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Western Blotting

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PHYSICAL PROPERTIES OF PROTEINS

What Do You Know about Your Protein?

In order to make informed choices among the bewildering range of available transfer and detection methods, it is best to have as clear an idea as possible of your own particular requirements. In large part these choices will depend on the nature of your target protein. Even limited knowledge can be used to advantage.

How abundant is your protein? It isn't necessary to answer the question in rigorously quantitative terms: an educated guess is sufficient. Are your samples easy to obtain and plentiful, or limited and precious? Is the sample likely to be rich in target protein (e.g., if the protein is overexpressed) or poor in target (perhaps a cytokine)? Obviously low protein concentration or severely limited sample size would require a more sensitive detection method.

What is the molecular weight of your target protein? Low MW proteins (12 kDa or less) are retained less efficiently than higher molecular weight proteins. Membranes with a pore size of 0.1 or 0.2 micron are recommended for transfer of these smaller proteins, and PVDF will tend to retain more low MW protein than nitrocellulose. The ultimate lower limit for transfer is somewhere around 5 kDa, although this depends largely on the protein's shape and charge.

The transfer of high molecular weight proteins (more than 100 kDa) can benefit from the addition of up to 0.1% SDS to the transfer buffer (Lissilour and Godinot, 1990). Transfer time can also be increased to ensure efficient transfer of high molecular weight proteins.

What Other Physical Properties Make Your Protein Unusual?

In cases where proteins are highly basic (where the pI of the protein is higher than the pH of the transfer buffer) the protein

will not be carried toward the anode, since transfer takes place on the basis of charge. In these cases it is best to include SDS in the transfer buffer. Alternatively, the transfer sandwich can be assembled with membranes on both sides of the gel.

CHOOSING A DETECTION STRATEGY: OVERVIEW OF DETECTION SYSTEMS

Detection systems range from the simplest colorimetric systems for use on the benchtop to complex instrument-based systems (Table 13.1). The simplest is radioactive detection: a secondary reagent is labeled with a radioactive isotope, usually the low-energy gamma-emitter iodine-125. After the blot is incubated with the primary antibody, the labeled secondary reagent (usually Protein A, but it can be a secondary antibody) is applied, the blot

Table 13.1 Comparison of Detection Methods

Method	Features	Limitations	Sensitivity
Radioactive	Can quantitate through film densitometry; can strip and reprobe blots; no enzymatic development step	Use of radioactive material can be difficult and expensive; requires licensing and radiation safety training	1 pg
Colorimetric	Easy to perform; hard copy results directly on blot; minimal requirements for facilities and equipment	Relatively insensitive	200 pg
Chemiluminescent	Highly sensitive; can quantitate using film densitometry; can strip and reprobe	Requires careful optimization	1 pg (luminol) 0.1 pg (acridan)
Fluorescent	Good linear range for quantitation; data stored digitally	Equipment expensive; stringent membrane requirements; stripping and reprobing possible but difficult	1 pg

washed and exposed to film for hours or days. Radioactive blots can more quickly be detected using storage phosphor plates instead of film; the plates are read on a specialized scanning instrument. Detailed discussions about the features and benefits of detection by film and scanners are included in Chapter 14, Nucleic Acid Hybridization.

Enzymatic reactions are used in a number of different systems to indicate the presence of bound antibody. The simplest type of enzymatic detection is chromogenic. Here the secondary reagent is conjugated to an enzyme, either horseradish peroxidase (HRP) or alkaline phosphatase (AP). After incubation with the secondary reagent and washing, the blot is incubated with a substrate. The enzyme catalyzes a reaction in which the substrate is converted to a colored precipitate directly on the membrane, essentially coloring the band on which the primary antibody has bound. While not as sensitive as other methods, colorimetric detection is fast and simple, and requires no special facilities.

Chemiluminescent detection combines characteristics of both radioactive and chromogenic detection. Again, an enzyme label is used (commonly HRP, but there are systems for use with AP as well), but in this case the reaction produces light rather than a colored product as a result of reaction. The light is usually captured on X-ray film, just like a radioactive blot. Specialized imaging equipment for chemiluminescent blots is also available. Chemiluminescent detection is very sensitive, and the blots are easily stripped for subsequent reprobing.

There are significant differences in the various available chemiluminescent detection systems. The most widely used are the luminol-based HRP systems. These typically emit usable signals for an hour or two. There are also newer, higher-sensitivity HRP-based systems that emit light for more than 24 hours; however, these substrates are more expensive and require even more careful optimization than the luminol-based systems. AP-based chemiluminescent systems are also available. They are not widely used in Western blotting, but they are highly sensitive and also emit light for extended periods. Those systems producing extended light output have the advantage that several exposures can be taken from the same blot.

With the availability of fluorescence-scanning instruments, new methods for detection have come into use. It may seem at first glance that a secondary antibody could simply be coupled to a fluorescent molecule and the detection performed directly. Although this is possible, this method is not sufficiently sensitive for most purposes. The approach usually taken uses an enzyme-coupled

secondary reagent (in this case usually AP) and a substrate that produces an insoluble, fluorescent product. The enzymatic reaction results in amplification of the signal, giving much better sensitivity than a fluorescently tagged secondary reagent. The blot is read on a fluorescent scanner and recorded as a digitized image.

What Are the Criteria for Selecting a Detection Method?

Sensitivity

There is a natural tendency to choose the most sensitive method available. High-sensitivity systems are desirable for detection of low-abundance proteins, but they are also desirable in cases where primary antibody is expensive or in limited supply, since these systems allow antibodies to be used at high dilutions. On the other hand, low-sensitivity systems, especially chromogenic systems, are easy to work with, require less exacting optimization, and tend to be less prone to background problems. Sensitivity overkill can be more trouble than it is worth.

What can you conclude from commercial sensitivity data? It can be difficult to compare the claims of sensitivity made by commercial suppliers. Although there is nothing wrong with the way these values are established, comparison between different systems can be difficult because the values depend on the exact conditions under which the determination was made. The primary antibody has a large effect on the overall sensitivity of any system, so comparisons between systems using different primary antibodies are less meaningful than they may seem at first glance. In order to compare two different detection systems, the target protein, the primary antibody, and, where possible, the secondary reagent should be the same. Such direct comparisons are hard to come by. Also sensitivity claims are usually made with purified protein rather than with crude lysate. For these reasons commercial sensitivity claims should be considered approximate, and it may be unrealistic to expect to attain the same level of sensitivity in your own system as that quoted by the manufacturer.

