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Detection of Proteins on Blot Membranes

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Abstract

Staining of blot membranes enables the visualization of bound proteins. Proteins are usually transferred to blot membranes by electroblotting, by direct spotting of protein solutions, or by contact blots. Staining allows the efficiency of transfer to the membrane to be monitored. This unit describes protocols for staining proteins after electroblotting from polyacrylamide gels to blot membranes such as polyvinylidene difluoride (PVDF), nitrocellulose, or nylon membranes. The same methods can be used if proteins are directly spotted, either manually or using robotics. Protocols are included for seven general protein stains (amido black, Coomassie blue, Ponceau S, colloidal gold, colloidal silver, India ink, and MemCode) and three fluorescent protein stains (fluorescamine, IAEDANS, and SYPRO Ruby). Also included is an in-depth discussion of the different blot membrane types and the compatibility of different protein stains with downstream applications, such as immunoblotting or N-terminal Edman sequencing.

Keywords

protein stain; membrane stain; electroblots; electrotransfer efficiency

INTRODUCTION

Staining of blot 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. A variation of transferring protein from gels to membranes is a contact blot, although the extent of transfer is usually far less than with the electrotransfer method. An alternative method of loading proteins onto membranes involves direct spotting of liquid samples onto the membrane either manually or robotically.

The first seven Basic Protocols describe the use of general protein stains—amido black, Coomassie blue, Ponceau S, colloidal gold, colloidal silver, India ink, and MemCode. In addition, the fluorescent stains fluorescamine and IAEDANS, which covalently react with amines or sulfhydryls on bound proteins are described in Basic Protocol 8 and Alternative

Protocol 1. Another commonly used fluorescent dye, SYPRO Ruby, forms strong non-covalent interactions with proteins and is described in Alternative Protocol 2. Table 10.8.1 lists approximate detection limits for each non-fluorescent 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. Furthermore, some stains are directly compatible with subsequent immunoblotting, e.g., Ponceau S, MemCode, and SYPRO Ruby.

NOTE: High-purity water such as 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 using clear 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.

BASIC PROTOCOL 1: 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 (*UNIT 10.7*)

Amido black 10B stain: 0.1% (w/v) amido black (naphthol blue black 10B, Sigma-Aldrich) 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.

BASIC PROTOCOL 2: 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 (e.g. Bio-Rad, Sigma-Aldrich, ThermoFisher Scientific, etc.) 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 or other protein chemistry methods where maximizing protein yield is important, acetic acid should be omitted from the staining and destaining solutions to minimize protein extraction from the membrane.
3. Destain membrane with 50% methanol/7% acetic acid for 5 to 10 min.
4. Rinse with water several times, then air dry.

BASIC PROTOCOL 3: 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 easily 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 (e.g. Sigma-Aldrich, ThermoFisher Scientific) in 1% (v/v) acetic acid

200 μ M 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 over-destain 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 μ M NaOH/20% acetonitrile for 1 min.
6. Wash membrane with water three times for 5 min each, then air dry.

BASIC PROTOCOL 4: COLLOIDAL GOLD STAINING

Colloidal gold is a highly sensitive stain and commercial, ready to use stains are available. 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)

Colloidal gold reagent (e.g. Bio-Rad)

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 colloidal gold stain for 1 to 2 hr (until desired color formation).
Use only enough stain to cover the membrane completely.
6. Rinse membrane thoroughly with water, then air dry.

BASIC PROTOCOL 5: 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.

BASIC PROTOCOL 7: MEMCODE STAINING

Like Ponceau S, MemCode Reversible Protein Stain is used for assessing protein transfer efficiency and potential imaging of total protein patterns before immunoblot development, but it is more sensitive than Ponceau S. This stain is ideal for downstream applications because proteins are unaffected by the staining process and the stain is entirely removed by the end of the protocol, as described in steps 7 and 8. Transferred proteins (>25 ng/band) appear as blue bands on a white background.

Materials

Blot transfer membrane (*UNIT 10.7*)

Pierce Reversible Protein Stain Kit for PVDF or nitrocellulose membranes (ThermoFisher Scientific)

Destain/Methanol solution (1:1 mixture of reagent grade methanol and MemCode Destain)

Eraser/Methanol solution (1:1 mixture of reagent grade methanol and MemCode Eraser)

Plastic box

1. Place blot transfer membrane in a plastic box. Briefly rinse with water three times.
2. Incubate membrane with MemCode Sensitizer and shake for 2 min at room temperature on a rotary shaker.

Note that there are different kits for nitrocellulose or PVDF membranes and the kits are not interchangeable.

3. Stain membrane with MemCode Reversible Stain for 1 min at room temperature on a rotary shaker.
4. Briefly rinse membrane with MemCode Destain
5. Destain membrane with Destain/Methanol solution for 5 min at room temperature on a rotary shaker
6. Briefly rinse with water five times. Scan or photograph membrane before proceeding.
7. Remove stain with Eraser/Methanol solution for 10 min for PVDF and 2 min for nitrocellulose at room temperature on a rotary shaker.

