

TRANSFER OF PROTEINS FROM SDS-POLYACRYLAMIDE GELS TO SOLID SUPPORTS: IMMUNOLOGICAL DETECTION OF IMMOBILIZED PROTEINS (WESTERN BLOTTING)

Western blotting (Towbin et al. 1979; Burnette 1981) is to proteins what Southern blotting is to DNA. In both techniques, electrophoretically separated components are transferred from a gel to a solid support and probed with reagents that are specific for particular sequences of amino acids (western blotting) or nucleotides (Southern hybridization). In the case of proteins, the probes usually are antibodies that react specifically with antigenic epitopes displayed by the target protein attached to the solid support. Western blotting is therefore extremely useful for the identification and quantitation of specific proteins in complex mixtures of proteins that are not radiolabeled. The technique is almost as sensitive as standard solid-phase radioimmunoassays and, unlike immunoprecipitation, does not require that the target protein be radiolabeled. Furthermore, because electrophoretic separation of proteins is almost always carried out under denaturing conditions, any problems of solubilization, aggregation, and coprecipitation of the target protein with adventitious proteins are eliminated.

The critical difference between Southern and western blotting lies in the nature of the probes. Whereas nucleic acid probes hybridize with a specificity and rate that can be predicted by simple equations (see Chapter 10), antibodies behave in a much more idiosyncratic manner. As discussed earlier in this chapter, an individual immunoglobulin may preferentially recognize a particular conformation of its target epitope (e.g., denatured or native). Consequently, not all monoclonal antibodies are suitable for use as probes in western blots, where the target proteins are thoroughly denatured. Polyclonal antisera, on the other hand, are undefined mixtures of individual immunoglobulins, whose specificity, affinity, and concentration are often unknown. Consequently, it is not possible to predict the efficiency with which a given polyclonal antiserum will detect different antigenic epitopes of an immobilized, denatured target protein.

Although there is an obvious danger that comes from using undefined reagents to assay a target protein that may also be poorly characterized, most problems that arise with western blotting in practice can be solved by designing adequate controls. These include the use of (1) antibodies (i.e., preimmune sera or irrelevant monoclonal antibodies) that should not react with the target protein and (2) control preparations that either contain known amounts of target antigen or lack it altogether.

Often, there is little choice of immunological reagents for western blotting—it is simply necessary to work with whatever antibodies are at hand. However, if a choice is available, either a high-titer polyclonal antiserum or a mixture of monoclonal antibodies raised against the denatured protein should be used. Reliance on a single monoclonal antibody is hazardous because of the high frequency of spurious cross-reactions with irrelevant proteins. If, as is usually the case, monoclonal and polyclonal antibodies have been raised against native target protein, it will be necessary to verify that they react with epitopes that either (1) resist denaturation with SDS and reducing agents or (2) are created by such treatment. This can be

done by using denatured target antigen in a solid-phase radioimmunoassay (see pages 18.19–18.23) or in western dot blots.

In western blotting, the samples to be assayed are solubilized with detergents and reducing agents, separated by SDS-polyacrylamide gel electrophoresis, and transferred to a solid support (usually a nitrocellulose filter), which may then be stained. The filter is subsequently exposed to unlabeled antibodies specific for the target protein. Finally, the bound antibody is detected by one of several secondary immunological reagents (^{125}I -labeled protein A or anti-immunoglobulin, or anti-immunoglobulin or protein A coupled to horseradish peroxidase or alkaline phosphatase). As little as 1–5 ng of an average-sized protein can be detected by western blotting.

Preparation and Electrophoresis of Samples

Two methods are used to extract proteins for western blotting from cells: Either the intact cells are dissolved directly in sample buffer or an extract is made as described earlier for samples to be immunoprecipitated (see pages 18.30–18.41). Which of these methods is best in any individual case depends on the type of cells and on the properties of the antigen.

- In general, bacteria expressing the target protein are lysed directly in SDS gel-loading buffer as described on pages 18.40–18.41.
- Yeasts are first lysed by vortexing in the presence of glass beads or enzymatically (see pages 18.35–18.36), and the resulting extracts are then prepared (see pages 18.35–18.39).
- Mammalian tissues are usually dispersed mechanically and then dissolved directly in SDS gel-loading buffer.
- Mammalian cells in tissue culture may be lysed gently with detergents as described on page 18.34, or, alternatively, the protocol on pages 18.62–18.63, in which the cells are lysed directly in SDS gel-loading buffer, may be used if the target antigen is resistant to this type of extraction.