Signal Duration

Will your research situation require extended signal output in order to prepare several exposures from the same blot?

Ability to Quantitate

Film-based systems (chemiluminescent and radioactive) as well as fluorescence-scanning methods, allow quantitation of target proteins. Results on film are quantified by densitometry, while the

digital raw data from fluorescence scanners (and storage-phosphor scanners for radioactive detection) is inherently quantitative. The linear range of film-based systems (limited by the response of the film) is a little better than one order of magnitude, while the manufacturers of fluorescent scanners claim something closer to two orders of magnitude.

There are several cautions to bear in mind when considering protein blot quantitation. Standards (known amounts of purified target protein—not to be confused with molecular weight standards) must be run on every blot, since even with the most consistent technique there can be blot-to-blot variation. The standard should be loaded on the gel, electrophoresed, and transferred in exactly the same way your samples are.

The determination of quantity can only be made within the range of standards on the blot: extrapolation beyond the actual standard values is not valid. This together with the limited linear range means that several dilutions of the unknown sample usually must be run on the same blot. Given all the lanes of standards and sample dilutions, the amount of quantitative data that can be extracted from a single blot is somewhat limited. Protein blot quantitation can be useful, but it is not a substitute for techniques such as ELISA or RIA.

Antibody Requirements

Typically the choice of available primary antibodies is not as wide as that of the other elements of the detection system. Primary antibodies can be obtained from commercial suppliers, non-profit repositories, and even other researchers. Tracking down a primary antibody can be time-consuming, but publications such as Linscott's Directory (Linscott, 1999, and *http://www.linscottsdirectory.com/index2.html*), the "Antibody Resource Page" (*http://www.antibodyresource.com*), the Usenet newsgroup "Methods and Reagents" (*bionet.molbio.methds-reagnts*), and Stefan Dubel's recombinant antibody page (*www.mgen.uni-heidelberg.de/SD/SDscFvSite.html*) and *www.antibody.com* can help.

If no antibodies against your target protein exist, your only options are to raise the antibody yourself or to have someone else do it. Companies such as Berkeley Antibody Company, Genosys, Rockland, and Zymed (among many others) can do this kind of work on a contract basis. Whichever route you choose, this is a time-consuming and potentially expensive undertaking.

Ability to Strip and Reprobe

Radioactive and chemiluminescent systems are ideally suited to stripping and reprobing. Other systems (chemifluorescent and chromogenic) leave insoluble precipitates over the bands of interest; these precipitates can be removed only with the use of solvents, which is an unpleasant extra step and can be hard on blots. Not all targets survive this treatment. (See below for important cautions regarding stripping.)

Equipment and Facility Requirements

Radioactivity can be used only after fulfilling stringent training and licensing requirements. Radioactive methods, like chemiluminescent methods, also require darkroom facilities (unless storage phosphor equipment is available). Fluorescent methods require specialized scanning equipment. Chromogenic methods do not require any specialized facilities or equipment.

What Are the Keys to Obtaining High-Quality Results?

Careful choice of materials, an understanding of the questions your experiments are intended to answer, and an appreciation of the fact that every new system requires optimization are all necessary for obtaining good results. Optimization takes time, but it will pay off in the final result. It is also important to develop consistency in technique from day to day, and to keep detailed and accurate records. Consistency and good record-keeping will make it much easier to isolate the source of any problem that may come up later.

Which Transfer Membrane Is Most Appropriate to Your Needs?

The same considerations go into the choice of membrane that go into the choice of any other component of your detection strategy. What is the molecular weight of your protein? What detection method will you use, and does this method have special membrane requirements? Do you intend to strip and reprobe your blots? (See Table 13.2.)

Nitrocellulose wets easily and gives clean backgrounds. Unfortunately, it is physically fragile (liable to tear and crack), especially when dry. This fragility makes nitrocellulose undesirable for use in stripping and reprobing. The problem of physical fragility has been overcome with the introduction of supported nitrocellulose, which has surfaces of nitrocellulose over a core or "web" of physically stronger material. The added physical strength comes at the cost of slightly higher background.

Table 13.2 Characteristics of Transfer Membranes

Membrane	Characteristics
Nitrocellulose	Low background. Easy to block. Physically fragile.
Supported nitrocellulose	Binding properties similar to nitrocellulose. Higher background than pure nitrocellulose. Physically strong.
PVDF	High protein binding capacity. Physically strong. Highly hydrophobic: requires methanol pre-wetting and dries easily. Good for stripping and reprobing.

PVDF (polyvinylidene difluoride) membranes are physically stronger and have higher protein-binding capacity than nitrocellulose. However, they are highly hydrophobic: so much so that they need to be pre-wetted with methanol before they can be equilibrated with aqueous buffer. When handling PVDF, you should take special care to ensure the membrane does not dry out, since uneven blocking, antibody incubation, washing, or detection can result. If the membrane does dry out, it should be re-equilibrated in methanol and then in aqueous buffer. The high affinity of PVDF for protein gives efficient transfer and high detection efficiency, but it can make background control more difficult. PVDF is the membrane of choice for stripping and reprobing.

Transfer membranes are available in several pore sizes. The standard pore size, suitable for most applications, is 0.45 micron. Membranes are also commonly available in 0.2 and even 0.1 micron pore size: these smaller pore sizes are suitable for transfer of lower molecular weight proteins, below about 12 kDa. Transfer efficiency is not good with membranes with a pore size of less than 0.1 micron.

BLOCKING

All transfer membranes have a high affinity for protein. The purpose of blocking is simply to prevent indiscriminate binding of the detection antibodies by saturating all the remaining binding capacity of the membrane with some irrelevant protein. (For a detailed discussion, see Amersham, n.d., from which much of the following is drawn.)

Which Blocking Agent Best Meets Your Needs?

The protein most commonly used for the purpose is nonfat dry milk, often referred to as "blotto," used at 0.5% in PBS containing 0.1% Tween-20. Any grocery-store brand of nonfat dry milk can be used.

Gelatin is isolated from a number of species, but fish skin gelatin is usually considered the best for Western blotting. Fish gelatin is usually used at a concentration of 2%. It is an effective blocker, and will not gel at this concentration at 4°C.

Bovine serum albumin (BSA) is available in a wide range of grades. Usually a blotting or immunological grade of BSA is appropriate. It is less expensive than fish skin gelatin, and can be used at 2%.

