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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

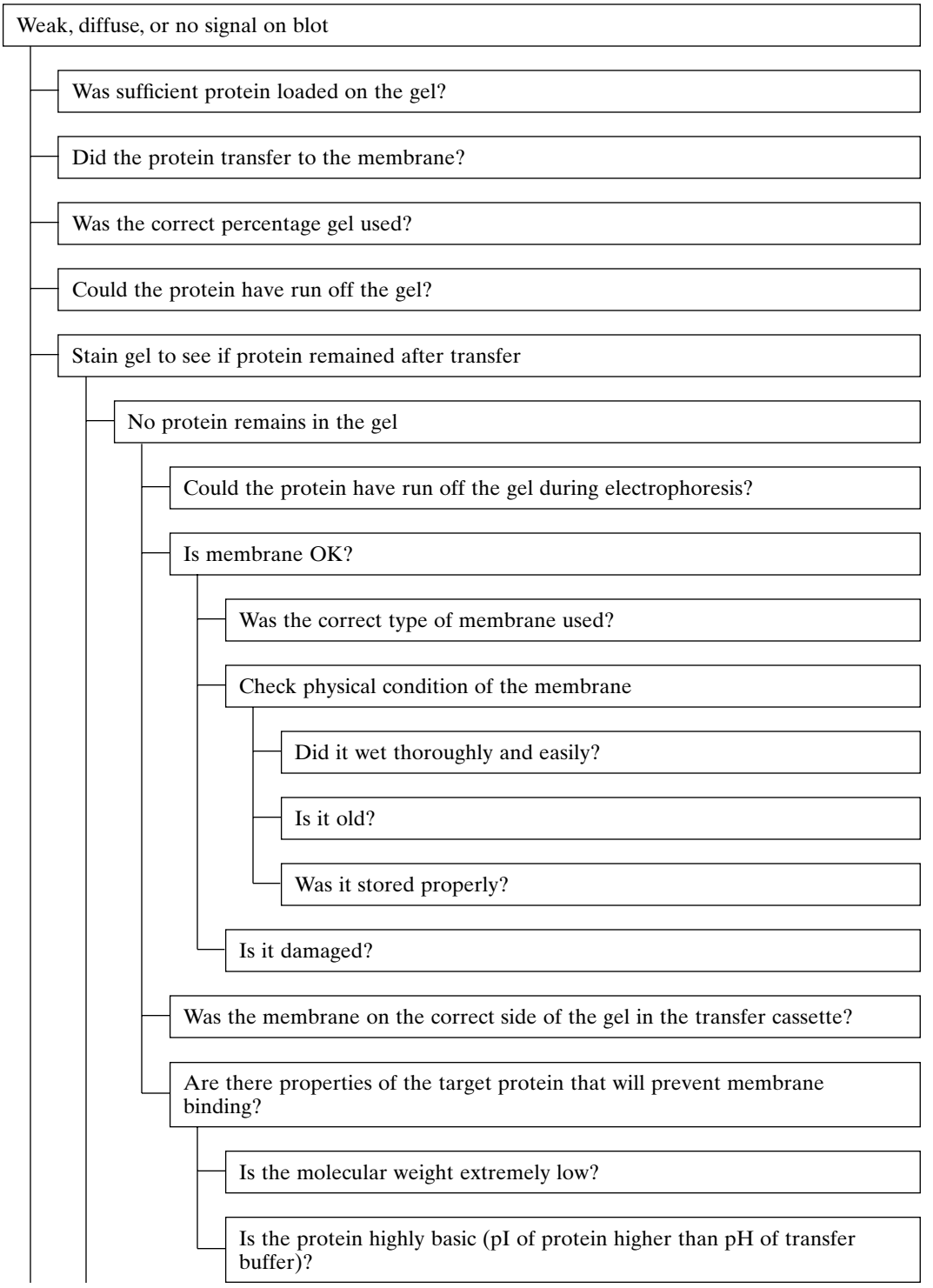


Table 13.4 (*Continued*)

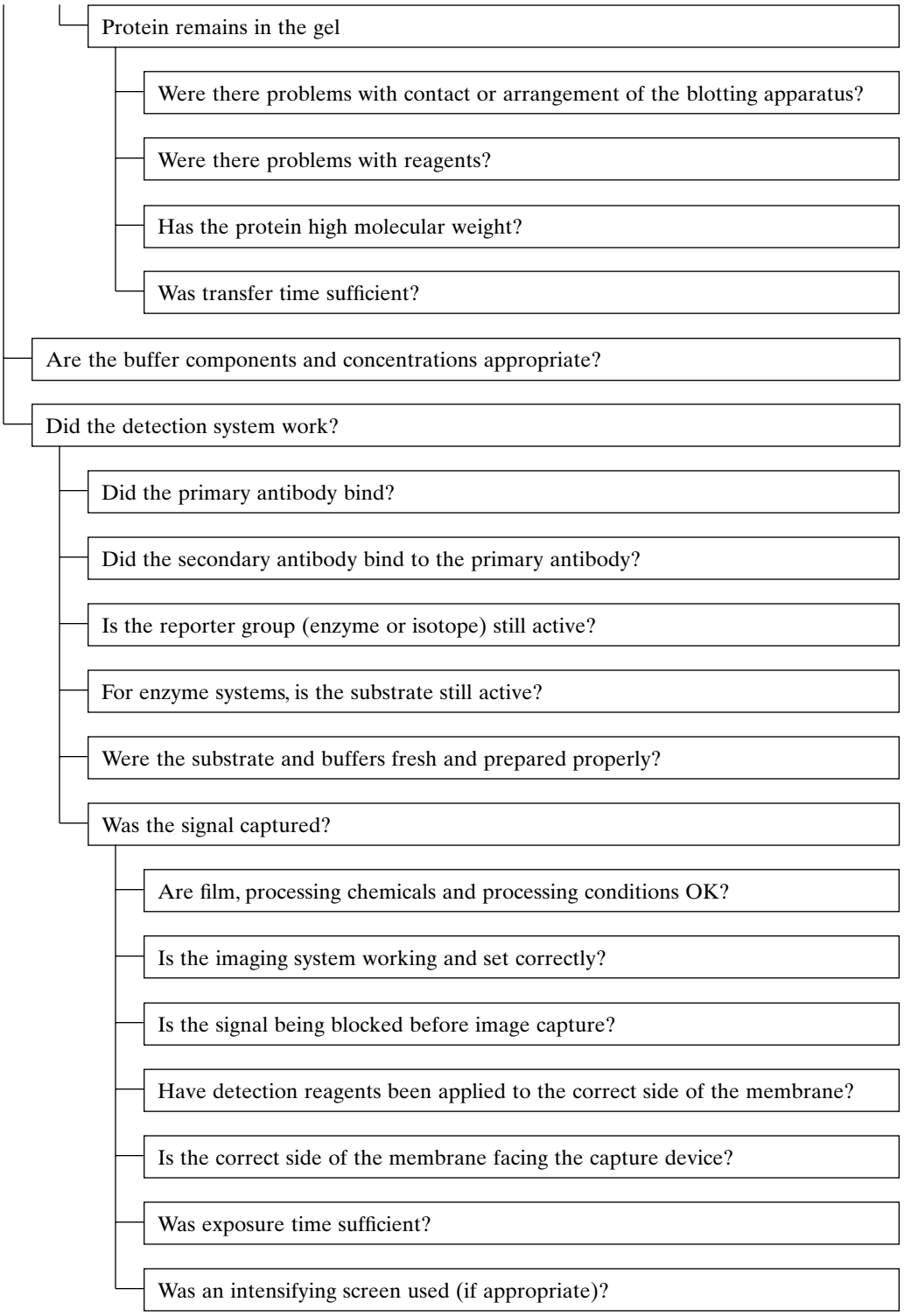
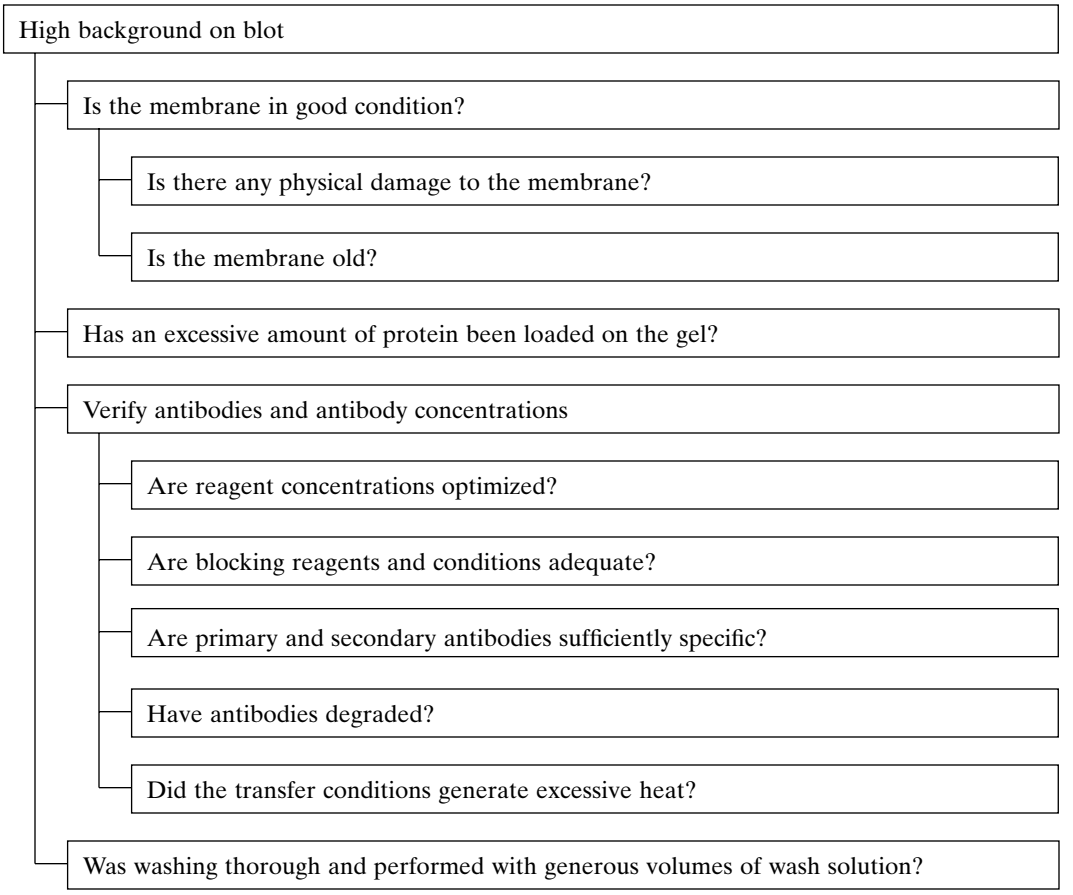


Table 13.4 (*Continued*)



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-

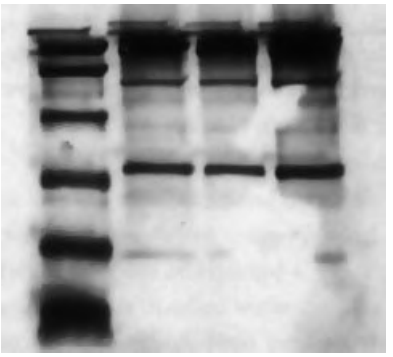


Figure 13.1 Western blot of fluorescein labeled Brome Mosaic Viral proteins prepared using a rabbit reticulocyte in vitro translation system, detected using an anti-fluorescein peroxidase conjugate and ECL. This effect is caused by poor contact between the polyacrylamide gel and the membrane in the electroblotting 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.

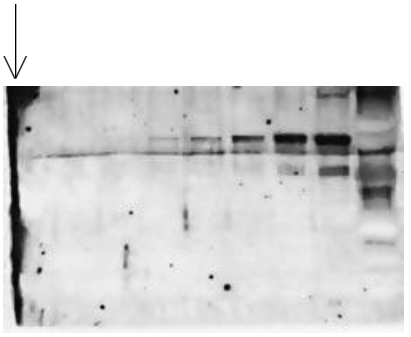


Figure 13.2 Rat brain homogenate Western blot immunodetected using an anti-transferrin antibody and ECL. This effect is caused by damage at the cut edge of the membrane resulting in a high level of nonspecific binding of the antibodies used during the immunodetection procedure. Membranes should be prepared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

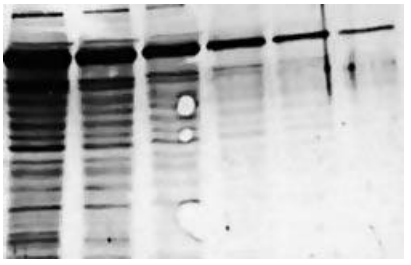


Figure 13.3 K562 cell lysate Western blot immunodetected using an anti-transferrin antibody and ECL. Air bubbles trapped between the gel and the membrane prevent transfer of the proteins, so no signal is produced. Air bubbles should be removed by rolling a clean pipette or glass rod over the surface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

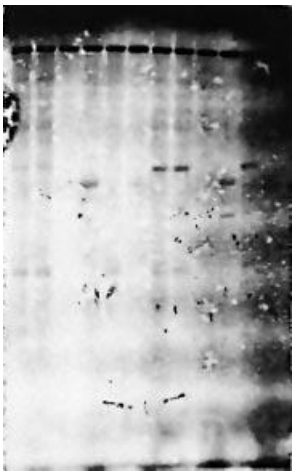


Figure 13.4 Western blot of fluorescein labelled Brome Mosaic Viral proteins prepared using a rabbit reticulocyte in vitro translation system, detected using an anti-fluorescein-peroxidase conjugate and ECL. This effect is caused by using dirty fiber pads in the electroblotting apparatus. The fiber pads 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.