In any case, the samples are analyzed by SDS-polyacrylamide gel electrophoresis as described on pages 18.47–18.54.

LYSIS OF MAMMALIAN CELLS AND TISSUE IN GEL-LOADING BUFFER

1. *For cells growing in monolayers*

- a. Wash the cells twice with phosphate-buffered saline (PBS; see Appendix B) and drain thoroughly. Remove the last traces of PBS by aspiration.
- b. For a 90-ml petri dish, add 100–200 μl of hot (85°C) 1 \times SDS gel-loading buffer to lyse the cells. Scrape the viscous lysate into a microfuge tube with a policeman and proceed with step 2.

For cells growing in suspension and fragments of tissue

- a. Wash the cells or tissue fragments thoroughly in PBS at 0°C. After centrifugation at 3000g for 5 minutes at 4°C, estimate the volume of the pellet in the bottom of the centrifuge tube.
- b. Remove all of the supernatant by aspiration. Use a disposable pipette tip attached to a vacuum line to remove any droplets of fluid that adhere to the walls of the centrifuge tube.
- c. Disperse the cells or tissue fragments in 5 volumes of ice-cold suspension buffer.

Suspension buffer

0.1 M NaCl
0.01 M Tris · Cl (pH 7.6)
0.001 M EDTA (pH 8.0)
1 $\mu\text{g}/\text{ml}$ aprotinin
100 $\mu\text{g}/\text{ml}$ phenylmethylsulfonyl fluoride (PMSF)

Caution: PMSF is extremely destructive to the mucous membranes of the respiratory tract, the eyes, and skin. PMSF may be fatal if inhaled, swallowed, or absorbed through the skin. In case of contact, immediately flush eyes or skin with copious amounts of water. Discard contaminated clothing.

PMSF, which is labile in aqueous solutions, should be added from a stock solution just before the suspension buffer is used. The rate of inactivation in aqueous solution increases with pH and is faster at 25°C than at 4°C. The half-life of a 20 μM aqueous solution of PMSF is about 35 minutes at pH 8.0 (James 1978). This means that aqueous solutions of PMSF can be safely discarded after they have been rendered alkaline (pH > 8.6) and stored for several hours at room temperature. PMSF is usually stored as a 10 mM or 100 mM stock solution (1.74 or 17.4 mg/ml in isopropanol) at -20°C .

The cells are suspended in buffer to prevent the formation of an insoluble mass when the 2 \times SDS gel-loading buffer is added. This step should be carried out as quickly as possible, using ice-cold suspension buffer, to minimize proteolytic degradation. Most types of mammalian tissue can be rapidly teased apart with forceps or cut into small pieces with scissors beneath the surface of the suspension buffer.

- d. As soon as possible, add an equal volume of $2 \times$ SDS gel-loading buffer.

2 \times SDS gel-loading buffer

100 mM Tris · Cl (pH 6.8)
200 mM dithiothreitol
4% SDS (electrophoresis grade)
0.2% bromophenol blue
20% glycerol

$2 \times$ SDS gel-loading buffer lacking dithiothreitol can be stored at room temperature. Dithiothreitol should then be added, just before the buffer is used, from a 1 M stock (see Appendix B).

2. Place the sample in a boiling-water bath for 10 minutes.

The sample should become highly viscous as a consequence of the release of high-molecular-weight chromosomal DNA.

3. Shear the chromosomal DNA by sonication, using either a sonicator with an immersible tip or a sonicator that can process several samples simultaneously in a chilled cup. Depending on the power output of the sonicator and its state of tuning, between 30 seconds and 2 minutes at full power should be sufficient to reduce the viscous lysate to manageable levels.

Samples that are too large to be sonicated conveniently can be sheared by repeated passage through a 23-gauge hypodermic needle.

4. Centrifuge the sample at 10,000g for 10 minutes at room temperature. Transfer the supernatant to a fresh tube, and discard the pellet.

5. If possible, calculate the amount of sample that will be required to detect the target protein by western blotting.

The lowest amount of an average-sized protein that can be detected by western blotting is approximately 1–5 ng. Approximately 100 μ g of total cellular protein can be applied to a lane in a 0.75-mm-thick SDS-polyacrylamide gel without overloading. To be detected by western blotting, an average-sized target protein must therefore comprise at least 1 part in 10^5 by weight of the total protein in the extract. If it is feasible to do so, reconstruction experiments should be carried out in which known amounts of the target protein are added to total extracts of cells and analyzed by western blotting. These experiments should serve as positive controls when unknown samples are being analyzed, and they should provide an accurate estimate of the sensitivity of the method with the antibodies that are available.