Normal serum (fetal calf or horse) is used sometimes, at a concentration of 10%. It can be an effective blocking agent, but is quite expensive. Since serum contains immunoglobulins, it is not compatible with Protein A and some secondary antibodies.

Casein can be used at 1%, but it is very difficult to get dry casein into solution. Casein and casein hydrolysate are the basis of some commercial blocking agents.

Different primary antibodies work better with different blocking agents: nonfat dry milk is usually a good first choice, but when setting up a new method, it is a good idea to evaluate different blockers.

It has been claimed that some blocking agents, nonfat dry milk in particular, can hide or "mask" certain antigens. Of course, there must be no component of the blocking agent that the primary or secondary antibodies can specifically react with.

Some researchers include a second blocking step prior to secondary antibody incubation. However, if the initial blocking is sufficient and reagent dilutions are optimal, this should not be necessary.

A more specific kind of blocking may be needed when avidin or streptavidin is used as a detection reagent and the sample contains biotin-bearing proteins. Because of this "endogenous biotin" the avidin or streptavidin will pick up these undesired proteins directly. If you suspect this may be a problem, a control reaction can be run with no primary antibody but with the avidin or streptavidin detection. The presence of bands in this control reaction will indicate that the avidin or streptavidin is binding to the endogenous biotin.

The remedy for such a situation is to treat the blot prior to antibody incubation first with avidin (to bind all the endogenous

biotin) and then with free biotin (to block all remaining free binding sites on the added avidin). The free biotin is washed away, and antibody detection can proceed (Lydan and O'Day, 1991).

WASHING

Thorough washing is critical to obtaining clean blots, so washing times and solution volumes should always be generous. It is important to realize that protein binding and antibody interactions do not all occur at the surface but rather throughout the entire thickness of the membrane. For this reason, thorough soaking and equilibration of the membrane is critical at every step.

Washing should always be performed at room temperature and with thorough agitation. The exact volume of wash buffer will depend on the container used for washing, but the depth of the solution should be about 1 cm. When protocols call for changing wash solution, this should not be ignored. The higher the sensitivity of the detection method, the more important is scrupulous washing technique.

What Composition of Wash Buffer Should You Use?

Standard wash buffer simply consists of PBS or TBS with added detergent: Tween-20 is routinely used at 0.1%, although Tween concentrations can be raised to as high as 0.3% to help reduce background. Concentrations higher than this tend to disrupt antibody binding. Triton, NP-40 and SDS should not be used, as they may strip off bound antibodies or target proteins.

Another method sometimes used to increase the effectiveness of washing is increasing the concentration of salt in the wash solution. High salt reduces charge-mediated effects, which tend to be less specific, and favors hydrophobic interactions, which are more specific. The usual upper limit for NaCl concentration in wash buffers is 500 mM. (Standard PBS and TBS contain 130 mM NaCl.)

What Are Common Blot Size, Format, and Handling Techniques?

Small blots, or larger blots cut into strips for analysis with several different antibodies, can be incubated in large centrifuge tubes or specialized strip-incubation trays. Larger blots should be incubated in trays. Centrifuge tubes are convenient and allow small reagent volumes to be used. Even with trays, there only needs to be sufficient blocking or antibody solution to submerge

the blot completely and allow free flow of the solution. Be generous, however, with volumes of stripping and washing solutions.

Incubations and washes should be performed with constant agitation. For tubes, a tube-roller or tilting platform can be used. For trays, an orbital platform shaker with adjustable speed is ideal. Antibody incubations are typically carried out for 30 minutes to 1 hour at room temperature; however, they can also be carried out at 4°C overnight. Overnight incubation allows lower antibody concentrations to be used and in some cases results in increased sensitivity. It is important that antibody concentrations be optimized under the same incubation conditions that will be used in the final procedure.

Membranes should never be handled with fingers. A forceps is best, but powder-free gloves can also be used. There is some evidence that residual powder from powdered gloves can mask chemiluminescent signals (Amersham Pharmacia Biotech, 1998).

Blots can be stored directly after transfer in buffer at 4°C overnight. Alternatively, the blocking step can be allowed to go overnight at 4°C without agitation. Blots should not be stored wet for longer than two days, as bacterial growth may occur.

After transfer or after stripping, blots can be air-dried and stored in airtight containers at 4°C. Do not air-dry blots without stripping them first if you intend to reprobe: dried-on antibody is almost impossible to strip.

THE PRIMARY ANTIBODY

Are All Antibodies Suitable for Blotting?

Successful blotting depends largely on the quality of the primary antibody. Not all primary antibodies that react with a target protein in solution will react with that same protein once it is bound to a membrane. During electrophoresis and transfer, proteins become denatured and reduced. This change in the target protein may render it nonreactive with some antibodies, particularly monoclonals. Before starting out, you should make sure that the primary antibody you intend to use is suitable for blotting. This information can be obtained from the originator or suppler of the antibody, or it can be determined by running control blots.

Polyclonal antibodies can be used simply as diluted raw sera, but in many cases (especially with low titer sera) the use of an Ig fraction can reduce background. Affinity purification is ideal, though not always feasible. Ammonium sulfate purification can also provide sufficient purity.

The same purification requirements hold for monoclonal antibodies, but given the small quantities available, especially when obtained from commercial sources, purification is not always practical. You should know the isotype of your primary antibody so you can choose an appropriate secondary reagent. IgMs are often considered less desirable as primary antibodies because they are more difficult to purify and require more specialized secondary reagents.

How Should Antibodies Be Handled and Stored?

Antisera and monoclonal antibodies should be divided into small aliquots, flash-frozen by plunging in a dry ice/ethanol or liquid nitrogen bath, and stored at –70°C. Under these conditions they are stable for years. Once thawed, aliquots should not be frozen and thawed again, but stored at 4°C. Sera and purified monoclonals are stable at 4°C (sometimes for as long as a year), but ascites fluids can contain proteases, so storage at 4°C is not recommended. Repeated freeze–thawing can cause aggregation of antibodies and loss of reactivity. Sodium azide may be used as a preservative at 0.02%.

Antibodies should always be diluted in buffer containing carrier protein. The actual antibody concentration in working solutions is so low that without added carrier, much of the antibody would be lost to adsorption to the walls of containers. Using 0.1% BSA is sufficient. Nonfat dry milk is not recommended, since it is not as clean as laboratory grade albumin and is prone to bacterial growth.