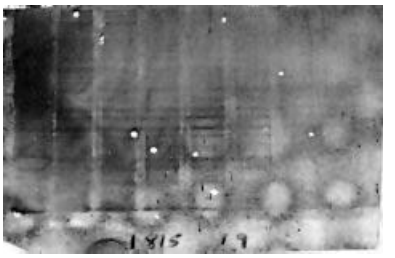


Figure 13.5 Rat brain homogenate Western blot stained with AuroDye Forte, a total protein stain. This effect is caused by fiber pads that are too thick for the electroblotting apparatus. Published by kind permission of Amersham Pharmacia Biotech UK Limited.

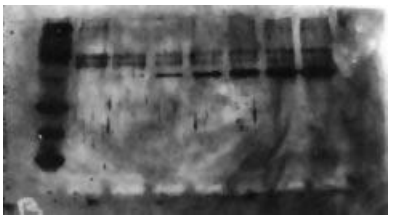


Figure 13.6 Rat brain homogenate Western blot detection of β-tubulin with the ECL Western blotting system. This effect is caused by too strong a dilution of secondary antibody. Antibodies and streptavidin conjugates should be titrated for optimum results. Published by kind permission of Amersham Pharmacia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

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14

Nucleic Acid Hybridization

Sibylle Herzer and David F. Englert

PLANNING A HYBRIDIZATION EXPERIMENT

Hybridization experiments usually require a considerable investment in time and labor, with several days passing before you obtain results. An analysis of your needs and an appreciation for the nuances of your hybridization event will help you select the most efficient strategies and appropriate controls.

The Importance of Patience

Hybridization data are the culmination of many events, each with several effectors. Modification of any one effector (salt concentration, temperature, probe concentration) usually impacts several others. Because of this complex interplay of cause and effect, consider an approach where every step in a hybridization procedure is an experiment in need of optimization. Manufacturers of hybridization equipment and reagents can often provide strategies to optimize the performance of their products.

What Are Your Most Essential Needs?

Consider your needs before you delve into the many hybridization options. What criteria are most crucial for your research—speed, cost, sensitivity, reproducibility or robustness, and qualitative or quantitative data?

Visualize Your Particular Hybridization Event

Consider the possible structures of your labeled probes and compare them to your target(s). Be prepared to change your labeling and hybridization strategies based on your experiments.

What Do You Know About Your Target?

The sensitivity needs of your system are primarily determined by the abundance of your target, which can be approximated according to its origin. Plasmids, cosmids, phagemids as colony lifts or dot blots, and PCR products are usually intermediate to high-abundance targets. Genomic DNA is considered an intermediate to low-abundance target. Most prokaryotic genes are present as single copies, while genes from higher eukaryotes can be highly repetitive, of intermediate abundance, or single copy (Anderson, 1999). However, sensitivity requirements for single-copy genes should be considered sample dependent because some genes thought to be single copy can be found as multiples. Lewin (1993) provides an example of recently polyploid plants whose genomes are completely repetitive. The RNA situation is more straightforward; 80% of RNA transcripts are present at low abundance, raising the sensitivity requirements for most Northerns or nuclease protection assays (Anderson, 1999).

If you're uncertain about target abundance, test a series of different target concentrations (van Gijlswijk, Raap, and Tanke, 1992; De Luca et al., 1995). Manufacturers of detection systems often present performance data in the form of target dilution series. Known amounts of target are hybridized with a probe to show the lowest detection limit of a kit or a method. Mimic this experimental approach to determine your sensitivity requirements and the usefulness of a system. This strategy requires knowing the exact amount of target spotted onto the membrane.

What Do You Know about Your Probe or Probe Template?

The more sequence and structural information you know about your probe and target, the more likely your hybridization will deliver the desired result (Bloom et al., 1993). For example, the size and composition of the material from which you will generate your probe affects your choice of labeling strategy and hybridization conditions, as discussed in the question, *Which Labeling Strategy Is Most Appropriate for Your Situation?* GC content, secondary structure, and degree of homology to the target should be taken into account, but the details are beyond the scope of this chapter. (See Anderson, 1999; Shabarova, 1994; Darby, 1999; Niemeyer, Ceyhan, and Blohm, 1999; and *http://www2.cbm.uam.es/jlcastrillo/lab/protocols/hybridn.htm* for in-depth discussions.)

Is a More Sensitive Detection System Always Better?

Greater sensitivity can solve a problem or create one. The more sensitive the system, the less forgiving it is in terms of background. A probe that generates an extremely strong signal may require an extremely short exposure time on film, making it difficult to capture signal at all or in a controlled fashion.

Femtogram sensitivity is required to detect a single-copy gene and represents the lower detection limit for the most sensitive probes. Methods at or below femtogram sensitivity can detect 1 to 5 molecules, but this increases the difficulty in discerning true positive signals when screening low-copy targets (Klann et al., 1993; Rihn et al., 1995). Single-molecule detection is better left to techniques such as nuclear magnetic resonance or mass spectrometry.

The pursuit of hotter probes for greater sensitivity can be an unnecessary expense. Up to 56% of all available sites in a 486 nucleotide (nt) transcript could be labeled with biotinylated dUTP, but 8% was sufficient to achieve similar binding levels of Streptavidin than higher-density labeled probes (Fenn and Herman, 1990). Altering one or more steps of the hybridization process might correct some the above-mentioned problems. The key is to evaluate the true need, the benefits and the costs of increased sensitivity.

What Can You Conclude from Commercial Sensitivity Data?

Manufacturers can accurately describe the relative sensitivities of their individual labeling systems. Comparisons between labeling systems from different manufacturers are less reliable because each manufacturer utilizes optimal conditions for their system. Should you expect to reproduce commercial sensitivity claims? Relatively speaking, the answer is yes, provided that you optimize your strategy. However, with so many steps to a hybridization experiment (electrophoresis, blotting, labeling, and detection), quantitative comparisons between two different systems are imperfect. Side-by-side testing of different detection systems utilizing the respective positive controls or a simple probe/target system of defined quantities (e.g., a dilution series of a housekeeping gene) is a good approach to evaluation.

LABELING ISSUES

Which Labeling Strategy Is Most Appropriate for Your Situation?

Each labeling strategy provides features, benefits, and limitations, and numerous criteria could be considered for selecting the

most appropriate probe for your research situation (Anderson, 1999; Nath and Johnson, 1998; Temsamani and Agrawal, 1996; Trayhurn, 1996; Mansfield et al., 1995; Tijssen, 2000). The questions raised in the ensuing discussions demonstrate why only the actual experiment, validated by positive and negative controls, determines the best choice.

The purpose of the following example is to discuss some of the complexities involved in selecting a labeling strategy. Suppose that you have the option of screening a target with a probe generated from the following templates: a 30 base oligo (30 mer), a double-stranded 800 bp DNA fragment, or a double-stranded 2 kb fragment.

30-mer

The 30-mer could be radioactively labeled at the 5′ end via T4 polynucleotide kinase (PNK) or at the 3′ end via Terminal deoxynucleotidyl transferase (TdT). PNK attaches a single molecule of radioactive phosphate whereas TdT reactions are usually designed to add 10 or less nucleotides. PNK does not produce the hottest probe, since only one radioactive label is attached, but the replacement of unlabeled phosphorous by ^{32}P will not alter probe structure or specificity. TdT can produce a probe containing more radioactive label, but this gain in signal strength could be offset by altered specificity caused by the addition of multiple nucleotides. A 30 mer containing multiple nonradioactive labels could also be manufactured on a DNA synthesizer, but the presence of too many modified bases may alter the probe's hybridization characteristics (Kolocheva et al., 1996).

800 bp fragment

The double-stranded 800 bp fragment could also be end labeled, but labeling efficiency will vary depending on the presence of blunt, recessed, or overhanging termini. Since the complementary strands of the 800 bp fragment can reanneal after labeling, a reduced amount of probe might be available to bind to the target. Unlabeled template will also compete with labeled probes for target binding reducing signal output further. However, probe synthesis from templates covalently attached to solid supports might overcome this drawback (Andreadis and Chrisey, 2000).

Random hexamer- or nanomer-primed and nick translation labeling of the 800 bp fragment will generate hotter probes than end labeling. However, they will be heterogeneous in size and specificity, since they originate from random location in the tem-

plate. Probe size can range from about 20 nucleotides to the full-length template and longer (Moran et al., 1996; Islas, Fairley, and Morgan, 1998). However, the bulk of the probe in most random prime labeling reactions is between 200 and 500 nt.

If the entire 800 bp fragment is complementary to the intended target, a diverse probe population may not be detrimental. If only half the template contains sequence complementary to the target, then sensitivity could be reduced. Any attempt to compensate by increasing probe concentration could result in higher backgrounds. However, the major concern would be for the stringency of hybridization. Different wash conditions could be required to restore the stringency obtained with a probe sequence completely complementary to the target.

2 kb DNA Fragment

The incorporation of radioactive label into probes generated by random-primer labeling does not vary significantly between templates ranging from 300 bp to 2 kb, although the average size of probes generated from larger templates is greater (Ambion, Inc., unpublished data). Generating a probe from a larger template could be advantageous if it contains target sequence absent from a smaller template.

The availability of different radioactive and nonradioactive labels could further complicate the situation, but the message remains the same. Visualize the hybridization event before you go to the lab. Consider the possible structures of your labeled probes and compare them to your target(s). Be prepared to change your labeling and hybridization strategies based on your experiments.

What Criteria Could You Consider When Selecting a Label?

One perspective for selecting a label is to compare the strength, duration, and resolution of the signal. One could also consider the label's effect on incorporation into the probe, and the impact of the incorporated label on the hybridization of probe to target. The quantity of label incorporated into a probe can also affect the performance of some labels and the probe's ability to bind its target. Many experienced researchers will choose at least two techniques to empirically determine the best strategy to generate a new probe (if possible).

Signal Strength and Resolution

Signal strength of radioactive and nonradioactive labels is inversely proportional to signal resolution.

Radioactive

Isotope signal strength diminishes in the order: $^{32}P > ^{33}P > ^{35}S > ^{3}H$. When sensitivity is the primary concern, as when searching for a low-copy gene, ^{32}P is the preferred isotope. Tritium is too weak for most blotting applications, but a nucleic acid probe labeled with multiple tritiated nucleotides can produce a useful, highly resolved signal without fear of radiolytic degradation of the probe.

^{3}H and ^{35}S are used for applications such as in situ hybridization (ISH) where resolution is more essential than sensitivity. The resolution of ^{33}P is similar to ^{35}S, but Ausubel et al. (1993) cites an improved signal-to-noise ratio when ^{33}P is applied in ISH.

Nonradioactive

Signal strengths of nonradioactive labels are difficult to compare. It is more practical to assess sensitivity instead of signal strength. The resolution of nonradioactive signals is also more complicated to quantify because resolution is a function of signal strength at the time of detection, and most nonradioactive signals weaken significantly over time. Therefore the length of exposure to film must be considered within any resolution discussion. Background fluorescence or luminescence from the hybridization membrane has to be considered as well. Near-infrared dyes are superior due to low natural near-infrared occurrence (Middendorf, 1992). Some dyes emit in the far red ≥700 nm (Cy7, Alexa Fluor 549, allophycocyanin).

Older nonradioactive, colorimetric labeling methods suffered from resolution problems because the label diffused within the membrane. Newer substrates, especially some of the precipitating chemifluorescent substrates, alleviate this problem. Viscous components such as glycerol are often added to substrates to limit diffusion effects. Colorimetric substrates and some chemiluminescent substrates will impair resolution if the reaction proceeds beyond the recommended time or when the signal is too strong. Hence background can increase dramatically due to substrate diffusion.

Detection Speed

Mohandas Ghandi said that there is more to life than increasing its speed (John-Roger and McWilliams, 1994), and the same holds true for detection systems. Most nonradioactive systems deliver a signal within minutes or hours, but this speed is useless if the system can't detect a low-copy target. Searching for a single-copy gene with a ^{32}P labeled probe might require an exposure of several weeks, but at least the target is ultimately identified.

Signal Duration

Will you need to detect a signal from your blot several times over a period of hours or days? Are you pursuing a low-copy target that requires an exposure time of days or weeks? Would you prefer a short-lived signal to avoid stripping a blot prior to re-probing?

Some nonradioactive detection systems allow for the quick inactivation of the enzyme that generates the signal, eliminating the stripping step prior to re-probing (Peterhaensel, Obermaier, and Rueger, 1998). The effects and implications of stripping are discussed in greater detail later in the chapter.

The practical lifetime of common radiolabels is several days to weeks, and is dependent on the label, the ligand, and its environment, as discussed in Chapter 6, "Working Safely with Radioactive Materials." Some nonradioactive systems based on alkaline phosphatase can generate signals lasting 10 days without marked reduction (personal observation). Some chemifluorescent systems generate a fluorescent precipitate capable of producing a cumulative signal, much like isotopes.

The functional lifetime of fluorescent labels will vary with the chemical nature of the fluorescent tag and the methodology of the application. For example, signal duration of a fluorescent tag could be defined by the number of times the chromophore can be excited to produce a fluorescent emission. Some tags can only be excited/scanned once or a few times, while others are much more stable. Consult the manufacturer of the labeled product for this type of stability information. In systems where an enyzme catalyzes the production of a reagent required for fluorescence, the enzyme's half-life and sufficient presence of fresh substrate can limit the duration of the signal.