6. Analyze an aliquot of the sample(s) by SDS-polyacrylamide gel electrophoresis and western blotting. The gel is loaded and run in the conventional manner as described on pages 18.47–18.54.

Electrophoresis is usually carried out using SDS-polyacrylamide gels and the Laemmli discontinuous buffer system (Laemmli 1970) (see page 18.47). However, many other types of gels have been used successfully, including urea-polyacrylamide gels and nondenaturing polyacrylamide gels. For details of the methods used to cast and run such gels, see Hames and Rickwood (1981).

- c. Place the nitrocellulose filter on the stack of 3MM paper. Make sure that the filter is exactly aligned and that no air bubbles are trapped between it and the 3MM paper.
 - d. Remove the glass plates holding the SDS-polyacrylamide gel from the electrophoresis tank. Transfer the gel briefly to a tray of deionized water, and then place it exactly on top of the nitrocellulose filter. Orient the gel so that the mark on the filter corresponds to the bottom left-hand corner of the gel. Squeeze out any trapped air bubbles with a gloved hand.

Important: To avoid the possibility of a short circuit, do not cut off the bottom left-hand corner of the gel.
 - e. Place the final three sheets of 3MM paper on the gel, again making sure that they are exactly aligned and that no air bubbles are trapped.
6. Place the upper electrode (which will become the cathode) on top of the stack, graphite side down. Connect the electrical leads (positive or red lead to the bottom graphite electrode). Apply a current of 0.65 mA/sq. cm. of gel for a period of 1.5–2 hours.
 7. Turn off the electric current and disconnect the leads. Disassemble the transfer apparatus from the top downward, peeling off each layer in turn. Transfer the gel to a tray containing Coomassie Brilliant Blue, and stain it as described on page 18.55. This will allow you to check whether electrophoretic transfer is complete.
 8. *Optional:* Remove the nitrocellulose filter from the sandwich and transfer it to a clean piece of 3MM paper. Allow the filter to dry for 30–60 minutes at room temperature.

Drying is claimed to improve the retention of proteins on the filter during subsequent processing. However, it may also result in further denaturation and consequent alteration in immunoreactivity. Drying may therefore be advantageous for some protein/antibody combinations and disadvantageous for others. This can be established empirically for the target proteins of interest.
 9. Cut off the bottom left-hand corner of the filter. This serves as insurance against obliteration of the pencil mark (step 2). Stain the filter with Ponceau S or India ink as described on pages 18.67–18.68. India ink should only be used if the western blot is to be probed with a radiolabeled antibody or probe.

Staining Proteins Immobilized on Nitrocellulose Filters

Of the several procedures available to stain proteins immobilized on nitrocellulose filters, only one, staining with Ponceau S, is completely compatible with all methods of immunological probing because the stain is transient and is washed away during processing of the western blot. Staining with Ponceau S therefore does not interfere with the subsequent detection of antigens by chromogenic reactions catalyzed by antibody-linked enzymes such as alkaline phosphatase or lactoperoxidase. However, because the pink-purple color of Ponceau S is difficult to capture photographically, the stain does not provide a permanent record of the experiment. Instead, staining with Ponceau S is used to provide visual evidence that electrophoretic transfer of proteins has taken place and to locate molecular-weight markers, whose positions on the nitrocellulose filter are then marked with pencil or indelible ink.

If the western blot is to be probed with radiolabeled antibody or radiolabeled protein A, the proteins immobilized on the nitrocellulose filter may be stained with India ink, which is cheaper and more sensitive than Ponceau S and provides a permanent record of the location of proteins on the nitrocellulose filter. Note that proteins transferred to nylon membranes cannot be stained with India ink, since the colloidal particles become irreversibly bound.

STAINING WITH PONCEAU S

1. If the nitrocellulose filter has been dried, float it on the surface of a tray of deionized water and allow it to wet from beneath by capillary action. Then, submerge the filter in the water for at least 5 minutes to displace trapped air bubbles.
2. Transfer the filter to a tray containing a working solution of Ponceau S stain. Incubate the filter for 5–10 minutes with gentle agitation.

For a stock solution of Ponceau S, mix:

Ponceau S	2 g
trichloroacetic acid	30 g
sulfosalicylic acid	30 g
H ₂ O to 100 ml	

Dilute one part of the stock solution with nine parts of deionized water to make a working solution. Discard the working solution after use.