SECONDARY REAGENTS

A wide variety of secondary reagents can be used to detect primary antibodies. Besides secondary antibodies, there are the immunoglobulin-binding proteins Protein A and Protein G, as well as avidin and streptavidin. Some considerations apply to all secondary reagents. In general, secondary reagents are less stable than primary antibodies, since not just antibody binding activity but reporter activity must be retained. In fact stability of the reporter group is the main determinant in secondary antibody stability. Iodinated conjugates are stable for weeks, while enzyme conjugates typically are stable for months. These reagents usually should not be frozen, as repeated freeze–thaw cycles can result in aggregation or loss of reporter activity. Several labs, however, have

reported good results in flash-freezing enzyme conjugates and storing them in single-use aliquots at –70°C.

How Important Is Species Specificity in Secondary Reagents?

The species in which a secondary antibody is raised is not usually important—goats and donkeys are often used because it is possible to obtain large amounts of serum from these animals. "Goat anti-rabbit" is simply an antibody raised against rabbit Ig, produced by immunizing a goat.

A good secondary antibody for blotting should be affinity purified: for example, a raw goat anti-rabbit antiserum is run over a column containing immobilized rabbit Ig. Everything in the serum that doesn't bind to rabbit Ig washes through the column and is discarded. Everything that does bind is then dissociated, eluted, and collected. This affinity-purified secondary antibody will have much less protein than the raw serum: the irrelevant proteins would only contribute to background without increasing the signal.

A further purification step is often performed to ensure species specificity. Cross-adsorption, as the process is known, is in some ways the mirror image of affinity purification. Anti-rabbit Ig is run through a column containing, for example, mouse Ig. Everything that washes through the column without binding is collected, thus removing any antibodies that react with mouse Ig. This process can be repeated with a number of columns containing Ig from different species, ensuring that the resulting antibody will only react with the Ig of a single species. Depending on the nature of your study, this species specificity may or may not be important. If there is not likely to be Ig from other species present in your sample, it is unnecessary. Furthermore no cross-adsorbed secondary reagent is completely species specific: there is enough homology between species that even a cross-adsorbed antibody will pick up a "foreign" Ig if enough of it is present. It is impossible to attain 100% species, class, or isotype specificity in secondary reagents, since there will always be some small degree of homology between the wanted and unwanted target.

Why Are Some Secondary Antibodies Offered as $F(ab')_2$ Fragments?

In blotting, there is usually no advantage to the use of these reagents. The only rare case in which an $F(ab')_2$ fragment would be advantageous would be one in which samples contained Fc

Table 13.3 Reactivity of Protein A and Protein G

Immunoglobulin	Protein A	Protein G
Mouse IgG1	+/–	++
Mouse IgG2a	++	++
Mouse IgG2b	++	++
Mouse IgG3	++	+++
Mouse IgA	–	?
Mouse IgM	+/–	?
Rat IgG1	+/–	?
Rat IgG2a	+/–	+++
Rat IgG2b	+/–	++
Rat IgG2c	+	++
Rat IgM	+/–	?
Goat Ig	+/–	+++
Sheep Ig	–	++
Rabbit Ig	+++	+++
Horse Ig	–	+++

Source: Adapted, with permission, from data provided by Amersham Pharmacia Biotech.
Note:
+++ Strong binding
++ Acceptable binding
+ Weak binding
– No binding
? No data

receptors (as do some bacteria and lymphocytes): the use of $F(ab')_2$ fragments would prevent the background binding of antibodies to these receptors through the Fc portion.

Protein A and Protein G

Protein A and Protein G are bacterial proteins that bind specifically to immunoglobulins from a variety of species. Table 13.3 lists some common immunoglobulins and their reactivity. Why use Protein A and Protein G rather than a secondary antibody? A species-specific secondary antibody will usually give stronger signal and better specificity than Protein A or G. The advantage of Protein A or G is versatility: the same secondary reagent can be used with a variety of primary antibodies. This is especially important for radioactive detection, since a stock of several different secondary antibodies would have to be constantly replenished because of radioactive decay.

Avidin and Streptavidin

Avidin, isolated from egg white, and streptavidin, a bacterial protein, bind biotin with extremely high affinity and specificity. Primary antibodies can be covalently conjugated to biotin, used

on a blot, then detected with avidin or streptavidin. A wide range of avidin and streptavidin conjugates is commercially available. Since any avidin or streptavidin conjugate can be used with any biotinylated reagent, avidin and streptavidin are close to being universal detection reagents.

Some primary antibodies are available in biotinylated form, and there are also kits and reagents available for performing biotinylation in the lab. Coupling is usually accomplished through an *N*-hydroxy-succinimidyl ester, an amine-reactive functional group (Haugland and You, 1998). Ideally antibodies to be labeled by this chemistry should be free of carrier protein, since all proteins in the solution will react. Subsequent purification by column or dialysis is necessary, which means that you need to start with a large enough amount of protein to ensure a reasonable recovery.

Avidin and streptavidin can be used interchangeably. However, streptavidin is not charged at neutral pH and not glycosylated. It therefore tends to yield slightly lower backgrounds and better specificity than avidin.

One very useful application of biotin/streptavidin detection is in the determination of molecular weights. Biotinylated molecular weight markers are commercially available, and they can be run on gels and transferred just like normal molecular weight markers. The blot is treated as usual through primary antibody incubation and washing, but when the secondary antibody incubation is performed, labeled streptavidin is added to the solution so that incubation with secondary antibody (to bind the primary antibody) and streptavidin (to bind the biotinylated markers) take place simultaneously. The streptavidin should be labeled with the same reporter group as the secondary antibody. In this way both the molecular weight markers and the band of interest will show on the blot, without having to separate the blot into different pieces. Determination of molecular weight by electrophoresis is, however, always approximate.

AMPLIFICATION

Several strategies have been used to increase the signal on Western blots by increasing the amount of reporter group that binds to a given amount of target protein. If one primary antibody bound to its target protein results in the binding of, say, 50 HRP molecules rather than 2 or 3, this will clearly result in increased signal.

This approach is often taken through the use of the biotin-streptavidin system. The simplest way to accomplish this would be

a three layer system: primary antibody-biotinylated secondary antibody-streptavidin reporter. The idea is that the binding of the second and third layer takes place on something better than a one-to-one basis; the additional layer multiplies this effect.

The same concept can be carried further through the use of special reporter groups: for example, multimeric complexes of enzyme. Such complexes are commercially available. The guiding idea is to bind as much reporter group as possible to a single primary antibody molecule.