Will the Label Be Efficiently Incorporated into the Probe?

The effects of label size, location, and linkage method on the incorporation of nucleotides into DNA or RNA are enzyme-dependent and can be difficult to predict. Small side chains can inhibit nucleic acid synthesis (Racine, Zhu, and Mamet-Bratley, 1993), while larger groups such as biotin might have little or no effect (Duplaa et al., 1993; Richard et al., 1994). In general, nucleotides labeled with isotopes of atoms normally present in nucleotides (^{32}P, ^{33}P, ^{3}H, ^{14}C) will be incorporated by DNA and RNA polymerases more efficiently than nucleotides labeled with isotopes of nonnative atoms. Commercial polyermases are frequently engineered to overcome such incorporation bias.

Some applications will exploit impaired incorporation of labeled nucleotides (Alexandrova et al., 1991). Fluorescein attached to position 5 of cytosine in dCTP inhibits terminal transferase and causes addition of only one to two labeled dNTPs at the 3′ end of DNA. Fluorescein- or biotin-riboUTP have been similarly applied (Igloi and Schiefermayr, 1993).

If no specific data exist regarding incorporation efficiency of your labeled nucleotide–labeling enzyme combination, contact the manufacturer of both products. They will likely be able to provide you with a starting point from which you can optimize your labeling reactions.

Will the Label Interfere with the Probe's Ability to Bind to the Target?

Hybridization efficiency can be altered by a label's chemical structure, its location within the probe, the linker that connects the label to the ligand, and the quantity of label within the probe. Isotopes of elements present in nucleic acids in vivo might not directly alter the probe's structure, but as described below (and in Chapter 6), a label's radioactive emissions can fragment a probe. The importance of label location is demonstrated by comparing the hybridization efficiencies of probes labeled with Cy5™ originating at either C5 or the primary amine attached to C4 of dCTP. Probes labeled throughout their sequence with the C5-linked label hybridize efficiently, and are commonly applied in microarray applications (Lee et al., 2000). Probes containing the label attached to the amine group at C4 do not hybridize efficiently to their targets. The purpose of the C4 amine-label is the addition of a single molecule of Cy5 dCTP to the 3′ end of a sequencing primer (Ansorge et al., 1992). A molecular model to accurately predict the effects of labels on analog conformation, hydrogen bonding, stacking interactions, and hybrid helical geometry has been proposed (Yuriev, Scott, and Hanna, 1999).

The C5 position is also preferred for dUTP (Petrie, 1991; Oshevskii, Kumarev, and Grachev, 1989). C5 is such an attractive labeling site because it does not contribute to base-pairing by hydrogen bonding, and at least some linkers seem to allow positioning of the label attached in position 5 so that helix formation is not impaired. But bulky tags linked to pyrimidines in the C5 position still interfere to some degree with hybridization because of steric hindrance. Other sites on purines and pyrimidines have been used as tag or label attachment points. However, they have only been shown to work as primers, not for internal labeling strategies (Srivastava, Raza, and Misra, 1994).

Linker length and sequence have been shown to have a major impact as well. Very short linkers (4–10 atoms) inhibit incorporation and affect hybridization (Haralambidis, Chai, and Tregear, 1987). Above a certain linker length (>20 atoms), incorporation and hybridization are impaired, and a model of steric hindrance has been postulated to explain this effect (Zhu, Chao, and Waggoner, 1994).

Overlabeling

With few exceptions, radioactive and nonradioactive probes should not be internally labeled to 100% completion. Strong beta-emitters (^{32}P, ^{33}P) will degrade extensively labeled probes. A random primer-generated probe labeled with ^{32}P-dCTP to a specific activity of 10^9 cpm/μg will have an average size of about 300 to 500 nucleotides immediately after labeling. After storage at 4°C for 24 hours, the average size falls to 100 nucleotides or less (Amersham Pharmacia Biotech, unpublished observations). Maximally diluting the probe immediately after labeling can reduce this radiolytic degradation.

High densities of large nonradioactive tags can interfere with duplex formation and strand extension due to steric hindrance (Zhu and Waggoner, 1997; Lee et al., 1992; Day et al., 1990; Mineno et al., 1993). Although linker chains that connect label to nucleotide are designed to minimize interference (Petrie et al., 1991), steric hindrance cannot be completely circumvented.

High label densities can also cause quenching. Quenching effects for fluorescein densities greater than 1 in 10 have been described (Makrigiorgos, Chakrabarti, and Mahmood, 1998). Manufacturers of nonradioactive labeling kits optimize protocols to avoid interference from the label. Consult your manufacturer before you alter a recommended procedure.

RADIOACTIVE AND NONRADIOACTIVE LABELING STRATEGIES COMPARED

The decision to apply radioactive or nonradioactive labeling and detection systems can be based on issues of sensitivity, high-throughput, cost, safety, and ease of use, to name a few criteria discussed in this chapter. While it is feasible on paper to evaluate your research needs against these criteria, the decision must usually be determined at the lab bench. In lieu of the need to test individual systems, several studies compared the sensitivity of nonradioactive probes to the ^{32}P gold standard: Yang et al. (1999),

Plath, Peters, and Einspanier (1996), Nass and Dickson (1995), Moore and Margolin (1993), Puchhammer-Stoeckl, Heinz, and Kunz (1993), Engler-Blum et al. (1993), Bright et al. (1992), Kanematsu et al. (1991), Lion and Haas (1990), Jiang, Estes, and Metcalf (1987), Tenberge et al. (1998), Holtke et al. (1992), Pollard-Knight et al. (1990), Hill and Crampton (1994), Dubitsky, Brown, and Brandwein (1992), and Nakagami et al. (1991).

What Are the Criteria for Considering Direct over Indirect Nonradioactive Labeling Strategies?

Direct labeling strategies utilize probes that are directly conjugated to a dye or an enzyme, which generates the detection signal. Indirect labeling systems utilize probes that contain a hapten that will bind to a secondary agent generating the detection signal; the probe itself does not generate signal. Typical secondary agents are dye- or enzyme-linked antibodies, and enzyme-linked avidin complexes.

Sensitivity

Comparing the sensitivities of indirect and direct strategies is a difficult process. The fluorescent tags or dyes incorporated directly into probes usually have lower sensitivity. Detection limits will vary with tag or label incorporation efficiency, amplification level introduced by the secondary agent, and amplification level added by substrate or dye. In one instance, simply increasing the duration of the labeling reaction within a direct labeling strategy produced more sensitive probes than an indirect labeling strategy which in previous experiments had produced the more sensitive probe (Herzer, P., unpublished observations).

Comparisons are further complicated because direct and indirect labeling or detection strategies demand very different hybridization, washing, and detection procedures. Additionally the performances of these different strategies can vary with the size and structure of a probe or template from which the probe will be synthesized, further complicating any prediction of performance.

Manufacturers of labeling and detection systems can usually provide sensitivity comparisons of their different products that are qualitatively, if not always quantitatively reproducible.

Flexibility

A directly labeled probe can be detected after hybridization and washes; no further blocking or antibody steps are required. Direct, nonradioactive techniques limit choices for hybridization, wash

buffer, and detection options. Optimization of your signal-to-noise ratio might be more difficult. Indirect nonradioactive detection systems are usually compatible with the common hybridization and wash buffers, but subsequent antibody incubation and detection steps can be difficult to optimize.

What Is the Storage Stability of Labeled Probes?

Radioactive

The effect of high and low emissions from radioactive labels, and methods to minimize their impact are discussed in Chapter 6. Ideally probes labeled with ^{32}P, ^{33}P, or ^{35}S should be prepared fresh for each experiment. If you choose to store a radiolabeled probe, the unincorporated label should be removed prior to storage. Damage from radioactive emissions can be minimized by dilution and the addition of free radical scavengers such as ethanol or reducing agents, but the probe must then be re-purified before reuse. As discussed in Chapter 6, it is crucial to fast-freeze radiolabeled probes to avoid complications from clustering.

Nonradioactive

The chemical nature of the tag will dictate specific storage conditions, but in general, nonradioactively labeled probes may be aliquoted and stored in the dark in –20 or –70°C non-frost-free freezers. Multiple freeze–thaws should be avoided. Stability varies depending on storage buffer formulation and the nature of the tag (e.g., fluorescent label and enzyme), and can vary from one month to one year.

Nucleic acids labeled by direct crosslinking of enzymes are supposed to be stable if stored in 50% glycerol at –20°C for several months, but this cannot be guaranteed. Since it is difficult to quantitate the remaining enzyme activity after storage, it is recommended that fresh probes be prepared to ensure that results over time are comparable. For probes labeled with chromophores or fluorophores, it is crucial to contact the manufacturer for the most contemporary storage information. In a system dependent upon a enzyme-labeled antibody, the storage conditions must maintain the integrity of the antibody, the enzyme and the probe.

If you plan to apply any probe (radioactive or not) over the long term, a positive control that can be used to evaluate the probe's effectivenss is highly recommended. Probe stability is also a function of the required sensitivity. If an old preparation of a labeled probe generates the desired signal, the probe was sufficiently stable.

Should the Probe Previously Used within the Hybridization Solution of an Earlier Experiment Be Applied in a New Experiment?

Does Sufficent Probe Remain?

Most blotting applications require and use probe in excess over target and depending on the amount of probe bound to the blot, and on decay and decomposition effects, sufficient probe might remain in the hybridization solution. A dot blot of a dilution series of the target DNA can determine if sufficient probe remains.

Label Potency

Consider the many issues regarding the lifetime and storage stability of labels and tags mentioned above. I (S.H.) have successfully reused ^{32}P random primer-labeled probes up to five days after the initial hybridization experiment. Storage of radiolabeled probes at –20°C in hybridization buffer for a few days or at 4°C overnight is usually not problematic. Reuse of radiolabeled probes is not recommended for high-sensitivity and/or quantitative applications. The storage issues discussed above for radioactive and nonradioactive labels should also be considered here.

It may be worthwhile to re-purify some probes from the buffer for optimal storage. Peptide nucleic acids (PNA) probes are an example where the probe expense may justify the expense of re-purification.

How Should a Probe be Denatured for Reuse?

Probes are stored in hybridization buffer prior to reuse. Such buffers may contain components that will aid the denaturation of probe (e.g., formamide) so that boiling is not required. Heating to temperatures above T_m is sufficient, since at T_m half of the nucleic acid is denatured. Boiling can also destroy buffer components such as blocking reagents, SDS, volume excluders, and label. Heating the hybridization buffer containing the probe to temperatures of 10 to 20°C above the hybridization temperature would be ideal, but 20 to 30°C below the boiling point has to suffice.

Is It Essential to Determine the Incorporation Efficiency of Every Labeling Reaction?

Labeling reactions are straightforward, and with the advent of commercial labeling kits, unlikely to fail. Hence we do not measure incorporation efficiency of every probe that we label. We only begin to question labeling efficiency when hybridization

results are unsatisfactory, a point at which it might be too late to determine incorporation efficiency.

Before skipping any control steps, consider the implications. Minimally, measure incorporation efficiency when working with a new technique, a new probe, a new protocol, or a new kit. Radiolabeled probes need to be purified or at least Trichloroacetic acid (TCA) precipitated to determine labeling efficiency, as discussed in Chapter 7, "DNA Purification." Determining the efficiency of nonradioactive labeling reactions can be more time-consuming, often involving dot blots and/or scanning of probe spots. Follow manufacturer recommendations to determine labeling efficiency of nonradioactive probes.

Is It Necessary to Purify Every Probe?

Unincorporated nucleotides, enzyme, crosslinking reagents, buffer components, and the like, may cause high backgrounds or interfere with downstream experiments. Hybridization experiments where the volume of the probe labeling reaction is negligible in comparison to the hybridization buffer volume do not always require probe cleanup. If you prefer to minimize these risks, purify the probe away from the reaction components.

While there are some labeling procedures (i.e., probes generated by random primer labeling with ^{32}P-dCTP), where unpurified probe can produce little or no background (Amersham Pharmacia Biotech, unpublished observations), such ideal results can't be guaranteed for every probe. When background is problematic, researchers have the option to repurify the probe preparation. Admittedly, this approach wouldn't be of much use if the experiment producing the background problem required a five day exposure. (Purification options are discussed in Chapter 7, "DNA Purification.")

HYBRIDIZATION MEMBRANES AND SUPPORTS

What Are the Criteria for Selecting a Support for Your Hybridization Experiment?

Beyond the information listed below and your personal experience, the most reliable approach to determine if a membrane can be used in your application is to ask the manufacturer for application and or quality control data. Whether a new membrane formulation will provide you with superior results is a matter that can usually be decided only at the bench, and the results can vary for different sets of targets, probes, and detection strategies.

Physical Strength

Nitrocellulose remains popular for low to medium sensitivity (i.e., screening libraries) applications and for situations that require minimal handling. The greater mechanical strength of nylon makes it superior for situations that require repeated manipulation of your blot. Nylon filters may be probed 10 times or more (Krueger and Williams, 1995; Li, Parker, and Kowalik, 1987). Even though nitrocellulose may be used more than once, brittleness, loss of noncovalently bound target during stripping, and decreased stability in harsh stripping solutions make nitrocellulose a lesser choice for reusable blots. Glass supports and chips can be stripped, but stripping efficiency and aging of target on these supports may impair reuse of more than two to three cycles of stripping and reprobing. Supported nitrocellulose is sturdier and easier to handle than pure nitrocellulose, but remember that it needs to be used in the proper orientation.