Ponceau S (Sigma P 7767) is 3-hydroxy-4-(2-sulfo-4-[4-sulfophenylazo]-phenylazo)-2,7-naphthalenedisulfonic acid.

3. When the bands of protein are visible, wash the nitrocellulose filter in several changes of deionized water at room temperature.
4. Mark the positions of proteins used as molecular-weight standards with waterproof black ink.
5. Proceed with immunological probing as described on pages 18.69–18.75.

STAINING WITH INDIA INK

This method of staining should only be used when radiolabeled antibody or radiolabeled protein A is used as a probe.

1. If the nitrocellulose filter has been dried, float it on the surface of a tray of deionized water and allow it to wet from beneath by capillary action. Then, submerge the filter in the water for at least 5 minutes to displace trapped air bubbles.
2. Transfer the filter to a tray containing 0.4% Tween 20 in phosphate-buffered saline (PBS; see Appendix B). Incubate for 5 minutes at room temperature with gentle shaking, change the wash solution, and continue to incubate for a further 5 minutes.

The Tween 20 prevents the colloidal particles of India ink from attaching to sites on the nitrocellulose filter.

3. Transfer the filter to a tray containing diluted India ink (100 μ l of ink in 100 ml of 0.4% Tween 20 in PBS).

Some brands of India ink work far better than others. Higgins India Ink and Pelikan Fount India Drawing Ink are both highly recommended.

4. Cover the tray with Saran Wrap and incubate the filter at room temperature in the solution of India ink until the protein bands reach the desired intensity. Depending on the amount of protein loaded on the gel, this may take from 15 minutes to several hours.
5. Wash the filter for several hours in PBS, changing the buffer every hour.
6. Proceed with immunological probing as described on pages 18.69–18.73.

Blocking Binding Sites for Immunoglobulins on the Nitrocellulose Filter

Just as proteins transferred from the SDS-polyacrylamide gel can bind to the nitrocellulose filter, so can proteins in the immunological reagents used for probing. The sensitivity of western blotting depends on reducing this background of nonspecific binding by blocking potential binding sites with irrelevant proteins. Of the several blocking solutions that have been devised, the best and least expensive is nonfat dried milk (Johnson et al. 1984). It is easy to use and is compatible with all of the immunological detection systems in common use. There is only one circumstance under which nonfat dried milk should not be used: when western blots are probed for proteins that may be present in milk.

1. Place the nitrocellulose filter in a heat-sealable plastic bag (Sears Seal-A-Meal or equivalent), and add 0.1 ml of blocking solution per square centimeter of filter. Seal the bag, leaving as few air bubbles as possible, and incubate the filter for 1–2 hours at room temperature with gentle agitation on a platform shaker.

Blocking solution

5% (w/v) nonfat dried milk
0.01% antifoam A
0.02% sodium azide in phosphate-buffered saline (PBS; see Appendix B)

Caution: Sodium azide is poisonous. It should be handled with great care wearing gloves, and solutions containing it should be clearly marked.

If the background of nonspecific binding of immunological probes is unacceptably high, try adding Tween 20 to a final concentration of 0.02%. In most cases, the presence of this detergent will not affect specific binding of antibodies to the target antigen.

2. Cut open the plastic bag and discard the blocking solution. Immediately incubate the filter with an antibody directed against the target protein as described on pages 18.70–18.71.

Binding of the Primary Antibody to the Target Protein

Virtually all western blots are probed in two stages: An unlabeled antibody specific to the target protein is first incubated with the nitrocellulose filter in the presence of blocking solution. The filter is then washed and incubated with a secondary reagent—anti-immunoglobulin or protein A that is either radiolabeled or coupled to an enzyme such as horseradish peroxidase or alkaline phosphatase. After further washing, the antigen-antibody-antibody or antigen-antibody-protein A complexes on the nitrocellulose filter are located by autoradiography or in situ enzymatic reactions.

Indirect or two-stage probing has the major advantage of allowing a single secondary reagent to be used to detect a wide variety of primary antibodies, thereby eliminating the tedious task of purifying and labeling each individual primary antibody. Since secondary immunological reagents can be purchased quite inexpensively from commercial sources, the resulting savings of time and money can be considerable.

INCUBATING THE NITROCELLULOSE FILTER WITH THE PRIMARY ANTIBODY DIRECTED AGAINST THE TARGET PROTEIN

1. To the plastic bag containing the nitrocellulose filter prepared as described on page 18.69, add 0.1 ml of blocking solution per square centimeter of filter and an appropriate quantity of the primary antibody.