Before chemiluminescent detection systems became widely available, this approach was about the only one used for obtaining very high sensitivity. The amplification methods can still be helpful in boosting the sensitivity of chromogenic detection systems. They can also be used with chemiluminescent systems, but here, the increase in sensitivity may not be balanced out by the higher background: with three layers the optimization becomes much more complex and demanding.

STRIPPING AND REPROBING

It is often an advantage to be able to perform detection on the same blot with more than one antibody. This can be done by dissociating or stripping antibodies off the blot after detection is complete so that the blot can be probed with a new set of antibodies.

Stripping is only feasible in cases where the detection system leaves no precipitate on the blot: colorimetric and chemifluorescent methods are not really suitable. (It is actually possible to strip such blots after treatment with organic solvents to dissolve the precipitate, but this is not recommended since membranes vary in their resistance to solvents and subsequent redetection is often not successful.) An alternative in cases where stripping is not practical is to run duplicate sets of lanes on the same gel and then to cut up the blot after transfer: the different portions of the blot can then be probed with different antibodies.

Will the Stripping Procedure Affect the Target Protein?

While stripping can be very useful, there are limitations to the technique. Treatment harsh enough to dissociate antibodies can be harsh enough to damage or dissociate target proteins. Loss of some target protein in each stripping cycle is inevitable. Sometimes a single treatment can result in complete loss of target protein (or at least its immunoreactivity). Even in favorable cases, 25% or more of the target can be lost in one stripping cycle. For

this reason it is a good practice to probe for the least abundant target protein first, and then to move on to increasingly abundant proteins where more target loss can be tolerated.

The most common stripping technique uses 2% SDS and 100 mM 2-mercaptoethanol (2-ME) or dithiothreitol (DTT) and heating with agitation at 50 to 65°C, preferably in a fume hood (Amersham Pharmacia Biotech, 1998). This method is effective but can result in pronounced target loss. Another method is incubation at room temperature with glycine buffer at pH 2. This is more gentle but may not be as effective. With either method, thorough washing is necessary afterward. Reblocking is also necessary, as the stripping treatment tends to remove the blocking agent.

The effectiveness of stripping can be verified by repeating the secondary antibody incubation and detection steps (i.e., with no primary antibody). This should be done at least at the outset to confirm that the chosen stripping method is effective.

Can the Same Stripping Protocols Be Used for Membranes from Different Manufacturers?

In most cases the same protocols can be used with membranes of the same kind from different manufacturers. Unless there is something unique about a particular membrane, standard protocols can be followed.

Is It Always Necessary to Strip a Blot before Reprobing?

There are some situations in which blots can be redetected without first stripping. When peroxidase is used as a reporter group in chemiluminescent blots, the blot can be treated with dilute hydrogen peroxide (30 minutes in 15% H_2O_2 in PBS, followed by thorough washing). The radicals formed in the peroxidase reaction will irreversibly inactivate the enzyme. The blot can then be washed and carried through subsequent redetection with another primary antibody. This method, however, is only suitable in cases in which two different, non-cross-reacting secondary reagents are used. Otherwise, the secondary reagent used in the second detection cycle will pick up both the original and the new primary antibodies.

TROUBLESHOOTING

It is important to develop rational troubleshooting strategies (see Table 13.4). Problems are inevitable, so taking a systematic approach to troubleshooting will, in the long run, save time,

Table 13.4 Western Blotting Troubleshooting Logic Tree

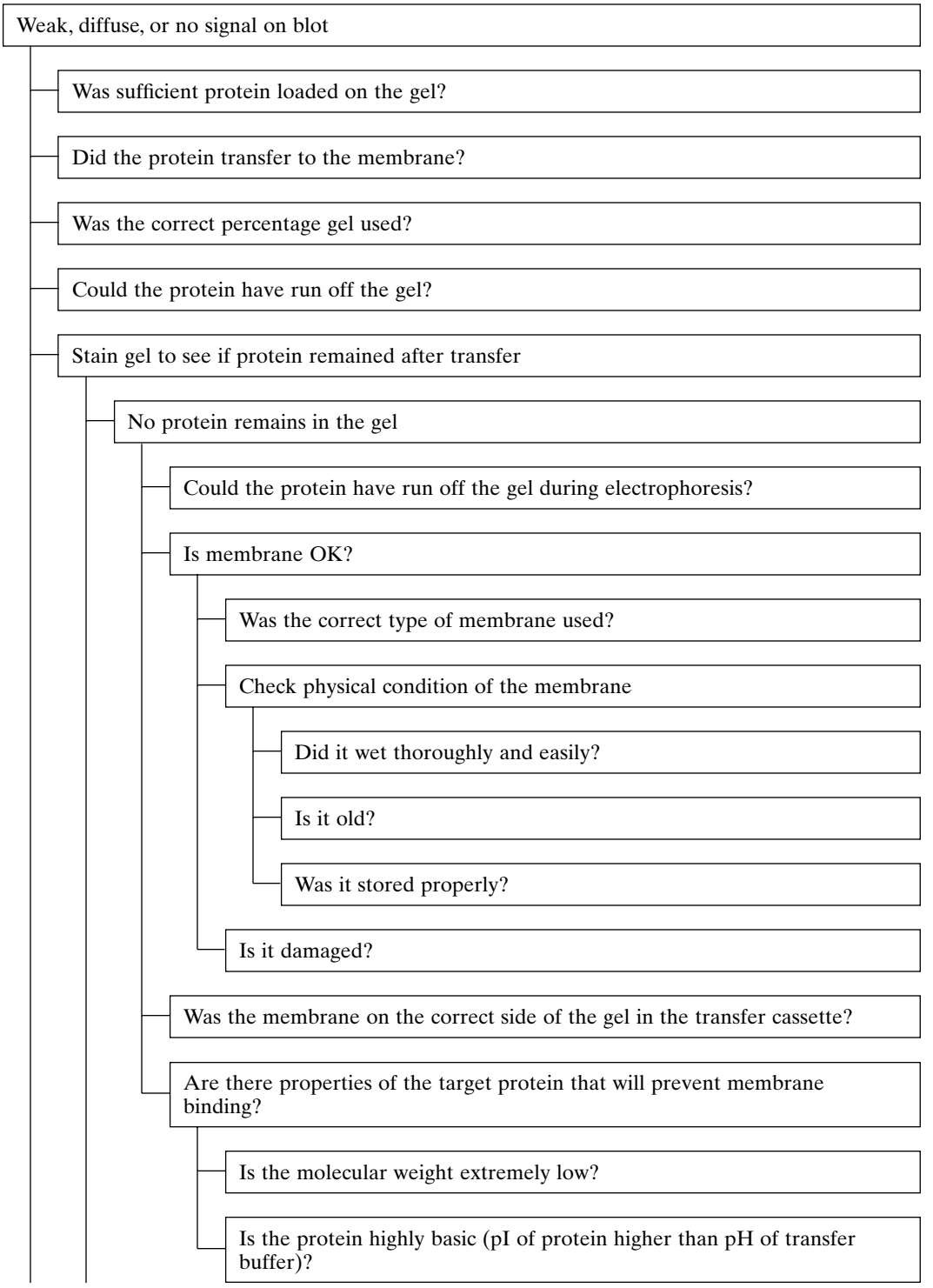


Table 13.4 (*Continued*)