Binding Capacity

Nylon and PVDF (polyvinylidene difluoride) membranes bind significantly more nucleic acid than nitrocelluose; hence they can generate stronger signals after hybridization. Nucleic acids can be covalently attached to nylon but not to nitrocellulose, as discussed below. Positively charged nylon offers the highest binding capacities. As is the case with detection systems of greater sensitivity, the greater binding capacity of positively charged membranes could increase the risk of background signal. However, optimization of hybridization conditions, such as probe concentration and hybridization buffer composition, will usually prevent background problems. If such optimization steps do not prevent background, a switch to another membrane type, such as to a neutral nylon membrane, might be required. If your signal is too low, try a positively charged nylon membrane. Positively charged nylon is often chosen for nonradioactive applications to ensure maximum signal strength. The quantity of positive charges (and potential for background) can vary by 10-fold between manufacturers. The lower binding capacity of nitrocellulose decreases the likelihood of background problems under conditions that generate a detectable signal.

Thickness

Most membranes are approximately 100 to 150 μm thick. Thickness influences the amount of buffer required per square

centimeter. Thicker membranes soak up more buffer, wet more slowly, and dry application of thicker filters to the surface of a gel can be more difficult.

Pore Size

Pore size limits the size of the smallest fragment that can be bound and fixed onto a membrane, but bear in mind that pore size is an average value. In general, 0.45 μm micron pore sizes can bind oligonucleotides down to 50 bases in length, but the more common working limit is 100 to 150 nucleotides or base pairs. Membranes comprised of 0.22 μm micron pores are recommended for work with the smallest single- and double-stranded DNA fragments. Custom manufacturing of membranes with 0.1 μm pore size is also available. Table 14.1 compares membrane characteristics.

Specialized Application

Microarrays

Glass slides stand apart from membrane supports because glass allows for covalent attachment of oriented nucleic acid, is nonporous and offers low autofluorescence. On a nonporous support, buffer volumes can be kept low, which decreases cost and allows increased probe concentration. Unlike nylon and nitrocellulose membranes, background isn't problematic under these aggressive hybridization conditions. Probes are labeled with different dyes and allow detection of multiple targets in a single hybridization experiment; nylon arrays are often restricted to serial or parallel hybridization, although examples of simultaneous detection on nylon membranes can be found in the literature. (Some references for multiple probes on nylon are Kondo et al. (1998), Holtke et al. (1992), and Bertucci et al. (1999).) These features make glass slides ideal for nonradioactive detection in micro arrays.

Macroarrays

Background problems, high buffer volumes, and hence cost, limit the usefulness of nonradioactive labels for macroarrays on nylon filters. Macroarrays employ thin charged or uncharged nylon membranes to reduce buffer consumption but suffer from low sensitivity due to the high autofluorescence of nylon. Stronger signals derived from enzyme-substrate driven signal amplification compromise resolution and quantitation. Radioactive labels such as ^{33}P are preferred for macroarrays (Moichi et al., 1999; Yano et al., 2000; Eickhoff et al., 2000).

Table 14.1 Characteristics of Membranes Used in Nucleic Acid Blotting Applications

Membrane	Physical Characteristics	Strip or Reprobe	Benefits	Limitations	Recommended Use
Nitrocellulose	Hydrophilic membrane of low solvent resistance and physical strength	Not recommended	Low cost, low background, easy to block	More difficult to handle, flammable, low binding capacity not good for nonrad systems because it binds proteins, not for RNA	Radioactive colony/ plaque lifts, library screens, low sensitivity applications
Nitrocellulose supported		Possibly 2–6 times via gentle conditions	Low cost, low background, easy to block	As for unsupported nitrocellulose	As for unsupported nitrocellulose
PVDF	Hydrophobic membrane of fair solvent resistance/ physical strength with a thermal stability <135C	Yes, strip with SDS, water, formamide	Intermediate binding capacity and hence sensitivity without nitrocellulose drawbacks of brittleness	Fairly new and hence not so well documented method that might be more difficult to optimize	some nonradioactive applications, some mixed protein/ nucleic acid applications
Nylon neutral	Hydrophilic, hygroscopic membrane of good solvent resistance and physical strength	Up to 10 times if under conditions that don't hydrolyze the target		Irreversibly binds many stains, staining often lowers signal	Where nitrocelluose inappropriate and background is problematic with charged nylon
Nylon negatively charged	Hydrophilic, hygroscopic membrane of good solvent resistance and physical strength	As for uncharged nylon	Lowest background of all nylon membranes	Poor specificity of signal/retention	
Nylon positively charged	Hydrophilic, hygroscopic membrane of good solvent resistance and physical strength	As for uncharged nylon	Highest sensitivity; usually optimal for most nonradioactive systems, but some supercharged membranes can interfere with nonradioactive detection systems	Can give high backgrounds if not optimzed properly	

Which Membrane Is Most Appropriate for Quantitative Experiments?

The size of the nucleic acid being transferred, the physical characteristics of the membrane, and the composition of transfer buffer affect the transfer efficiency. There is no magic formula guaranteeing linear transfer of all nucleic acids at all times. Linearity of transfer needs to be tested empirically with dilution series of nucleic acid molecular weight markers.

What Are the Indicators of a Functional Membrane?

Membranes will record every fingerprint, drop of powder, knick, and crease. Always handle membranes with plastic forceps and powder-free gloves.

Membranes should be dry and uniform in appearance. They should be wrinkle- and scratch-free since mechanical damage may lead to background problems in these affected areas. Membranes should wet evenly and quickly. If membranes do appear blotchy or spotty, or seem to have different colors, it is best not to use them. Membranes are hygroscopic, light sensitive, and easily damaged, but as long as membranes are properly stored, may remain functional for years. Please note that manufacturers only guarantee potency for shorter time periods, usually six to twelve months. If the vitality of the membrane is in doubt, a quick dot blot or test of the binding capacity may help. Manufacturers can provide guidelines for assessing binding capacity. Including an untreated, target-free piece of membrane to evaluate background in a given hybridization buffer or wash system can help to troubleshoot background problems.

Can Nylon and Nitrocellulose Membranes Be Sterilized?

Researchers performing colony hyrbidizations often ask about membrane sterilization. While membranes might not be supplied guaranteed to be sterile, they are typically produced and packaged with extreme care, minimizing the likelihood of contamination.

Theoretically it is possible to autoclave membranes, but cycles should be very short (two minutes at 121°C in liquid cycle). Note that such short durations cannot guarantee sterility. Membranes should be removed as soon as the autoclave comes down to a safe temperature, and dried at room temperature. Multiple membranes should be separated by single sheets of Whatman paper. Note that filters may turn brown, become brittle, may shrink and warp and become difficult to align with plates, but this does not interfere with probe hybridization.

Treatment of membranes with 15% peroxide or 98% ethanol at room temperature after crosslinking can also sterilize filters. Peroxide may be more harmful to nucleic acid and filter chemistry over time.

NUCLEIC ACID TRANSFER

What Issues Affect the Transfer of Nucleic Acid from Agarose Gels?

This discussion will focus on the transfer of nucleic acids from agarose gels onto a membrane via passive transfer. Details on the transfer of DNA from polyacrylamide gels are presented in Westermeier (1997).

Active or Passive Techniques

Vacuum, electrophoretic, and downward gravity transfer methods are fast (less than 3 hours) and efficient (greater than 90% transfer). Transfer efficiency depends on thickness and percentage of the gel and nucleic acid concentration or size. Transfer time increases with percentage of agarose, gel thickness, and fragment size. Capillary blotting of RNA larger than 2.5 kb takes more than 12 hours, and downward transfer only 1 to 3 hours (Ming et al., 1994; Chomczynski, 1992; Chomczynski and Mackey, 1994). Speed, low cost, no crushing of gel, and efficient alkaline transfer of RNA are the main reasons why downward transfer is gaining popularity for RNA transfer (Inglebrecht, Mandelbaum, and Mirkov, 1998).

Transfer Buffer

Manufacturers of filter or blotting equipment provide transfer protocols that serve as a starting point for transfer buffer formulation. If nucleic acids are of unusual size or sequence, modified protocols might be required. RNA, small DNA fragments (<100 bp), and nitrocellulose membranes usually require greater salt concentrations. Keep in mind that RNA has a very low affinity for nitrocellulose even at high salt.

The effects of pH on transfer efficiency and subsequent detection of target are many and complex. Transfer buffer pH can directly affect the stabilities of the membrane and the nucleic acid target. Nitrocellulose and some nylon membranes are not stable at pH > 9, and nitrocellulose will not bind DNA at pH above 9 (Ausubel et al., 1993). Some nylon membranes are not stable at acidic pH (Wheeler, 2000). Transfer buffer pH

can also affect signal output and background levels, especially when working with nylon membranes (Price, 1996; McCabe et al., 1997).

Transfer buffer pH can also affect the surface charge of the membrane. Nylon membranes are polyamides. The net charge of unmodified nylon is zero, but the polyamide backbone will become more positive when lowering the pH. Different side groups are introduced into the nylon precursors for the purpose of increasing the positive or negative charge of the membrane. These side chains may alter the membrane's response to the pH of the transfer buffer, which might ultimately affect the ability of a probe to bind to the target nucleic acid. When using an acidic or alkaline transfer buffer, you may want to verify the expected impact of pH on a particular membrane. For further effects of pH and salt concentration, see Khandjian (1985).

Alkaline transfer conditions will fragment and denature nucleic acids, and these effects have been exploited to crosslink DNA after transfer. Prolonged exposure of RNA to mildly alkaline conditions (pH > 9) will degrade RNA, but Inglebrecht, Mandelbaum, and Mirkov (1998) applied alkaline pH for short periods to enhance the transfer of large, problematic RNA. Some membrane manufacturers warn against alkaline transfer of RNA and DNA because of nonuniform results. If the gel is depurinated prior to alkaline or nonalkaline transfer, omission of the neutralization step prior to transfer can reduce signal. Without a neutralization step, depurination continues in the gel.

Depurination

Breakdown of nucleic acids via depurination increases transfer efficiency. Transfer of targets larger than 5 kb, agarose concentrations greater than 1%, and gels thicker than 0.5 cm improve upon depurination. Depurination beyond recommended times will result in reduced sensitivities on hybridization.

Stains

Gels and/or membranes can be stained in order to monitor transfer efficiency, but it is impossible to make an absolute statement regarding whether stains interfere with transfer and subsequent hybridization. Intercalating dyes, such as ethidium bromide or methylene blue, can influence transfer and hybridization efficiency (Thurston and Saffer, 1989; Ogretmen et al., 1993), yet others report no effect of ethidium bromide utilized in Southern hybridization experiments (Booz, 2000). In another instance,

ethidium bromide interfered with transfer onto supercharged nylon membrane (Amersham Pharmacia Biotech, unpublished observation). DNA stains are usually intercalating cations; hence intercalation will be affected by salt concentration. Therefore salt concentration of the transfer buffer might also affect transfer and subsequent hybridization. Tuite and Kelly (1993) also show the interference of methylene blue staining upon subsequent hybridization.

Some newer dyes (SYBR® Gold and SYBR® Green, Molecular Probes Inc.) are promoted as noninterfering stains. Otherwise, in light of the inconsistencies described above, it is best to destain the gel prior to transfer, or to stain a marker lane only. Visualization of DNA on membranes by UV shadowing has been done, but concerns exist about insufficient sensitivity and overfixation of nucleic acids and (Thurston and Saffer, 1989; Herrera and Shaw, 1989).

Staining details are provided in Wilkinson, Doskow, and Lindsey (1991), Wade and O'Conner (1992), Correa-Rotter, Mariash, and Rosenberg, 1992) and at *http://www.mrcgene.com/met-blue.htm*, *http://www.cbs.umn.edu/~kclark/protocols/transfer.html*, *http://www.bioproducts.com/technical/visualizingdnainagarosegels.shtml.*

Physical Perturbations

Air bubbles between gel and membrane, between membrane and filters, and between gel and support will interfere with transfer. Crushed gel sections trap nucleic acids, as does a gel whose surface has dried out. Moving a membrane in contact with a gel after transfer has begun causes stamp or shadow images and/or fuzzy bands.

Should Membranes Be Wet or Dry Prior to Use?

It is best to follow the recommendations from the manufacturer of your particular blotting equipment or membrane; strategies from different suppliers are not always identical.

In general, capillary transfer can benefit from pre-equilibration of membrane and gel. Free floating of gel and membrane in excess (transfer) buffer pre-equilibrates them to the conditions necessary for good transfer, and can reduce transfer time. Another factor to consider is ease of membrane application; some researchers prefer applying a wet membrane to the gel, but this is a matter of personal preference.

If pre-wetting is preferred, nitrocellulose as well as nylon should be pre-wet in distilled water first. Both membranes will wet more quickly and evenly if no salt is present.

Most membranes need not be wet for dot blots. Dots may spread more if the membrane has been pre-wet. Dots and/or slot blot-applied samples will soak more evenly onto dry membranes. Uneven dot spreading due to unevenly wet membrane or damp membrane can lead to asterisk shapes instead of circles or squares.

What Can You Do to Optimize the Performance of Colony and Plaque Transfers?