Certain proteins may require more time (up to 20 min) to completely remove MemCode stain.
8. Briefly rinse membrane with water five times then proceed with immunoblotting.

BASIC PROTOCOL 8: FLUORESCAMINE LABELING

Fluorescamine, or 4-phenylspiro[furan-2(3*H*),1'-phthalan]-3,3'-dione, is used to introduce a fluorescent label on electroblotted 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 similar to Ponceau S ([Basic Protocol 3](#)) or MemCode ([Basic Protocol 7](#)). However, unlike these alternative stains, fluorescamine covalently reacts with available amino groups (i.e., lysines and the protein N terminus if it was not previously blocked and irreversibly modifies the protein.

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-Aldrich) 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.

ALTERNATE PROTOCOL 1: IAEDANS LABELING

N-iodoacetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine (IAEDANS or 1,5-I-AEDANS) is used for fluorescent labeling of electroblotted 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-Aldrich, ThermoFisher Scientific, etc.; 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.

ALTERNATE PROTOCOL 2: SYPRO RUBY LABELING

SYPRO Ruby protein blot is used for the fluorescent labeling of proteins on blot transfer membranes. SYPRO Ruby has excellent sensitivity that rivals colloidal gold (2–8 ng protein/band) and is generally compatible with downstream immunoblotting and N-terminal sequencing. Transferred protein bands appear as orange-red fluorescence under UV light.

Materials

Blot transfer membrane (*UNIT 10.7*)

SYPRO Ruby protein blot stain (Life Technologies)

7% acetic acid/10% reagent grade methanol

Plastic box

1. Place blot transfer membrane in a plastic box. Cover in 7% acetic acid/10% methanol solution for 15 minutes at room temperature.

For PVDF, allow the membrane to dry after electrotransfer before beginning this procedure. When performing incubation and wash steps, place membrane face-down in the indicated solution. For nitrocellulose, completely immerse the membrane in the indicated solution.

2. Rinse with water four times for 5 min each.
3. Stain with SYPRO Ruby protein blot stain for 15 min

For PVDF, transfer the membrane to a new dish already containing the SYPRO Ruby stain then place the membrane face-down.

4. Rinse with water four to six times for nitrocellulose and two to three times for PVDF for 1 min each, then air-dry.

COMMENTARY

Background Information

The common use of electrophoretic transfer of proteins from 1D or 2D gels 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 protein chemistry analyses such as N-terminal sequencing and some other protein chemistry methods. However, they are most often used in parallel with antibody reactivity to either evaluate efficiency of electrotransfer or to precisely correlate immunoreactivity with specific stained bands in protein patterns. For the latter purpose an advantage of on-blot staining is that duplicate sets of lanes on the membrane can be cut apart and one section can be used for immunoblotting while the duplicate lanes can be 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 during fixing and staining. For other applications, proteins are loaded onto blot membranes by direct spotting of proteins in solution. This can be accomplished by manually pipetting small numbers of proteins onto a membrane, vacuum deposition in a 96-well format, or by the robotic printing of high density protein arrays.

In most studies unstained proteins are denatured and separated on SDS gels prior to electrotransferring or spotting them onto blot membranes with subsequent staining of the

blot using one of the general protein stains described above. However, for some applications proteins are stained prior to gel electrophoresis and the stained proteins are then transferred to the blot. For example, Shimazaki and Michhiro (2013) separated plasma proteins using non-denaturing 2D gels, transferred the native proteins to PVDF membranes, stained the proteins with Ponceau S and measured activities of protease inhibitors on the blot membranes. Another approach is to use blue native polyacrylamide gel electrophoresis, which is a powerful method of separating native protein complexes. After blue native gel electrophoresis the separated protein complexes can either be transferred directly to a PVDF membrane for immunoblotting, or the complexes can be separated into components using an SDS gel electrophoresis second dimension followed by electroblotting to a membrane (Kikuchi, Bedard, Nakai, 2011). Even the successful transfer of Coomassie blue stained peptides to PVDF membranes has been reported (Lee, Chang, 2015). This study showed that the bound stain actually increased retention on the membrane and improved detectability of small proteins and peptides. Even stained peptides less than 2 kDa could be retained on the blot membrane.

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 become brittle and break easily when dry. A number of PVDF membranes are commercially available that have subtle but important differences in properties resulting from different proprietary manufacturing processes. For example, Immobilon-P, Immobilon-PSQ and Immobilon-FL (Sigma-Aldrich) are PVDF membranes that have been optimized for: 1) western blotting, 2) higher binding for applications such as Edman sequencing, or 3) fluorescence applications, respectively. Immobilon-PSQ and similar high retention PVDF membranes from other manufacturers generally show both higher protein binding capacities and higher binding affinities compared to Immobilon-P and similar PVDF membranes that have been optimized for western blotting (Mozdzanowski and Speicher, 1992). Use of these higher affinity 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-affinity PVDF membranes tend to exhibit higher staining backgrounds, and it is more difficult to extract proteins or peptides from such membranes. Similarly, if these membranes are used for western blotting they typically yield higher background staining

The protocols for staining with amido black, Coomassie blue, Ponceau S, AuroDye, and MemCode 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). MemCode is the most sensitive of the two completely reversible stains described here (the other being Ponceau S) with a sensitivity of 25 ng/band (Antharavall, 2004).