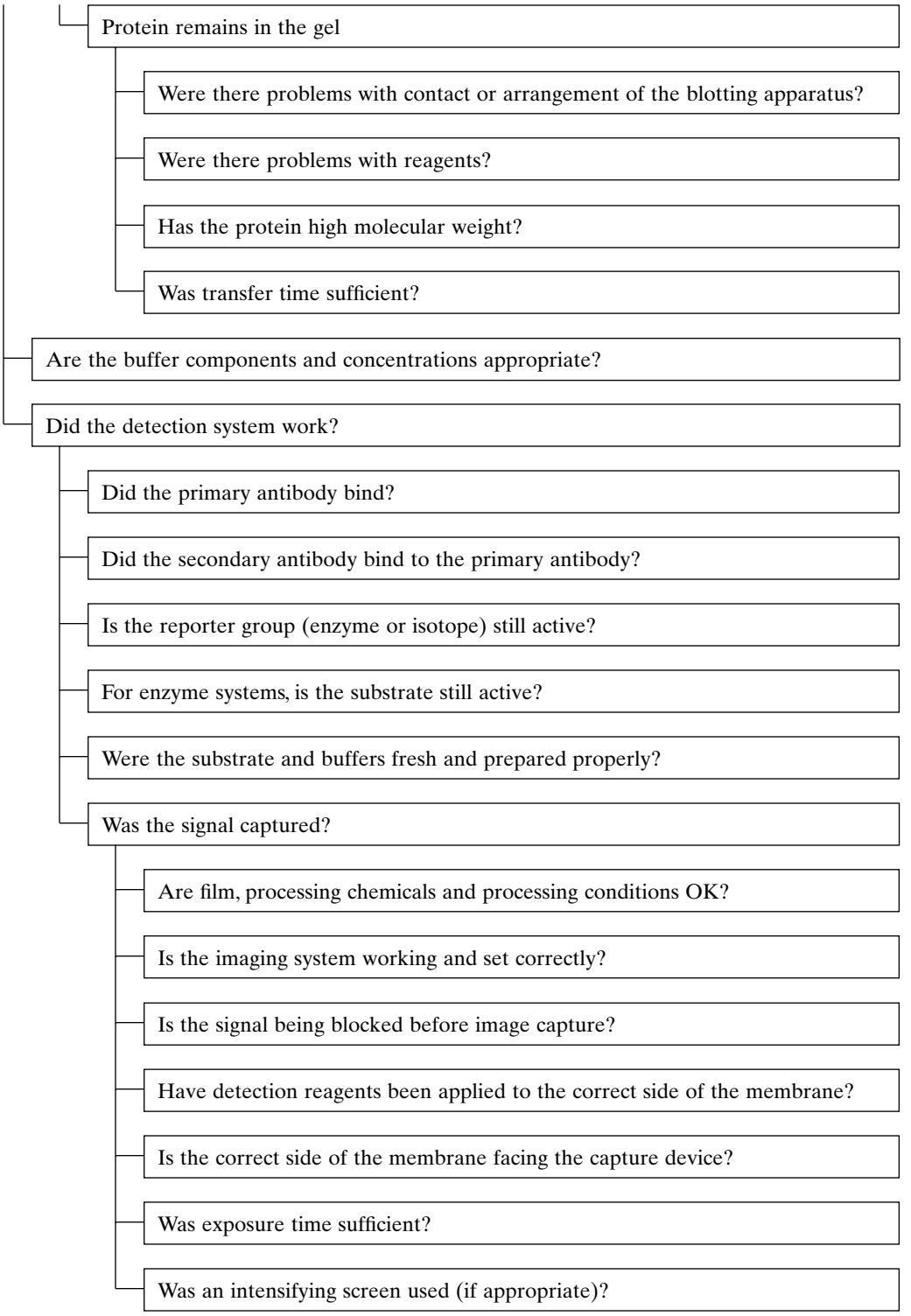
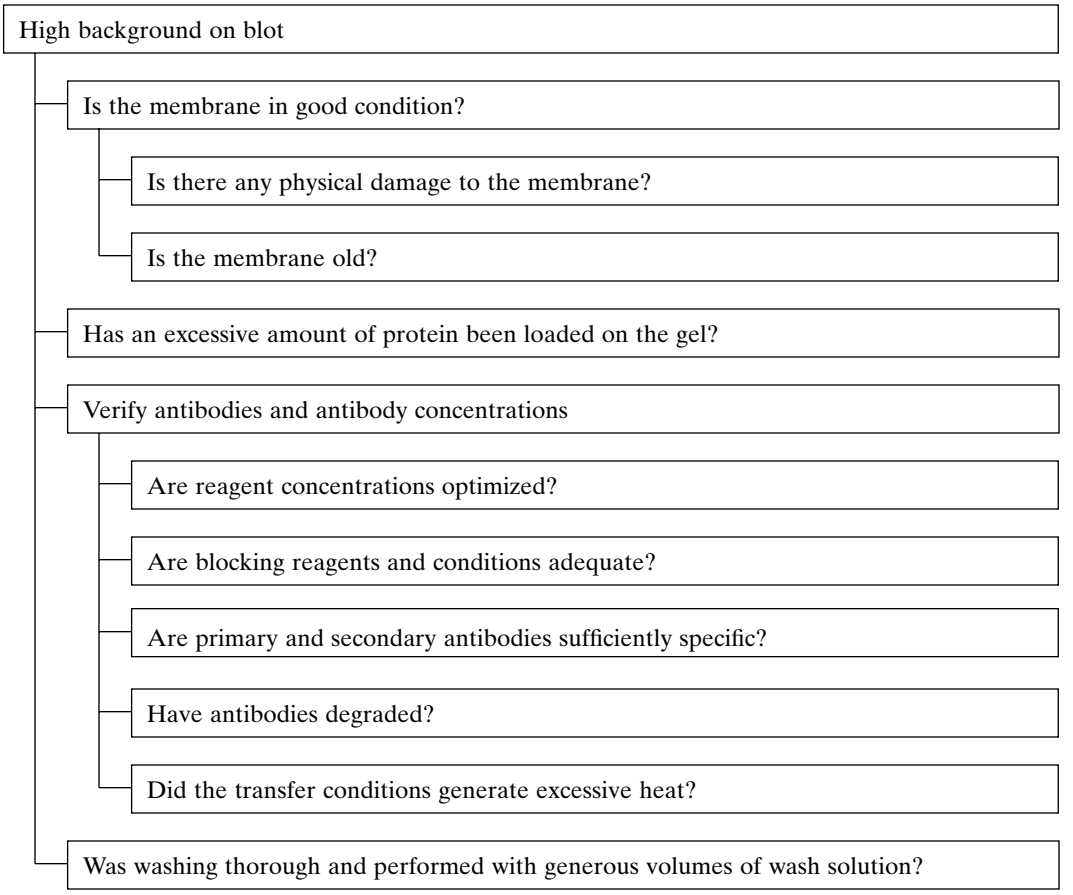