Single colonies or plaques usually contain millions of target copies, so transfer can afford to be less efficient. Cell lysis and DNA denaturation are achieved in a sodium hydroxide/SDS step. Fixation can also be achieved in this same step when using positively charged membranes. The blotting process is finished by a neutralization step and a filter equilibration step into salt buffers such as SSC prior to fixation. Transfer may be followed with a proteinase K digestion to remove debris and reduce background (Kirii, 1987; Gicquelais et al., 1990). Proteinase K treatment will reduce background signal when using nonradioactive detection systems, especially those based on alkaline phosphatase. Bacterial debris can also be removed mechanically by gentle scrubbing with equilibration buffer-saturated tissue wipes.

Ideally colonies or plaques should be no larger than 1 mm in diameter; colonies smaller than 0.5 mm deliver a more focused signal (*http://www.millipore.com/analytical/pubdbase.nsf/docs/TN1500ENUS.html*). Filters should be "colony side up" during denaturing/neutralization steps. Two different methods have been described for filter treatment: the bath method, where filters are floated or submerged in the buffers, and the wick method, where 3 MM Whatman paper is saturated with buffers. The wick method yields clearer, more focused dots; the "bath" method is less likely to lead to only partial denaturation and loss of signal. Newer protocols skip the denaturing/neutralization steps in favor of a microwaving step (*http://www.ambion.com/techlib/tb/tb_169.html*) or an autoclaving/crosslinking protocol (*http://www.jax.org/~jcs/techniques/protocols/ColonyLifts.html*). These techniques, though difficult to optimize, save time. However, microwaving can warp membranes, making it difficult to align filters with the original agar plate.

CROSSLINKING NUCLEIC ACIDS

What Are the Strengths and Limitations of Common Crosslinking Strategies?

Four different methods for crosslinking nucleic acids to membrane are commonly applied, but the efficiency will vary with the target and the type of membrane.

UV Crosslinking

UV light photoactivates uracil (U) or thymine (T) of RNA and DNA, respectively, such that they react with amine groups on the nylon membrane. Therefore short nucleic acids (<100 bases) with high GC content may bind less efficiently. If the duration of UV exposure is too long, or the UV energy output too high, the hybridization potential of the target is reduced, and so is any subsequent detection signal. Depending on the UV crosslinker and membrane used, membranes can be wet or dry, but settings will depend on the percentage of moisture on the membrane. Hence wet and dry crosslinking times or energy settings are not interchangeable. Nitrocellulose is flammable and may combust during UV crosslinking.

Crosslinking on transilluminators tends to produce inconsistent results because the delivered energy (in microjoules or Watts × time) fluctuates with these instruments. When crosslinking on a UV transilluminator, a 254 nm emission is required, and the optimal time needs to be determined empirically. Because the light source in a UV transilluminator is not calibrated for a preset energy output, one cannot predict how to compensate for an aging UV bulb by increasing the time of crosslinking. Exposing the nucleic acid side (side of membrane in direct contact with gel surface) to a multiple-user transilluminator increases the chance of target degradation and contamination.

Baking

Baking membranes at 80°C drives all water from the nucleic acid and membrane until the hydrophobic nucleotide bases form a hydrophobic bond to the aromatic groups on the membrane. As little as 15 minutes at 80°C may be sufficient. Vacuum baking is used for nitrocellulose to reduce the risk of combustion. Excessive temperature (>100°C) or extended exposure to heat (two hours) will destroy a membrane's ability to absorb buffers efficiently, leading to background problems, loss of signal, and membrane damage.

Alkaline Transfer

Alkaline transfer onto positively charged nylon membranes produces covalent attachment of the nucleic acid, but the process is slow (Reed and Mann, 1985). Transfers of short duration (few minutes versus hours) will not produce covalent attachment. Short transfer time applications, such as slot blots, dot blots, or colony filter lifts should be followed by a fixation step to secure linkage to the membrane. Opinions diverge whether crosslinking after longer alkaline transfer times is necessary. Some researchers skip crosslinking to avoid loss of signal due to overfixation. Others crosslink because loss of nucleic acids due to incomplete fixation is feared.

Alkali Fixation after Salt Transfer

DNA may also be covalently immobilized onto positively charged nylon by laying this membrane onto 0.4 M NaOH—soaked 3 MM Whatman paper for 20–60 minutes. The exact time needs to be determined empirically.

What Are the Main Problems of Crosslinking?

Avoid rinsing membranes prior to to crosslinking, especially with water. Washing with large volumes of low salt solutions, such as 2× SSC, is also risky. Ideally fix nucleic acids first, then stain, wash, and so forth.

UV crosslinking and baking are nonspecific fixation techniques, so any biopolymers present on the filter have the potential to bind, increasing the risk of background and errant signals. Therefore filters should be kept free of dirt and debris. Brown and/or yellow stains observed after alkaline transfer did not interfere with signal or add to background (personal observation). Standard electrophoresis loading dyes do not interfere with transfer and/or fixation.

What's the Shelf Life of a Membrane Whose Target DNA Has Been Crosslinked?

Membranes can be stored between reprobings for a few days in plastic bags or Saran wrap in the refrigerator in 2× SSC. For storage lasting weeks or months, dried blots, kept in the dark, are preferable (note that blots need to be stripped of their probe(s) prior to drying). Dry, dark conditions will minimize microbial contamination and nucleic acid degradation. Dried membranes may be stored in the dark at room temperature in a desiccator at 4°C, or at –20°C in the presence of desiccant.

One reference cited decreased shelf lives for storage at room temperature (Giusti and Budowle, 1992). Blots maintained dry (desiccant for long-term storage), dark, and protected from mechanical damage may be stored safely for 6 to 12 months.

THE HYBRIDIZATION REACTION

The hybridization step is central to any nucleic acid detection technique. Choices of buffer, temperature, and time are never trivial because these effectors in combination with membrane, probe, label, and target form a complex network of cause and effect. Determining the best conditions for your experiment will always require a series of optimization experiments; there is no magic formula. The role of the effectors of hybridization, recommended starting levels, and strategies to optimize them will be the focus of this section. Readers interested in greater detail on the intricacies and interplay of events within hybridization reactions are directed to Anderson (1999), Gilmartin (1996), Thomou and Katsanos (1976), Ivanov et al. (1978), and Pearson, Davidson, and Britten (1977).

How Do You Determine an Optimal Hybridization Temperature?

Hybridization temperature depends on melting temperature (T_m) of the probe, buffer composition, and the nature of the target:hybrid complex. Formulas to calculate the T_m of oligos, RNA, DNA, RNA-DNA, and PNA-DNA hybrids have been described (Breslauer et al., 1986; Schwarz, Robinson, and Butler, 1999; Marathias et al., 2000). Software that calculates T_m is described by Dieffenbach and Dveksler (1995).

The effects of labels on melting temperatures should be taken into consideration. While some claim little effect of tags as large as horseradish peroxidase on hybrid stability/T_m (Pollard-Knight et al., 1990a), others observed T_m changes with smaller base modifications (Pearlman and Kollman, 1990). It will have to suffice that nonradioactive tags may alter the hybridization characteristics of probes and that empiric determination of T_m may be quicker than developing a formula to accurately predict hybridization behavior of tagged probes. Hybridization temperatures should also take into account the impact of hybridization temperature on label stability. Alkaline phosphatase is more stable at elevated temperatures than horseradish peroxidase. Thermostable versions of enzymes or addition of thermal stabilizer such as trehalose

(Carninci et al., 1998) may provide alternatives to hybridization at low temperatures.

When switching from a DNA to an RNA probe, hybridization temperatures can be increased due to the increased T_m of RNA-DNA heteroduplexes. Because of concerns about instability of RNA at elevated temperatures, an alternative approach with RNA probes is the use of a denaturing formamide or urea buffer that allows hybridization at lower temperature.

A good starting point for inorganic (nondenaturing) buffers are hybridization temperatures of 50 to 65°C for DNA applications and 55 to 70°C for RNA applications. Formamide buffers offer hybridization at temperatures as low as 30°C, but temperatures between 37 and 45°C are more common. Enzyme-linked probes should be used at the lowest possible temperature to guarantee enzyme stability.

After hybridization and detection has been performed at the initially selected hybridization temperature, adjustments may be required to improve upon the results. A hybridization temperature that is too low will manifest itself as a high nonspecific background. The degree by which the temperature of subsequent hybridizations should be adjusted will depend on other criteria discussed throughout this chapter (GC content of the probe and template, RNA vs. DNA probe, etc.), and thus hybridization temperature can't be exactly predicted. Most hybridization protocols employ temperatures of 37°C, 42°C, 50°C, 55°C, 60°C, 65°C, and 68°C.

Note that sometimes a clean, strong, specific signal that is totally free of nonspecific background cannot be obtained. Background reduction, especially through the use of increased hybridization temperatures, will result in the decrease of specific hybridization signal as well. There is often a trade-off between specific signal strength and background levels. You may need to define in each experiment what amount of background is acceptable to obtain the necessary level of specific hybridization signal. If the results are not acceptable, the experiment might have to be redesigned.

What Range of Probe Concentration Is Acceptable?

Probe concentration is application dependent. It will vary with buffer composition, anticipated amount of target, probe length and sequence, and the labeling technique used.

Background and signal correlate directly to probe concentration. If less probe than target is present, then the accuracy of band quantities is questionable.

In the absence of rate-accelerating "fast" hybridization buffers, probe concentration is typically 5 to 10 ng/ml of buffer. Another convention is to apply 2 to 5 million counts/ml of hybridization buffer, which may add up to more than 10 ng/ml if the probe was end-labeled, as compared to a random primer-generated probe. The use of rate accelerators or "fast" hybridization buffers requires a reduction in probe concentration to levels of 0.1 to 5 ng/ml of hybridization buffer.

Another approach to select probe concentration is based on the amount of target. A greater than 20× excess of probe over target is required in filter hybridization (Anderson, 1999). Solution hybridization may not require excess amounts for qualitative experiments. To determine if probe is actually present in excess over target, perform replicate dot or slot blots containing a dilution series of immobilized target and varying amounts of input probe (Anderson, 1999). If probe is present in excess, the signal should reflect the relative ratios of the different concentrations of target. If you do not observe a proportional relationship between target concentration and specific hybridization signal at any of the probe concentrations used, you may need to increase your probe concentration even higher. Probe concentration cannot be increased indefinitely; a high background signal will eventually appear.

What Are Appropriate Pre-hybridization Times?

Prehybridization time is also affected by the variables of hybridization time. For buffers without rate accelerators, prehybridization times of at least 1 to 4 hours are a good starting point. Some applications may afford to skip prehybridization altogether (Budowle and Baechtel, 1990). Buffers containing rate accelerators or volume excluders usually do not benefit from prehybridization times greater than 30 minutes.

How Do You Determine Suitable Hybridization Times?

Hybridization time depends on the kinetics of two reactions or events: a slow nucleation process and a fast "zippering" up. Nucleation is rate-limiting and requires proper temperature settings (Anderson, 1999). Once a duplex has formed (after "zippering"), it is very stable at temperatures below melting, given that the duplex is longer >50 bp. Hybridizing overnight works well for a wide range of target or probe scenarios. If this generates a dissatisfactory signal, consider the following.

There are several variables that affect hybridization time. Double-stranded probes (i.e., an end-labeled 300 bp fragment)

require longer hybridization times than single-stranded probes (end-labeled oligonucleotide), because reassociation of double-stranded probes in solution competes with annealing events of probes to target. At 50% to 75% reassociation, free probe concentration has dwindled to amounts that make further incubation futile. Hybridization time for a double-stranded probe can therefore be deduced from its reassociation rate (Anderson, 1999). Glimartin (1996) discusses methods to predict hybridization times for single-stranded probes, as does Anderson (1999). Other variables of hybridization time include probe length and complexity, probe concentration, reaction volume, and buffer concentration.

Buffer formulations containing higher concentrations (≥10 ng/ml) of probe and/or rate accelerators or blots with high target concentrations may require as little as 1 hour for hybridization. Prolonged hybridization in systems of increased hybridization rate will lead to background problems. The shortest possible hybridization time can be tested for by dot blot analysis. Standard buffers usually require hybridization times between 6 and 24 hours. Plateauing of signal sets the upper limit for hybridization time. Again, optimization of hybridization time by a series of dot blot experiments, removed and washed at different times, is recommended. Plaque or colony lifts may benefit from extended hybridization times if large numbers of filters are simultaneously hybridized.

What Are the Functions of the Components of a Typical Hybridization Buffer?

Hybridization buffers could be classified as one of two types: denaturing buffers, which lower the melting temperatures (and thus hybridization temperatures) of nucleic acid hybrids (i.e., formamide buffers), and salt/detergent based buffers, which require higher hybridization temperatures, such as sodium phosphate buffer (as per Church and Gilbert, 1984).

Denaturants

Denaturing buffers are preferred if membrane, probe, or label are known to be less stable at elevated temperatures. Examples are the use of formamide with RNA probes and nitrocellulose filters, and urea buffers for use with HRP-linked nucleic acid probes. Imperfectly matched target : probe hybrids are hybridized in formamide buffers as well.

For denaturing, 30% to 80% formamide, 3 to 6 M urea, ethylene glycol, 2 to 4 M sodium perchlorate, and tertiary alkylamine

chloride salts have been used. High-quality reagents, such as deionized formamide, sequencing grade or higher urea, and reagents that are DNAse- and/or RNAse-free are critical.