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 colloidal gold and iron staining 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) for an unambiguous correlation of general protein staining patterns and immunoreactivity.

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 side chains) it yields a highly fluorescent product. IAEDANS is an iodoacetic acid analog containing a naphthalene ring and is fluorescent under UV light. When the protein sample 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. SYPRO Ruby contains ruthenium which interacts noncovalently with sulfonates (Berggren, 1999). SYPRO Ruby-stained proteins are generally compatible with immunoblotting and N-terminal sequencing.

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 using white light. Protein bands appear as clear areas. Sensitivity is usually comparable to that of Coomassie blue staining. This approach was recently demonstrated to also be compatible with the new low fluorescence PVDF membranes such as Immobilon-FL (Park, Mauchi, Sharma, 2015). This simple, fast method is a very attractive strategy for imaging blots prior to fluorescent immunodetection.

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 followed by thorough rinsing with water before use to avoid staining artifacts. Blot membranes should be handled by only by the edges while wearing gloves, or preferably, only 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, Ponceau S, and MemCode 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 ~15 minutes to 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.

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Literature Cited

- Antharavall BS, Carter B, Bell PA, Mallia AK. A high-affinity reversible protein stain for Western blots. *Anal Biochem.* 2004; 329:276–280. [PubMed: 15158487]
- Berggren K, Steinberg TH, Lauber WM, Carroll JA, Lopez MF, Chernokalskaya E, Zieske L, Diwua Z, Haugland RP, Patton WF. A luminescent ruthenium complex for ultrasensitive detection of proteins immobilized on membrane supports. *Anal Biochem.* 1999; 276(2):129–143. [PubMed: 10603235]
- Hancock K, Tsang VCM. India ink staining of proteins on nitrocellulose paper. *Anal Biochem.* 1983; 133:157–162. [PubMed: 6195938]
- Kikuchi S, Bedard J, Nakai M. One- and two-dimensional blue native-page and immunodetection of low-abundance chloroplast membrane protein complexes. *Methods Mol Biol.* 2011; 775:3–17. [PubMed: 21863435]
- Lee DY, Chang GD. Post-staining electrophoresis for efficient and reliable peptide blotting. *Methods Mol Biol.* 2015; 1312:185–195. [PubMed: 26044003]
- Moeremans M, Daneels G, De Mey J. Sensitive colloidal metal (gold or silver) staining of protein blots on nitrocellulose membranes. *Anal Biochem.* 1985; 145:315–321. Describes a method for preparation of colloidal metal stains. [PubMed: 2409835]
- Mozdzanowski J, Speicher DW. Microsequence analysis of electroblotted proteins. *Anal Biochem.* 1992; 207:11–18. [PubMed: 1489083]
- Park J, Mabuchi M, Sharma A. Visualization of unstained protein bands on PVDF. *Methods Mol Biol.* 2015; 1314:191–194. [PubMed: 26139268]
- Reig J, Klein D. Submicrogram quantities of unstained proteins are visualized on polyvinylidene difluoride membranes by transillumination. *Appl Theor Electrophor.* 1988; 1:59–60. [PubMed: 2484988]

- Shimazaki Y, Michhiro M. Analysis of trypsin inhibition activity in human plasma proteins after separation by non-denaturing two-dimensional electrophoresis. *Clinica Chimica Acta*. 2013; 425:48–53.
- Speicher, DW. Microsequencing with PVDF membranes: Efficient electroblotting, direct protein adsorption and sequencer program modifications. In: Hugli, TE., editor. *Techniques in Protein Chemistry*. Academic Press; San Diego: 1989. p. 24-35.
- Sutherland MW, Skerritt JH. Alkali enhancement of protein staining on nitrocellulose. *Electrophoresis*. 1986; 7:401–406.
- Vera JC, Rivas C. Fluorescent labeling of nitrocellulose-bound proteins at the nanogram level without changes in immunoreactivity. *Anal Biochem*. 1988; 173:399–404. Describes the use of multiple detection methods. [PubMed: 3142302]

Table 10.8.1Staining Sensitivities and Membrane Compatibilities for Non-fluorescent Stains^a

Stain	Minimum amount detected ^b	Membrane type ^c		
		PVDF	Nitrocellulose	Nylon
Amido black	50 ng	+	+	+
Coomassie blue	50 ng	+	–	+
Ponceau S	200 ng	+	+	+
Colloidal gold	2 ng	+	+	–
Colloidal silver	5 ng	+	+	–
India ink	5 ng	+	+	–
MemCode	25 ng	+	+	–

^a Sensitivity of the covalent fluorescent stains (fluorescamine, IAEDANS) is very dependent on protein amino acid composition. The sensitivity of Sypro Ruby is similar to colloidal gold and is compatible with PVDF and nitrocellulose membranes.

^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 (approximately 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.