Table 13.4 (*Continued*)



energy, and reagents. Examples of common and unusual problems are illustrated in Figures 13.1–13.6.

The guiding principle is to break the system into its component parts, and test each step in isolation. This ideal is not possible in every case. Rather, those components that can be isolated should be. Once validated, they can be used to test the other components.

Consider the case of weak or no signal. The first step would be to review your system overall and make sure there are no reagent incompatibilities. Certain detection reagents are incompatible with common buffers and buffer additives. Sodium azide is a powerful peroxidase inhibitor. Although it is often used as a buffer preservative, peroxidase conjugates must not be diluted in azide-containing buffer, nor should wash buffers containing azide be used with peroxidase conjugates. The presence of azide in con-

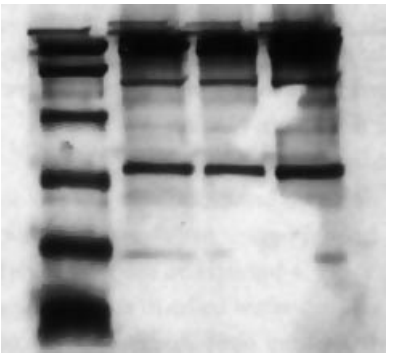


Figure 13.1 Western blot of fluorescein labeled Brome Mosaic Viral proteins prepared using a rabbit reticulocyte in vitro translation system, detected using an anti-fluorescein peroxidase conjugate and ECL. This effect is caused by poor contact between the polyacrylamide gel and the membrane in the electroblotting apparatus. Ensure that all fiber pads are of sufficient thickness; with use these pads will flatten. Periodically they must be replaced. Published by kind permission of Amersham Pharmacia Biotech UK Limited.

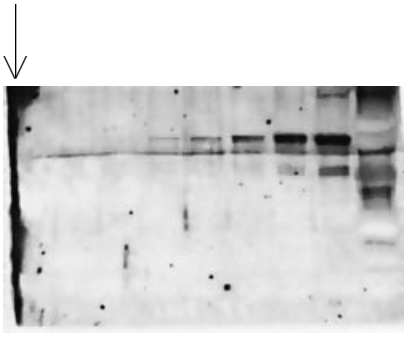


Figure 13.2 Rat brain homogenate Western blot immunodetected using an anti-transferrin antibody and ECL. This effect is caused by damage at the cut edge of the membrane resulting in a high level of nonspecific binding of the antibodies used during the immunodetection procedure. Membranes should be prepared using a clean sharp cutting edge, for example, a razor blade or scalpel. Published by kind permission of Amersham Pharmacia Biotech UK Limited.

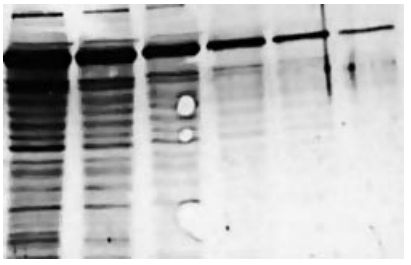


Figure 13.3 K562 cell lysate Western blot immunodetected using an anti-transferrin antibody and ECL. Air bubbles trapped between the gel and the membrane prevent transfer of the proteins, so no signal is produced. Air bubbles should be removed by rolling a clean pipette or glass rod over the surface of the polyacrylamide gel/membrane before assembling the electroblotting apparatus. Published by kind permission of Amersham Pharmacia Biotech UK Limited.

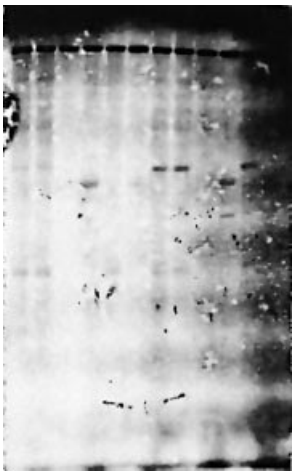


Figure 13.4 Western blot of fluorescein labelled Brome Mosaic Viral proteins prepared using a rabbit reticulocyte in vitro translation system, detected using an anti-fluorescein-peroxidase conjugate and ECL. This effect is caused by using dirty fiber pads in the electroblotting apparatus. The fiber pads should be cleaned after each use by soaking in Decon™ and rinsing thorougly in distilled water. Periodically the fiber pads must be replaced. Published by kind permission of Amersham Pharmacia Biotech UK Limited.

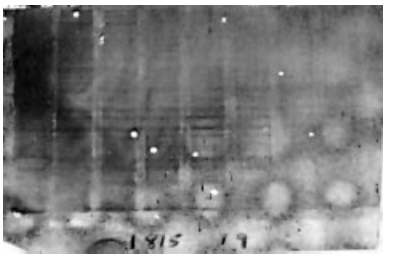


Figure 13.5 Rat brain homogenate Western blot stained with AuroDye Forte, a total protein stain. This effect is caused by fiber pads that are too thick for the electroblotting apparatus. Published by kind permission of Amersham Pharmacia Biotech UK Limited.