Formamide concentration can be used to manipulate stringency, but needs to be >20%. Hybrid formation is impaired at 20% formamide but not at 30 or 50% (Howley et al., 1979). 50% to 80% formamide may be added to hybridization buffers. 50% is routinely used for filter hybridization. 80% formamide formulations are mostly used for in situ hybridization (ISH) where temperature has the greatest influence on overall stability of the fixed tissue and probe, and in experiments where RNA:DNA hybrid formation is desired rather than DNA:DNA hybridization. In 80% formamide, the rate of DNA:DNA hybridization is much lower than RNA:DNA hybrid formation (Casey and Johnson 1977). Phosphate buffers are preferred over citrate buffers in formamide buffers because of superior buffering strength at physiological pH.

In short oligos 3 M tetramethylammonium chloride (TMAC) will alter their T_m by making it solely dependent on oligonucleotide length and independent of GC content (Bains, 1994; Honore, Madsen, and Leffers, 1993). This property has been exploited to normalize sequence effects of highly degenerate oligos, as are used in library screens. Note that some specificity may be lost.

Salts

Binding Effects

Hybrid formation must overcome electrostatic repulsion forces between the negatively charged phosphate backbones of the probe and target. Salt cations, typically sodium or potassium, will counteract these repulsion effects. The appropriate salt concentration is an absolute requirement for nucleic acid hybrid formation.

Hybrid stability and sodium chloride concentration correlate in a linear relation in a range of up to 1.2 M. Stability may be increased by adding salt up to a final concentration of 1.2 M, or decreased by lowering the amount of sodium chloride. It is the actual concentration of free cations, or sodium, that influences stability (Nakano et al., 1999; Spink and Chaires, 1999). Final concentrations of 5 to 6× SSC or 5 to 6× SSPE (Sambrook, Fritsch, and Maniatis, 1989), equivalent to approximately 0.8 to 0.9 M sodium chloride and 80 to 90 mM citrate buffer or 50 mM sodium phosphate buffer, are common starting points for hybridization buffers. At 0.4 to 1.0 M sodium chloride, the hybridization rate of

DNA:DNA hybrids is increased twofold. Below 0.4 M sodium chloride, hybridization rate drops dramatically (Wood et al., 1985). RNA:DNA and RNA:RNA hybrids require slightly lower salt concentrations of 0.18 to 1.0 M to increase hybridization by twofold.

pH Effects

Incorrect pH may impair hybrid formation because the charge of the nucleic acid phosphate backbone is pH dependent. The pH is typically adjusted to 7.0 or from 7.2 to 7.4 for hybridization experiments. Increasing concentrations of buffer substances may also affect stringency. EDTA is sometimes added to 1 to 2 mM to protect against nuclease degradation.

Detergent

Detergents prevent nonspecific binding caused by ionic or hydrophobic interaction with hydrophobic sites on the membrane and promote even wetting of membranes. 1% to 7% SDS, 0.05% to 0.1% Tween-20, 0.1% *N*-lauroylsarcosine, or Nonidet P-40 have been used in hybridization buffers. Higher concentrations of SDS (7%) seem to reduce background problems by acting as a blocking reagent (Church and Gilbert, 1984).

Blocking Reagents

Blocking reagents are added to prevent nonspecific binding of nucleic acids to sites on the membrane.

Proteinaceous and nucleic acid blocking reagents such as BSA, BLOTTO (nonfat dried milk), genomic DNA (calf thymus, herring, or salmon sperm), and poly A may be used. Denhardt's solution is often referred to as a blocking reagent, but it is really a mix of blocking reagents and volume excluder or rate accelerator. Screening tissue samples with nucleic acid probes labeled with enzyme-linked avidin might require additional blocking steps because of the presence of endogenous biotin within the sample. Vector Laboratories, Inc., manufacturers a solution for blocking endogenous biotin.

The best concentration of each of the blocking reagents for individual applications needs to be determined empirically. If nonspecific binding is observed, then increase the concentration of blocking agent or switch to a different blocking agent. Concentrations of BSA range from 0.5% to 5%; 1% is a common starting point. Other blocking agents include nonfat dry milk (BLOTTO) (1–5%), 0.1 to 1 mg/ml sonicated, denatured genomic

DNA (calf thymus or salmon sperm), or 0.1 to 0.4 mg/ml yeast RNA.

Hybridization Rate Accelerators

Agents that decrease the time required for hybridization are large, hydrophilic polymers that act as volume excluders. That is, they limit the amount of "free" water molecules, effectively increasing the concentration of probe per ml of buffer without actually decreasing the buffer volume. Common accelerators are dextran sulfate, ficoll, and polyethylene glycol. There are no hard and fast rules, but test a 10% solution of these polymers as accelerants. Rate accelerators can increase the hybridization rate several-fold, but if background is problematic, reduce the concentration to 5%. The performance of dextran sulfate (and perhaps other polymers whose size distribution changes between lots) can vary from batch to batch, so the concentration of this and perhaps other accelerators might have to be adjusted after ordering new materials.

Higher concentrations (30–40%) of Ficoll 400, polyethylene glycol, and dextran sulfate are difficult to dissolve, and microwaving or autoclaving may help. Carbohydrate polymers such as Ficoll and dextran sulfate will be ruined by standard autoclave temperatures; 115°C should be the temperature maximum, and allow solutions to cool slowly. Pipetting of stock solutions of any of these viscous polymers can be difficult. Pouring solutions into tubes or metric cylinders followed by direct dilution with aqueous buffer components may be easier than pipetting. An alternative approach to increase hybridization rate is the use of high salt concentrations and/or lower hybridization temperatures. This simply allows faster annealing of homologous probe/target duplexes that are significantly less than 100% homologous.

What to Do before You Develop a New Hybridization Buffer Formulation?

Check for Incompatibilities

Not every combination of the above components will be chemically compatible. Membranes blocked with milk may form precipitates in the presence of hybridization buffers containing high concentrations of SDS, as found in Church and Gilbert (1984). Most sodium, potassium, and ammonium salts are soluble, but mixing soluble magnesium chloride from one buffer component with phosphate buffers produces insoluble magnesium phosphate.

A proteinaceous blocking reagent could be salted out by ammonium sulfate.

Stock solutions of protein blocking agents may contain azide as a preservative. Undiluted azide may inhibit the horseradish peroxidase used in many nonradioactive detection systems.

Change One Variable at a Time

Unless you change to a totally different buffer system, optimization is usually faster if you alter one variable incrementally and monitor for trends.

Hybridization is an experiment within an experiment. The calculation of theoretical values that closely resemble your research situation may require more work than empiric determination, especially when selecting hybridization temperature and time.

Record-Keeping

At the very least, include a positive control to monitor your overall experimental performance. As described elsewhere in this chapter, the better you control for the different steps (labeling, transfer, etc.) in a hybridization reaction, the better informed your conclusions will be.

Consider equipment-related fluctuation when modifying a strategy. Glass and plastic heat at different rates, and heat exchange in water is quicker than in air. So the duration of washes may need to be prolonged if you switch from sealed polyethylene sleeves incubating in a water bath to roller bottles heated in a hybridization oven.

What Is the Shelf Life of Hybridization Buffers and Components?

Most hybridization buffers are viscous at room temperature, and floccular SDS precipitates are often observed that should go into solution upon pre-warming to hybridization temperature. Colors vary from colorless to very white to yellow. The yellowish tint often comes from the nonfat dried milk blocking agent.

An analysis of different hybridization buffers stored at room temperature for a year showed that the most common problem was formation of precipitates that would not go into solution when heated. No difference in scent or color of the buffer could be observed (S. Herzer, unpublished observations).

Blocking reagents were much less stable. DNA, nonfat dried milk and BSA were stable for a few weeks at 4°C, and stable for three to six months when frozen. A foul smell appeared in stored

solutions of protein blocking reagents, most likely due to microbial contamination.

What Is the Best Strategy for Hybridization of Multiple Membranes?

When simultaneously hybridizing several blots in a tub, box, or bag, the membranes can be separated by meshes, which are usually comprised of nylon. Additional buffer will be required to compensate for that soaked up by the mesh. The mesh should measure at least 0.5 cm larger than the blot. Meshes should be rinsed according to manufacturers instructions (with stripping solution if possible) before reuse because they may soak up probe from previous experiments. When working with radioactive labels, check meshes with a Geiger counter before reuse. Multiple filters may also be hybridized without separating meshes. Up to 40 20×20 cm could be hybridized in one experiment without meshes (S. Herzer, unpublished observation).

Filter transfer into hybridization roller bottles can be difficult. Dry membranes are not easy to place into a hybridization tube/roller bottle. Pre-wetting in hybridization buffer or 2× SSC may help. Membranes may be rolled around sterile pipettes and inserted with the pipette into the roller bottle. If several filters need to be inserted into the tube, consider inserting them one by one, because uniform and even wetting with prehybridization solution is important. If tweezers are to be used to handle filters, use blunt, nonridged plastic (metal is more prone to damage membrane) tweezers. Avoid scraping or wrinkling of the membrane. A second approach is to pre-wet the filters and stack them alternating with a mesh membrane, roll them up (like a crepe), and insert this collection into the roller bottle. A third approach is to insert filters into 2× SSC and then exchange to prewarmed prehybridization buffer. Rotate roller bottles slowly, allowing tightly wound filters to uncurl without trapping air between tube and filter, or between multiple filters.

Is Stripping Always Required Prior to Reprobing?

If a probe is stripped away, some target might be lost. If the probe is not stripped away prior to reprobing, will the presence of that first probe interfere with the hybridization by a second probe? There are too many variables to predict which strategy will generate your desired result. If faced with a situation where your prefer not to remove an earlier probe, consider the following options.

If different targets are to be probed, you can sometimes circumvent stripping of radioactively labeled probes by letting the signal decay. Make sure that a positive control for probe A does not light up with probe B if stripping has been skipped. Some nonradioactive systems may allow simple signal inactivation rather than stripping. Horseradish peroxidase activity can be inactivated by incubating the blot in 15% H_2O_2 for 30 minutes at room temperature (Amersham Pharmacia Biotech, Tech Tip 120). Other protocols circumvent stripping by employing different haptens or detection strategies for each target (Peterhaensel, Obermaier, and Rueger, 1998).

What Are the Main Points to Consider When Reprobing Blots?

Considering the amount of work involved in preparing a high quality blot, reuse of blots to gain additional information makes sense. As discussed previously, not all membranes are recommended for reuse. Nylon membranes are more easily stripped and reprobed. If you plan on reusing a blot many times, there are a few guidelines you could consider:

1. No stripping protocol is perfect; some target is always lost. Therefore start out by detecting the least abundant target first.
2. The number of times a blot can be restripped and reprobed cannot be predicted.
3. Never allow blots to dry out before stripping away the probe. Dried probes will not be removed by subsequent stripping procedures.
4. Store the stripped blot as discussed above in the question, *What's the Shelf Life of a Membrane Whose Target DNA Has Been Crosslinked?*
5. Select the most gentle approach when stripping for the first time in order to minimize target loss. Regarding the harshness of stripping procedures, formamide < boiling water < SDS < NaOH, where formamide is the least harsh. NaOH is usually not recommended for stripping Northern blots.
6. Excess of probe or target on blots can form complexes that are difficult to remove from a blot with common stripping protocols (S. Herzer unpublished observation). Avoid high concentrations of target and/or probe if possible when reuse of the blot is crucial.
7. UV crosslinking is preferred when blots are to be reprobed because they withstand harsher stripping conditions.

8. A comparison of stripping protocol efficiencies suggests that NaOH at 25°C led to a fourfold higher loss of genomic DNA compared to formamide at 65°C or a 0.1% SDS at 95°C (Noppinger et al., 1992). Formamide was found to be very ineffectual in stripping probes of blots (*http://www.millipore.com/analytical/pubdbase.nsf/docs/TN056.html*).

How Do You Optimize Wash Steps?

What Are You Trying to Wash Away?

Washes take advantage of the same salt effects described above for hybridization buffers. During removal of unbound or nonspecifically bound probe, sequential lowering of salt concentrations will wash away unwanted signal and background, but may also wash away specific signal if washing is too stringent. Since the required stringency of wash steps is often not known prior to the first experiment, always begin with low-stringency washes, and monitor wash efficiency whenever possible. You can always wash more, but you can never go back after washing with buffer whose stringency is too high.

When increasing the stringency of washes, ask yourself whether you are trying to remove nonspecific or specific background. It is easy to confuse the requirement of a more stringent wash with just more washing. An overall high background with a mismatched probe may not benefit from higher-temperature or lower-salt concentration in the wash steps because you are already at the limit of stringency. Instead, extended washes at the same stringency may be used to remove additional background signal. To summarize, increase the duration (time and/or number) of washing steps to remove more material of a particular stringency; increase temperature and/or decrease salt concentration if further homologous materials need to be removed.

The Wash Solutions

After removing the bulk of the hybridization buffer, a quick rinse of the membrane with wash buffer to remove residual hybridization buffer can drastically improve reproducibility and efficiency of subsequent wash steps. Efficient washing requires excess buffer. At least 1 to 2 ml/cm^2 of membrane or to 30% to 50% of total volume in roller bottles are required for each wash step. Washes may be repeated up to three times for periods of 5 to 30 minutes per wash.

Low-stringency washes start out at 2× SSC, 1% SDS and room temperature to 65°C; intermediate stringency can vary from

0.5× SSC to 1× SSC/0.5% SDS and room temperature to 70°C; high-stringency washes require 0.1% SDS/0.1× SSC at higher temperatures. Some of the newer wash buffers may include urea or other denaturants to increase the stringency (*http://www.wadsworth.org/rflp/Tutorials/DNAhybridization.html*); concentrations similar to those used in the hybridization buffer may be used. Detergent is added to ensure even wetting of filters.