Blocking solution

5% (w/v) nonfat dried milk
0.01% antifoam A
0.02% sodium azide in phosphate-buffered saline (PBS; see Appendix B)

Caution: Sodium azide is poisonous. It should be handled with great care wearing gloves, and solutions containing it should be clearly marked.

If the background of nonspecific binding of immunological probes is unacceptably high, try adding Tween 20 to a final concentration of 0.02%. In most cases, the presence of this detergent will not affect specific binding of antibody to the target antigen.

The appropriate amount of primary antibody should be determined empirically in pilot experiments. The recommended test dilutions are:

- Polyclonal antibodies: 1:100 to 1:5000
- Supernatants from cultured hybridoma cells: undiluted to 1:100
- Ascitic fluid from mice bearing hybrid myelomas: 1:1000 to 1:10,000

2. Seal the bag, leaving as few air bubbles as possible, and incubate the filter for 2 hours at 4°C with gentle agitation on a platform shaker.

Longer incubation times (up to 18 hours at room temperature) have been reported to increase the sensitivity of detection of target antigens. However, the background of nonspecific binding also increases as a function of the time and temperature of incubation.

3. Cut open the plastic bag, and discard the blocking solution and antibody. Wash the filter three times (10 minutes each time) with 250 ml of PBS.
4. Immediately incubate the filter with the secondary immunological reagent as described on pages 18.72–18.73.

INCUBATING THE NITROCELLULOSE FILTER WITH THE SECONDARY IMMUNOLOGICAL REAGENT

The secondary reagent (usually an anti-immunoglobulin or protein A) may be radiolabeled with ^{125}I as described on pages 18.24–18.25 or may be covalently coupled to an enzyme such as horseradish peroxidase or alkaline phosphatase. Covalently coupled immunoglobulin and protein A are sold commercially.

Although both radiolabeled and enzyme-coupled secondary reagents can work very well, antibodies are sometimes inactivated if the radiolabeling process is carried out too enthusiastically. Most workers therefore prefer to use enzyme-coupled reagents that have been tested by their commercial manufacturers.

Radiolabeled secondary reagents

1. Transfer the nitrocellulose filter from the final wash in phosphate-buffered saline (PBS; see Appendix B) (step 3, page 18.71) to a heat-sealable plastic bag (e.g., Sears Seal-A-Meal) containing 0.1 ml of fresh blocking solution per square centimeter of filter and add approximately 10^4 cpm of the radiolabeled secondary reagent per square centimeter of filter.

Blocking solution

5% (w/v) nonfat dried milk
0.01% antifoam A
0.02% sodium azide in PBS

Caution: Sodium azide is poisonous. It should be handled with great care wearing gloves, and solutions containing it should be clearly marked.

If the background of nonspecific binding of immunological probes is unacceptably high, try adding Tween 20 to a final concentration of 0.02%. In most cases, the presence of this detergent will not affect specific binding of antibody to the target antigen.

2. Incubate the filter for 1–2 hours at room temperature with gentle agitation on a platform shaker.
3. Cut open the bag, and quickly transfer the filter to a tray containing 250 ml of PBS. Discard the plastic bag and radioactive fluid in the radioactive waste.
4. Wash the filter in several changes of PBS (10 minutes each change). Continue washing until no radioactivity is detected by a hand-held minimonitor in regions of the filter that carry no protein.
5. Remove the filter from the last wash, and allow it to dry for 10 minutes on a stack of paper towels.
6. Mount the filter on a piece of Whatman 3MM paper, and attach radioactive markers to the paper.

Radioactive markers can be made by applying spots of radioactive ink to adhesive labels or tape, which are then attached to the surface of the 3MM paper. Radioactive ink can be made by adding a small amount of Tran³⁵S-label (ICN Radiochemicals) or ³²P to a microfuge tube containing India ink. The radioactive ink can be applied with a fiber-tip pen kept for that specific purpose and clearly marked with radioactive-warning tape.

7. When the radioactive ink is dry, wrap the 3MM paper in Saran Wrap and expose the filter to X-ray film at -70°C with an intensifying screen (see Appendix E).

Enzyme-coupled secondary reagents

1. Transfer the nitrocellulose filter from the final wash in PBS (step 3, page 18.71) to a tray containing 200 ml of 150 mM NaCl, 50 mM Tris · Cl (pH 7.5). Incubate the filter for 10 minutes at room temperature with gentle agitation.