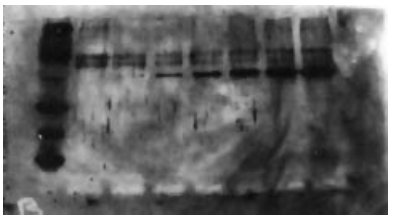


Figure 13.6 Rat brain homogenate Western blot detection of β-tubulin with the ECL Western blotting system. This effect is caused by too strong a dilution of secondary antibody. Antibodies and streptavidin conjugates should be titrated for optimum results. Published by kind permission of Amersham Pharmacia Biotech UK Limited.

centrated stocks of primary antibodies is not a problem, however, because the azide will be diluted and washed away before the HRP conjugate is applied.

Alkaline phosphatase should not be used with phosphate buffers. Use TRIS instead. The presence of phosphate will inhibit the phosphatase reaction.

Avidin and streptavidin should not be diluted in buffers containing nonfat milk. Nonfat milk contains free biotin, which will bind to avidin or streptavidin with high affinity, preventing binding with your biotinylated antibody (Hoffman and Jump, 1989).

If there are no problems with the choice of reagents, the next step is to demonstrate that all the components are functioning properly. Start by verifying the detection system. With many detection systems, function can be verified directly: chemiluminescent reagents can be quickly tested by adding enzyme conjugate to the prepared substrate in the darkroom and observing the production of light. In other systems, spots of diluted secondary antibody can be applied directly to membrane and carried through the detection step. If the secondary antibody shows up, the detection reagents are not at fault.

Backtracking further, the primary antibody can be spotted on membrane, the membrane blocked, incubated with the secondary antibody, and carried through the detection. This shows that the secondary antibody is able to detect the primary antibody. If this is not the problem, purified antigen or lysate can be serially diluted, dotted on the membrane, and carried through primary and secondary antibody incubations and detection. This shows the primary antibody is able to detect the target. If the problem still isn't apparent, then the transfer must be verified. The transfer of colored molecular weight markers does not always indicate efficient transfer of target proteins. It is best to verify transfer by use of a reversible stain like Ponceau S (Salinovich and Montelaro, 1986).

With the proliferation of high-sensitivity detection methods, high background is now probably the most common problem encountered in Western blotting. In trying to solve background problems, the first step is to stop and examine the offending blots carefully. Is the background occurring all over the blot (i.e., over the lanes and the areas between the lanes), or is it confined to the lanes themselves (i.e., extra bands, or in some cases, the entire lane showing up)?

Background over the entire blot suggests something general such as washing or blocking conditions. Check your procedures: Is your washing thorough and complete? Are you using sufficient volumes of wash solution? If you are already washing thoroughly, then it may be necessary to reassess your blocking conditions. Finally, greatly excessive antibody concentrations can cause generalized background: make sure you've optimized antibody concentrations.

Background confined to the lanes is more likely to be related to non-specific antibody binding. Again, be sure that you have optimized all your antibody concentrations. In order to pinpoint the problem, it may be a good idea to run a control blot with no primary antibody. If bands show up in the absence of primary antibody, the problem can be assigned to the secondary antibody; in most cases the concentration of secondary antibody is simply too high. Otherwise, your secondary antibody may have some specific affinity for something in your samples. If this is the case, the only choice is to switch to a different secondary antibody or even a different detection approach (e.g., Protein A or biotin/streptavidin).

With other problems the guiding principle is still the same: to try to glean as much information from the problem blot as possible, to isolate each step in the process, and change only one variable at a time. Holding each variable constant except for one makes each experiment decisive. This is the kind of situation in which detailed record-keeping is critical. When the performance of a system changes, carefully going back over records often will suggest the source of the trouble.

SETTING UP A NEW METHOD

When setting up a new method, it may appear that there is an impossible number of choices that need to be made all at once. Actually, it's not so difficult. Your decision to go with another method should be based on the properties of your protein of interest, the availability and nature of your samples, your needs for reprobing or quantitation, and the nature of your facilities. Read up on the relevant literature, and, at least in the beginning, base your protocol on a published method.

An important issue that needs to be addressed in setting up a new method is optimization of antibody concentrations. These concentrations will be different for every system. They can most easily be established through dot or slot blots: the target protein (either lysate or purified protein) is spotted on membrane and blocked. Detection is then carried out using varying dilutions of primary antibody. (To begin with, use the secondary antibody at the manufacturer's recommended dilution.) The maximum dilution of primary antibody that yields a usable signal should be your working dilution. The same process is repeated for the secondary antibody, using for the primary antibody the dilution you previously established. Again, the minimum concentration of secondary antibody that gives usable signal should be chosen. The use of

minimum concentrations of primary and secondary antibodies helps ensure the greatest specificity with the minimum background (while at the same time conserving reagents).

For blocking and washing conditions, start by following a published method. If your model method was developed for the same protein you are looking at, then you can simply follow these conditions exactly. If you are looking at a new protein, 0.5% nonfat dry milk with 0.1% Tween-20 is probably the best blocking agent to start with. If you experience high background or other unexpected results, then you may want to evaluate other blockers, look at other washing conditions, consider loading less protein on your gels, or re-examine the optimization of antibody concentrations.

BIBLIOGRAPHY

Amersham Life Science. n.d. *A Guide to Membrane Blocking Conditions with ECL Western Blotting.* Tech Tip 136. Amersham Life Science Inc., Arlington Heights, IL.

Amersham Pharmacia Biotech. 1998. *ECL Western Blotting Analysis System.* Amersham Pharmacia Biotech, Piscataway, NJ.

Haugland, R. P., and You, W. W. 1998. Coupling of antibodies with biotin. *Meth. Mol. Biol.* 80:173–183.

Hoffman, W. L., and Jump, A. A. 1989. Inhibition of the streptavidin-biotin interaction by milk. *Anal. Biochem.* 181:318–320.

Linscott, W. 1999. *Linscott's Directory of Immunological and Biological Reagents*, 10th ed. Linscott, Mill Valley, CA.

Lissilour, S., and Godinot, C. 1990. Influence of SDS and methanol on protein electrotransfer to Immobilon P membranes in semidry blot systems. *Biotech.* 9:397–398, 400–401.

Lydan, M. A., and O'Day, D. H. 1991. Endogenous biotinylated proteins in *Dictyostelium discoideum*. *Biochem. Biophys. Res. Commun.* 174:990–994.

Salinovich, O., and Montelaro, R. C. 1986. Reversible staining and peptide mapping of proteins transferred to nitrocellulose after separation by sodium dodecylsulfate-polyacrylamide gel electrophoresis. *Anal. Biochem.* 156:341–347.