Nonradioactive protocols often call for re-equilibration steps of blots in buffers that provide optimal enzyme activity or antibody binding. Contact the manufacturer of the detection system before you change these conditions.

Monitor Washing Efficiency

Where practical, it is recommended to measure the efficiency of the washing steps. Radioactive applications can be analyzed with handheld probes to check for localized rather than diffuse signal on a blot. Nonradioactive applications may benefit from a pre-experiment where a series of membrane samples containing dot blots is hybridized and washed where a sample is removed before each increase in wash stringency and signal-to-noise ratio is compared. It is crucial to include a negative control to ensure that detected signal is actually specific.

How Do You Select the Proper Hybridization Equipment?

Boxes (plastic or otherwise), plastic bags, and hybridization oven bottles are the common options. Buffer consumption in boxes is higher than in bags or bottles, but these larger volumes can help reduce background problems. Larger capacity also makes it feasible to simultaneously manipulate multiple filters, whereas bags accommodate one filter each.

Hybridization bottles can accommodate multiple membranes, but the membranes tend to stick together much more than in boxes, and the number of filters incubated in a bottle even when using separating meshes will be lower than in a box of the same volume. As described earlier under *What Is the Best Strategy for Hybridization of Multiple Membranes*, membranes are more easily inserted into hybridization bottles after rolling them around clean pipettes. Washing in boxes is more efficient than in bottles or bags, so an increase in number or duration of wash steps might be necessary with bottles or bags.

When working with radioactive probes, contamination of hybridization bottles and loss of probe is minimized by treating the glassware with a siliconizing agent. Bottle caps need to be

tightly sealed, nonporous, and fit snugly into the tube. Note that most hybridization buffers and wash solutions are prone to foaming upon gas exchange between the environment and heated air/buffer when the cap on top of the tube is removed, so open roller bottles in a safe area over absorbent paper. Plan for the possibility of minor spills and contaminations when working with plastic bags/sleeves, which don't always seal completely.

DETECTION BY AUTORADIOGRAPHY FILM

How Does An Autoradiography Film Function?

Autoradiography film is composed of a polyester base covered with a photographic emulsion of silver halide crystals. The emulsion may lie on one or both sides of the plastic base, and is usually covered with a material to protect the emulsion against scratches and other physical perturbation.

Photons of light and radioactive emissions can reduce a portion of the ionic silver in a silver halide crystal to silver atoms, forming a catalytic core (the latent image) that, upon development, causes the precipitation of the entire crystal. These precipitated crystals are the grains that form the images seen on the film.

One photon of light produces one silver atom, but a single silver atom in a crystal is unstable and will revert to a silver ion. A minimum of two silver atoms in a crystal are required to prevent reversion to the ionic form. In a typical emulsion, several photons of visible light must interact with an individual silver halide crystal in rapid succession to produce a latent image. In contrast, the energy of a single beta particle or gamma ray can produce hundreds of crystals capable of development into an image (Laskey, 1980 and Amersham International, 1992, *Guide to Autoradiography*).

Indirect Autoradiography

Indirect autoradiography involves the exposure of sample to film at –70°C in the presence of an intensifying screen (Laskey, 1980; Bonner and Laskey, 1974; Laskey and Mills, 1977). An intensifying screen is a flat plate coated with a material such as calcium tungstate, which, when bombarded with radiation, will phosphoresce to produce photons of light. The plates are typically placed on the inside of one side or both of a film cassette. In this way, the film is sandwiched in between. Indirect autoradiography creates a

composite signal consisting of radioactive and photon emissions. Exposure at –70°C is essential; a photon of light will generate only a single unstable silver atom (in a silver halide crystal) that will rapidly revert to a silver ion. –70°C stabilizes a single silver atom long enough to allow hits by additional photons of light, producing stable silver atoms and hence visible grains on the film.

Fluorographic chemicals are also utilized for indirect autoradiography. A fluorographic reagent is a solution (organic or aqueous) containing fluors, which will soak into a gel or accrete onto a membrane (Laskey and Mills, 1975; Chamberlain, 1979). When dried, the gel or membrane will have an even layer of fluors impregnated onto the surface. The fluors that are in proximity to the radioactivity fixed on the matrix will be activated by the radiation. These fluors give off light upon being activated, enhancing the signal coming from the radioactive sample. Fluorography requires film exposure at –70°C for the same reason as required by intensifying screens.

The additional sensitivity provided by intensifying screens is offset by a loss of resolution because the signals generated from the screen disperse laterally. In addition, the use of screens and fluorographic reagents also compromises the quantitative response of the film. Two or more silver atoms within a silver halide crystal are required to generate a visible grain on film, but a photon of light will generate only a single unstable silver atom that will rapidly revert to a silver ion. Because larger quantities of radioactivity are more likely than smaller quantities to produce sufficient photons to generate stable silver atoms, lesser amounts of radioactivity are under-represented when working with screens and flours.

When working with radioactive labels, this problem can be corrected by a combination of exposure at –70°C and sensitizing the film with a controlled pre-flash of light of the appropriate duration and wavelength (Laskey and Mills, 1975). Pre-flashing provides stable pairs of silver atoms to many crystals within the emulsion. The appropriate duration and intensity of the flash is crucial to restoring the linear response of the film (Amersham Review Booklet, 23).

Direct Autoradiography

Direct autoradiography refers to the exposure of sample to film at room temperature without use of intensifying screens or reagents.

What Are the Criteria for Selecting Autoradiogaphy Film?

Sensitivity and Resolution

There are two major aspects of film to bear in mind. There is sensitivity, or how much the investigator can see, and resolution, or how well defined the area of activity is. In most cases, higher sensitivity (less time for an image to come up on the film) rather than resolution is crucial. Resolution is more crucial to applications such as DNA sequencing, when probing for multiple bands indicative of mobile genetic elements and repetitive sequence, and when analyzing tissue sections, where location of activity is critical.

Sensitivity and resolution of films are based on the size and packing density of the silver halide crystals. A film is said to be more sensitive if its silver grains are larger (J. DeGregaro, Kodak Inc., Personal communication); Helmrot and Carlsson (1996) suggest that grain shape also affects sensitivity. Higher resolution is achieved when the grains are packed less densely in the emulsion. Some films eliminate the protective anti-scratch coating to improve sensitivity to labels that produce weak energy emissions.

Double and Single Coatings

Most double-coated films contain blue light-sensitive emulsion on both sides of the plastic base, allowing for added sensitivity with and without intensifying screens, albeit at the expense of resolution. High energy emitters such as ^{32}P and ^{125}I can be detected without screens, although the ^{125}I story is more complicated as described below. The emissions from medium emitters (^{14}C and ^{35}S) are essentially completely absorbed by the first emulsion layer, negating any benefit by the second emulsion. However, the use of a specialized intensifying screen (Kodak Transcreen LE) and double-coated film (Kodak Biomax MS) can increase the sensitivity and speed of detection of signal from ^{3}H, ^{14}C, ^{33}P and ^{35}S (J. DeGregaro, Kodak Inc., Personal communication).

Single-coated films allow for greater resolution. Radioactive and nonradioactive signals continue to spread (much like an expanding baloon) as they travel to the second emulsion of a double-coated film, resulting in a bleeding or fuzzy effect. Some emulsion formulations also allow for added speed and sensitivity.

Label

Weak Emitters

Very weak beta emitters such as tritium usually require special films and/or intensifying screens, as described above in the dis-

cussion about double-coated films. The tritium beta emission travels only a few microns through material. So, if the film has a coating over the emulsion, the beta particle will not come in contact with the silver grains. In cases of direct autoradiography, that is, without any fluorographic enhancement of signal, tritium samples are best recorded on film without a coating over the emulsion.

If you have the luxury of using fluorographic reagents (described above) and tritium, however, standard autoradiography film (single or double-coated) will work fine, since the film will be picking up the photons of light instead of the betas. This will generally tend to give much faster exposure times, about less than a week, although usually there will be a loss of resolution. This is not recommended for tissue section work, since definition would be compromised by the scattering photons. In this case a liquid nuclear emulsion can be applied.

Medium Emitters

When working with "medium" beta emitters, such as ^{35}S, ^{14}C, and ^{33}P, commonly available single- and double-coated autoradiographic film works well. However, there is no added sensitivity provided by the second emulsion layer without the use of specialized intensifying screens mentioned above. Fluorographic reagents will enhance the signals coming from these isotopes as well, but the impact is less dramatic than observed with tritium. Exposure times can vary greatly. They usually range 6 to 120 hours.

If you're considering the simultaneous use of a fluorographic reagent and an intensifying screen, perform a first experiment with the intensifying screen alone. The presence of a layer of fluorographic material can also attenuate a signal before it reaches the phosphor surface of the screen (Julie DeGregaro, Kodak Inc., Personal communication).

High-Energy Emitters

The most commonly used high-energy beta emitter is ^{32}P. Using standard autoradiographic film (single or double coated), it is not uncommon to have an image within a few minutes to a few hours. Because ^{32}P has such a high energy, the beta particles hitting the film can expose surrounding silver halide crystals and thus result in very poor resolution. At lower levels of counts in a given sample, ^{32}P does benefit from the use of intensifying screens.

^{125}I is a more complex isotope than those described above because it has gamma-ray emission, and a very low-energy X-ray emission. The low-energy X rays have an energy emission similar

to tritium. Specialty films used by investigators working with tritium can also easily detect ^{125}I. The high energy gamma rays will pass through the film and are less likely to expose the silver halide crystals. Standard film might detect a portion of the ^{125}I, but most of the signal will not be detected. Specialty films (i.e., Kodak BioMax MS) exist that will detect gamma rays.

The gamma rays from the ^{125}I are best detected by a standard autoradiography film with intensifying screens. Gamma rays are penetrating radiation, and as such are less likely to collide with anything in their path. In combination with intensifying screens on both sides of the cassette, you'll get a good signal from ^{125}I. A single Kodak Transcreen (HE) can also be applied to detect ^{125}I. The use of intensifying screens usually results in some loss of resolution.

Nonradioactive Emissions

Chemiluminescent signals and intensifying screens have a lambda max of light output (Durrant et al., 1990; Pollard-Knight, 1990a). Most double-coated films and intensifying screens are appropriate for chemiluminescent applications. Films dedicated to direct autoradiography are not always responsive to blue and ultraviolet light. They should not be used in fluorography, with intensifying screens, or with most chemiluminescent-based detection systems.

Speed of Signal Detection

The composition of some emulsions are designed for rapid signal generation.

Why Expose Film to a Blot at –70°C?

As described above, a single silver atom in a silver halide crystal is unstable and will revert to a silver ion. At low temperatures this reversion is slowed, increasing the time available to capture a second photon to produce a stable pair of silver atoms. When using intensifying screens or fluorographic reagents to decrease exposure times, keeping the film with cassette at –70°C can enhance the signal several fold. One report indicates that exposure at –20°C might be equally useful (Henkes and Cleef, 1988).

Chemiluminescent detection systems are enzyme driven, and should never be exposed to film at –70°C. Instead, nonradioactive signals can receive a short term boost by heating or microwaving the detection reaction within the membrane (Kobos et al., 1995; Schubert et al., 1995). Since enzymes will not survive this thermoactiviation, long-term signal accumulation is lost. Heating steps that dry the membrane while the probe is attached also make it

impossible to strip away that probe. For these reasons thermoactivation is considered a last resort.

Helpful Hints When Working with Autoradiography Film

Static electricity can produce background signals on film. A solution to this problems has been proposed by Register (1999). The use of fluorescent crayolas to mark the orientation of filters in the cassette has been described (Lee and Wevrik, 1997). A protocol for data recovery from underdeveloped autoradiographs has also been described (Owunwanne, 1984).

DETECTION BY STORAGE PHOSPHOR IMAGERS (David F. Englert)

Research has pushed the need for convenience and quantification to a point where autoradiography on film may no longer suffice.

How Do Phosphor Imagers Work?

Storage phosphor imaging is a method of autoradiography that works much like X-ray film. Energy from the ionizing radiation of radioisotopic labels is stored in inorganic crystals that are formed into a thin planar screen. The energy stored in the crystals can be released in the form of light when the crystals are irradiated with intense illumination. After contact exposure to the sample the screen is scanned in a storage phosphor imager with a focused laser beam, and the light emission (at a wavelength different from that of the laser) is recorded with a sensitive light detector. An image is constructed from the raster scan of the screen and is stored for viewing and analysis. The pixel values in the image are linearly proportional to the radioactivity in the sample, and spatial relationships between labeled materials can be determined within the spatial resolution of the system.

Is a Storage Phosphor Imager Appropriate for Your Research Situation?

Speed, Sensitivity, Resolution

Storage phosphor imaging is convenient for autoradiography with most radioisotopes used in biological research. It provides faster results than film autoradiography, and quantitative results in electronic form can be obtained much more readily with storage phosphor imaging than with film. Because of the relatively large

dynamic range with storage phosphor imaging, one has much greater latitude with the exposure time, and usually a single exposure will provide acceptable results with storage phosphor imaging. With film, it may be necessary to perform more than one exposure to get the dynamic range of the activity in the sample to correspond to the film's more limited dynamic range.

Better resolution can usually be obtained with film, so when very good resolution is more important than quantitative results, film autoradiography (or autoradiography with emulsions) may be a better choice. For imaging tritium, special storage phosphor screens are necessary which are much less durable than other screens. Thus storage phosphor autoradiography of tritium can be expensive compared to film.