It is important to remove azide and phosphate from the filter before the enzyme-coupled secondary reagent is added.

2. Transfer the filter to a heat-sealable plastic bag (e.g., Sears Seal-A-Meal), or to a shallow tray, containing 0.1 ml of phosphate-free, azide-free blocking solution per square centimeter of filter.

Phosphate-free, azide-free blocking solution

5% (w/v) nonfat dried milk
150 mM NaCl
50 mM Tris · Cl (pH 7.5)

3. Add the enzyme-coupled secondary reagent according to the manufacturer's instructions and seal the bag if a bag was used. Usually, it is recommended that the secondary reagent be diluted 1:200 to 1:2000 to yield a final concentration of 0.5–5.0 $\mu\text{g}/\text{ml}$.
4. Incubate the filter with the enzyme-coupled secondary reagent for 1 hour at room temperature with gentle agitation.
5. Transfer the filter to a tray containing 200 ml of 150 mM NaCl, 50 mM Tris · Cl (pH 7.5). Incubate the filter for 10 minutes at room temperature with gentle agitation. Repeat this step three more times using fresh NaCl/Tris · Cl solution each time.
6. Add the appropriate chromogenic substrates to the filter as described on pages 18.74–18.75.

USE OF CHROMOGENIC SUBSTRATES WITH ENZYME-COUPLED ANTIBODIES

Alkaline phosphatase

The substrate 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) is converted in situ into a dense blue compound by immunolocalized alkaline phosphatase.

1. Prepare the following three solutions:

NBT

Dissolve 0.5 g of NBT, which is available from several manufacturers, in 10 ml of 70% dimethylformamide.

BCIP

Dissolve 0.5 g of BCIP disodium salt, which is available from several manufacturers, in 10 ml of 100% dimethylformamide.

Alkaline phosphatase buffer

100 mM NaCl

5-mM MgCl₂

100 mM Tris · Cl (pH 9.5)

These solutions are stable when stored in closed containers at room temperature.

2. Mix 66 μ l of NBT stock with 10 ml of alkaline phosphatase buffer. Mix well, and add 33 μ l of BCIP stock. This chromogenic substrate mixture should be used within 30 minutes.
3. Transfer the washed nitrocellulose filter (step 5, page 18.73) to a shallow tray. Add 0.1 ml of chromogenic substrate mixture per square centimeter of filter. Incubate the filter at room temperature with gentle agitation.
4. Monitor the progress of the reaction carefully. When the bands are of the desired intensity (~20 minutes), transfer the filter to a tray containing 200 μ l of 0.5 M EDTA (pH 8.0) and 50 ml of phosphate-buffered saline (PBS; see Appendix B).
5. Photograph the filter to provide a permanent record of the experiment.

Horseradish peroxidase

The most sensitive substrate for immunocoupled horseradish peroxidase is 3,3'-diaminobenzidine, which is converted in situ into a brown precipitate. The color of the precipitate is enhanced and the sensitivity of the reaction is improved by carrying out the reaction in the presence of cobalt or nickel ions. However, it is impossible to suppress background staining completely with horseradish peroxidase, and it is therefore essential to monitor the course of the reaction very carefully. The reaction should be stopped as soon as the specifically stained bands are clearly visible.

1. Dissolve 6 mg of diaminobenzidine tetrahydrochloride in 9 ml of 0.01 M Tris · Cl (pH 7.6). Add 1 ml of 0.3% (w/v) NiCl₂ or CoCl₂.

The solution of diaminobenzidine must be freshly prepared.

2. Filter the solution through a Whatman No. 1 filter to remove any precipitate that may form.
3. Add 10 μl of 30% H₂O₂. Mix well, and use the mixture immediately.

H₂O₂ is supplied as a 30% solution. It should be stored in tightly closed brown bottles and discarded after a few weeks.

4. Transfer the washed nitrocellulose filter (step 5, page 18.73) to a shallow tray. Add 0.1 ml of the substrate solution per square centimeter of filter. Incubate the filter at room temperature with gentle agitation.
5. Monitor the progress of the reaction carefully. When the bands are of the desired intensity (~2–3 minutes), wash the filter briefly in water, and then transfer it to a tray containing 250 ml of phosphate-buffered saline (PBS; see Appendix B).
6. Photograph the filter to provide a permanent record of the experiment.

The peroxidase-stained bands will fade after several hours of exposure to light.