH 8.6 *(APPENDIX 2E)*
- \(N\)-iodoacetyl-\(N\)'-(5-sulfo-1-naphthyl)ethylenediamine (IAEDANS; Sigma; store desiccated in the dark at −20°C)
- Glass box

1. Place blot transfer membrane in a glass box. Wash with water three times for 5 min each.
2. Incubate membrane with DTT solution for 30 min to reduce disulfides in the sample.
3. Wash membrane with 100 mM Tris·Cl (pH 8.6) three times for 5 min each.
This washing step probably could be deleted if proteins have been electroblotted from a reducing gel (i.e., containing DTT and/or 2-mercaptoethanol). However, it is advisable to include this simple step routinely as some oxidation may occur during electrotransfer or on the blotted membrane itself during drying or storage.

4. Dissolve 86 mg IAEDANS (2 mM final concentration) in 100 ml of 100 mM Tris-Cl (pH 8.6).

   This step should be carried out in the dark (solution should be made fresh daily).

5. Add IAEDANS solution to the membrane and allow the reaction to proceed in the dark for 30 min with shaking.

6. Wash membrane with 100 mM Tris-Cl (pH 8.6) twice for 5 min each. Thoroughly rinse membrane with water to remove excess reagent.

7. Visualize the transferred proteins under UV light.

COMMENTARY

Background Information

The recent rapid expansion in the use of electrophoretic transfer of separated proteins to different types of membranes has necessitated the adaptation of existing protein staining techniques to transfer membranes. On-blot staining techniques serve multiple purposes, including detection of proteins for structural analysis and use in parallel with antibody reactivity to correlate precisely immunoreactivity with protein staining patterns. For the latter purpose an advantage of on-blot staining is that duplicate sections of membrane can be cut out and one section used for immunoblotting (UNIT 10.10) while a second section with duplicate lanes is stained with a general protein stain. The two pieces can then be precisely realigned. In contrast, a direct comparison of an immunoblot with a stained polyacrylamide gel is much less precise because of shrinking and swelling of the gel.

The most common membranes used for electroblotting are polyvinylidene difluoride (PVDF) and nitrocellulose. PVDF membranes have become increasingly popular because they are easy to handle and store, whereas nitrocellulose membranes are brittle and tend to break easily when dry. A number of PVDF membranes are now commercially available that have subtle but important differences in protein binding properties resulting from different proprietary manufacturing processes. For example, Bio-Rad Trans-Blot or Millipore Immobilon-P high-retention PVDF membranes generally show higher protein binding capacities and higher binding affinities compared to Immobilon-P (Mozdzanowski and Speicher, 1992). Use of high-retention PVDF membranes usually results in higher and more consistent electroblotting recoveries of most proteins than the use of either low-retention PVDF membranes or nitrocellulose. However, high-retention PVDF membranes tend to exhibit higher staining backgrounds, and it is more difficult to extract proteins or peptides from such membranes.

The protocols for staining with amido black, Coomassie blue, Ponceau S, and AuroDye follow the suppliers’ recommendations. It should be noted that when staining PVDF membranes with Coomassie blue before N-terminal sequencing, omitting acetic acid from both the stain and destain solution is recommended to minimize potential extraction of protein from the membrane (Speicher, 1989).

The protocol for India ink staining of electroblotted proteins is essentially that of Hancock and Tsang (1983). Different brands of India ink may be used, but staining sensitivity may vary as a result.

The colloidal gold stain is the most sensitive membrane stain described here. As an alternative to commercially available colloidal gold stains, Moeremans et al. (1985) describe methods for preparing gold and iron solutions.

Fluorescent labels are advantageous because they can be used not only for sequential detection methods on the same blot with minimal potential interference, but also for detection prior to protein extraction from the membrane. For example, after visualization of proteins with a fluorescent label, the blot can be photographed and specific bands marked with a pencil, either directly on the membrane or through a plastic bag. The latter method leaves a permanent indentation on the membrane. The blot can then be probed with antisera (i.e., immunoblotted; UNIT 10.10).
The protocol for fluorescamine labeling is based on the procedure described by Vera and Rivas (1988). Fluorescamine itself is not strongly fluorescent; however, when combined with the primary amines of proteins (i.e., N termini and lysine residues) it yields a highly fluorescent product. IAEDANS is an iodooacetic acid analog containing a naphthalene ring and is fluorescent under UV light. When the sample protein is reduced either prior to gel electrophoresis or with DTT after blotting, all cysteines are potentially available for reaction with IAEDANS. When the protein is not reduced, some cysteines may be involved in disulfide bonds and therefore not available for reaction. Additionally, some cysteines may be sterically inaccessible because of adsorption to the membrane and therefore will not react.

An additional visualization technique for PVDF membranes is transillumination, described by Reig and Klein (1988). In that technique, the membrane is dried at room temperature, then wet with 20% methanol and viewed on a white light box. Protein bands appear as clear areas. Sensitivity is usually comparable to that of Coomassie blue staining.

Critical Parameters and Troubleshooting

High-quality water (from a Milli-Q purification system or equivalent) should be used throughout these protocols. All plastic and glass boxes must be thoroughly cleaned by rinsing with water before use to avoid staining artifacts. Blot membranes should be handled by the edges only with gloves or, preferably, with forceps. This precaution is most critical for the more sensitive stains (i.e., colloidal gold, colloidal silver, and India ink). When using PVDF membranes, it is especially critical that the membrane does not dry between steps. If drying occurs, wet the PVDF membrane for 5 sec with 100% methanol, then rinse several times with water.

A brief alkali treatment can enhance staining with India ink or colloidal gold. In a procedure described by Sutherland and Skerritt (1986), the membrane is washed with 1% (w/v) KOH for 5 min followed by several rinses with PBS. The alkali treatment can easily be incorporated at the beginning of the procedures if desired.

Anticipated Results

Approximate detection limits and membrane compatibilities are listed in Table 10.8.1. It should be noted that detection limits may vary with gel size and the percentage of polyacrylamide in the gel used in preparation of the blot transfer membrane. The sensitivity of fluorescent stains is related to the number of reactive amino groups (fluorescamine) or cysteine residues (IAEDANS) present in the protein of interest.

Time Considerations

The total time required for staining with amido black, Coomassie blue, and Ponceau S is 30 min to 1 hr; colloidal gold requires 4 to 6 hr; colloidal silver requires 1 hr; whereas staining with India ink requires 2 hr to overnight. Fluorescent labeling requires ~1 hr. The optional alkali enhancement (see Critical Parameters and Troubleshooting) requires an additional 30 min at the beginning of the India ink and colloidal gold staining procedures.

Literature Cited


Key References


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