Dynamic Range

Dynamic range is the intensity range over which labels can be quantified in a storage phosphor image. This is equal to the net signal from the highest activity that can be measured (at the level of saturation) divided by the signal from the lowest activity that can be detected or measured. The noise level of the measurement determines the lowest signal that can be detected or measured. The noise level can be assessed with standard statistical tests for hypothesis testing, but generally, the lowest detectable signal is that which can be readily seen in an image with appropriate adjustment of image scaling and contrast levels.

The dynamic range of storage phosphor imaging is generally in the range of 10^4 to 10^5. The dynamic range of X-ray film is somewhat greater than two orders of magnitude or about 100 times less than storage phosphor imaging. This is important for two reasons: (1) a larger range of intensities can be quantified in a single image with storage phosphor imaging, and (2) a user has much greater latitude for the exposure time. The result is that one is much more likely to capture the desired information in a first exposure without saturating the image.

The dynamic range of computer monitors is only about 8 bit or 256 levels of gray, which is far less than the dynamic range that may exist in a storage phosphor image. The image data must be transformed in some way to match the dynamic range of the image data to the display device. The software provided with the storage phosphor imager usually allows one to adjust the way the image data are transformed.

The transformation may be linear, in which case all the detail of the intensity variations may not be visible because the incre-

ments of intensity of the computer display are larger than the increments of intensity in the image. The transformation between the image data and the computer display may be nonlinear, for example, exponential. Nonlinear transformation has an effect similar to a logarithmic scale on a graphical plot. Namely, intensity variations are evident over a large dynamic range, but the scale is compressed, providing a distorted view of the intensities in the image. It is also possible to clip the lowest or highest intensities in the image, for example, so that all intensities below a certain level are displayed as white, and the image background is eliminated from view. Alternatively, intensities above a certain level may be displayed as black, and high intensities effectively saturate the display. The software tools usually allow one to adjust the computer display interactively to optimize the display to emphasize the desired information in the image.

Although these manipulations of the image display may cause an apparent loss of image information, all the information is usually retained in the image file, so quantitative analysis of the image will provide accurate information, regardless of what is displayed on the computer monitor. Note that conventional photo-editing software may store modified versions of the image file in which there may be loss of information or distortions of the original information.

Quantitative Capabilities

With proper use of the analytical software, storage phosphor imaging provides accurate quantitative results. Although the response may appear nonlinear at very low activity because of inaccurate estimation of the background level or at very high activity because of saturation of the image, the response of storage phosphor imaging is linear over its entire dynamic range between these extremes. Other aspects of quantitating data by phosphor imaging are discussed below.

What Affects Quantitation?

Is the Reproducibility of Phosphor Imaging Instrumentation Sufficient for Microarray Applications Such as Expression Profiling?

Although there is some risk that local damage to the storage phosphor screen could affect results, storage phosphor imaging with a system that is in good condition will contribute insignificantly to the measurement error. Phosphor imaging is appropriate for microarray analysis.

Can One Accurately Compare the Results Obtained with Different Screens in the Same Experiment?

Different screens may have slightly different responses to the same level of activity, and the exposure times with different screens are difficult to control accurately. Therefore calibration is required for accurate comparison of results obtained on two or more screens. Since the response of storage phosphor imaging is linear, this is a simple matter. Calibration standards can be included during the exposure of all the screens, and the quantitative results within each image can be normalized to (divided by) the signal measured from the calibration standards. This normalization can be performed with a spreadsheet program or may be performed with the analytical software provided with the scanner. Of course, the normalization is only as accurate as the calibration standards. Several nominally identical standards can be used on each screen to determine the error associated with the standards.

Can Storage Phosphor Imaging Provide Results in Absolute Units such as Disintegrations per Minute or Moles of Analyte?

The units of the results reported by the analytical software are arbitrary and have no physical meaning except that they are proportional to the light intensity emitted from the screen during the scanning process. However, calibration standards can be included with samples in the exposure cassette to linearly transform the arbitrary units to units that have significance in a particular experiment. For example, aliquots of a solution containing a radioactive tracer could be dispensed within the same physical matrix as the sample and included with the sample. Other aliquots could be counted by liquid scintillation counting to determine the actual activity in disintegrations per minute. Then quantitative results obtained from the storage phosphor image can be multiplied by a factor to obtain results in disintegrations per minute. Either a spreadsheet program or the scanner software may be used to perform the calibration. For accurate calibration it is important that the calibration standards be within the same physical matrix as the sample, since detection efficiency depends on the sample matrix, especially for relatively low-energy radioisotopes.

Suppose That the Amount of Activity in Part of the Sample Exceeds the Range of the Instrument. What Effect Does This Have on Quantification and How Does One Know That This Has Occurred? Can Accurate Results Be Obtained If This Occurs?

High levels of activity in some part of the sample can result in signal levels greater than the instrument was designed to measure. This is referred to as "saturation." Pixel values in this part of the sample will usually be set to some maximum value, and if the activity in this part of the image is quantified, the results obtained will underestimate the true level of activity. Some instruments paint any pixels that saturate red to warn the user that saturation has occurred.

Saturation is a concern only if the user wishes to quantify the activity in the part of the image that saturated. Accurate results can be obtained by exposing the sample again for a shorter period of time. Another solution is to scan the storage phosphor screen again. When the screen is scanned by the laser, much, but not all, of the signal is erased, and a second scan will result in an image with intensities three to five times less than in the first scan. Parts of the sample that were saturated in the first image may not be saturated in the second image.*

What Should You Consider When Using Screens?

Does the Sensitivity of Storage Phosphor Imaging Increase Indefinitely with Increasing Screen Exposure Times?

No. Energy is stored in the storage phosphor screen throughout the exposure, but there is also a slow decay of the stored energy during the exposure. After a long exposure time, a relatively large amount of signal will be stored in the phosphor, and the decay of this stored signal becomes nearly as great as the accumulation of new signal. Hence the net increase of signal is small. The net increase in signal becomes marginal after a few days, but longer exposures are sometimes used.

**Editor's note: Some manufacturers strongly urge not to rescan the storage phosphor screen because subsequent scans will not produce quantitative data. A third alternative would be to rescan at different voltages where applicable.*

Is There Any Advantage to Exposing Storage Phosphor Screens at Low Temperatures?

There is a small improvement in signal intensity if the storage phosphor screen is kept at low temperature during exposure, probably because the slow decay of the stored signal is slower at lower temperature. This can be beneficial for very long exposures (more than one week), but it has little practical value for most routine work. Because of the marginal effect and the potential for damage due to condensation on the screen, low-temperature exposure should be considered only as a last resort.

Are the Storage Phosphor Screens Used for Tritium Reusable? What Precautions Can One Take to Get Multiple Uses from These Screens?

Because tritium screens are not coated to protect the storage phosphor crystals (any coating would "protect" the crystals from the weak beta radiation of tritium), the screens cannot be cleaned and are readily contaminated or damaged. Nevertheless, some investigators have been able to use the screens multiple times. To reuse tritium screens, samples must be very dry, must not stick to the screen, and must not contain loose material that could adhere. The screens should be stored in a dry place. To check for contamination between uses, the screens should be left in an exposure cassette for the same period of time that one would use to expose a sample and then scanned. Any contamination should be quantified to assess whether it is significant compared to the level of signal expected with a sample.

What Limits Resolution with Storage Phosphor Imaging? Do Some Screens Provide Better Resolution Than Others?

Resolution is limited largely by the isotropic spread of radiation within the storage phosphor screen. As is the case with autoradiography film, resolution is generally better with lower-energy radioisotopes, since their radiation is less penetrating. For example, resolution is better with ^{33}P than with ^{32}P (although the sensitivity with ^{32}P is better due to its higher energy and shorter half-life). Resolution is better with thinner layers of phosphor on the screen, and with thinner protective coatings. The resolution of screens varies between manufacturers, and between the screen types available from a single manufacturer. Resolution is also affected by the quality of the instrumentation, although the newer confocal scanners provide very good resolution and do not limit the resolution that can be achieved in autoradiography.

What Practices Should the Laboratory Use to Ensure That Storage Phosphor Screens Are Completely Erased before Exposure to a Sample?

Storage phosphor screens are erased by exposure to white light, and light boxes with bright fluorescent bulbs are usually used after scanning to completely erase the residual image. Since one cannot always be sure that the previous user has adequately erased the screen, it is a good practice to always erase a screen with white light just before beginning an exposure. This practice also minimizes any background signal on the screen due to prolonged storage in the presence of cosmic radiation or slight contamination of the screen surface.

How Can Problems Be Prevented?

Can These Machines Accidentally Generate Misleading Data?

Storage phosphor imagers could generate misleading data if the screen was contaminated or incompletely erased so that artifactual signals appear in the image. Storage phosphor imagers, like other imaging systems, can generate misleading or confusing results depending on how the image data are displayed on the computer monitor or in an exported or printed image. Important details might be overlooked or significant artifacts might be intentionally hidden by manipulation of the image display.

What Causes the Background with Storage Phosphor Imaging and How Can It Be Reduced?

Some of the background in storage phosphor images is due to instrument noise or very slight stimulated emission of light from the storage phosphor in the absence of stored energy. This component of the background is a property of the system and cannot be reduced. Another component of the background is due to the absorption of cosmic radiation during the exposure. Shielding the exposure cassette from cosmic radiation with lead bricks during the exposure can reduce this component of the background. This measure is worthwhile only for very long exposure times. For exposures up to a few days long, the background due to cosmic radiation is not very significant.

What Is "Flare" in Storage Phosphor Imaging? What Effect Does This Have on Results? How Can It Be Minimized?

Flare is an optical artifact due to the collection of light from adjacent regions of the screen during scanning. It can cause errors

if regions of high activity are close to regions of low activity. For example, in images of high-density arrays used for expression profiling, the activity resulting from a highly expressed gene could increase the apparent activity in nearby spots. Flare is an instrument effect that is evident in older storage phosphor imagers but is largely eliminated by the use of confocal optics in newer instruments. With confocal optics, light is collected only from the region (pixel) of the image that is currently being excited by the laser.

Is It Crucial to Avoid Exposing the Storage Phosphor Screen to Bright Light after Exposure and before Imaging?

Ambient light will erase the latent image on a storage phosphor screen. After exposure to radioactive samples, exposure of the storage phosphor screen to ambient light (e.g., the bright fluorescent lighting in many laboratories) should be minimized. Transfer the screen to the scanner without delay. Turn off overhead fluorescent lighting, and work in dim light to retain the maximum signal on the screen.

TROUBLESHOOTING

What Can Cause the Failure of a Hybridization Experiment?

What is the difference in appearance of hybridization data between an experiment where the probe-labeling reaction failed due to inactive polymerase, and an experiment where the gel filtration column trapped the labeled probe? Will the data above look different in a Northern hybridization where the mRNA was stored in a Tris buffer whose pH increased beyond 8.0 when stored in the cold, or in a Northern where the transfer failed? The answer is no. Where hybridization produced a weak signal, was it due to overly stringent hybridization conditions, insufficient quantity of probe, a horseradish peroxidase-linked probe that lost activity during six weeks of storage?

The take-home lessons from the above discussions and the information presented in Table 14.2 are two:

- Problems at any one or combination of steps can generate inadequate hybridization data.
- Problems at different stages of a hybridization experiment can generate data that appear identical.

Scrupulous record-keeping, thorough controls, an open mind, and a stepwise approach to troubleshooting as discussed for

Western blots (Chapter 13) will help you identify the true cause of a disappointing hybridization result. A gallery of images of hybridization problems is provided in Figures 14.1–14.9, and inhibitors of enzymes used to label probes are listed at *http//:www.wiley.com/go/gerstein.*

Table 14.2 Potential Explanations for a Failed Hybridization Experiment

Type of Failure	Possible Causes
Probe Labeling	Template quality
	Template quantity
	Reaction components; enzyme, nucleotides, etc.
	Label integrity
Probe Purification	Inappropriate purification strategy
	Failed purification reaction
Target-related	Target quantity and quality
	Target transfer
	Crosslinking
Hybridization failure	Probe quantity
	Hybridization conditions; prehybridization, blocking, hybridization buffer, washing
Detection failure	Film
	Developer
	Imaging instrumentation

Figure 14.1 Human genomic Southern blot hybridized with the proto-oncongene N-ras DNA probe (1.5 kb), labeled using the ECL random prime system. Exposed to Hyperfilm™ ECL for 30 minutes. Poorly dissolved agarose during preparation of the gel has swirls of high background. Ensure that the agarose is completely dissolved before casting the gel, or invert the gel before blotting. Published by kind permission of Amersham Pharmacia Biotech, UK Limited.

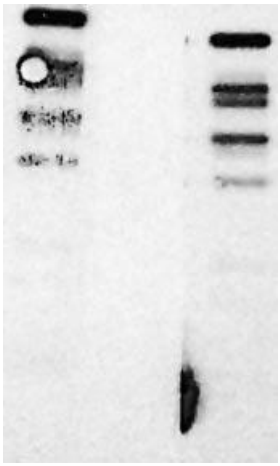

Figure 14.2 Lambda Hind III Southern blot hybridized with a lambda DNA probe, labeled using ECL direct. Exposed to Hyperfilm™ ECL for 60 minutes. Air bubbles trapped between the gel and the membrane have prevented transfer of the nucleic acid; the result is no visible signal. These may be removed by rolling a clean pipette or glass rod over the surface. Published by kind permission of Amersham Pharmacia Biotech, UK Limited.

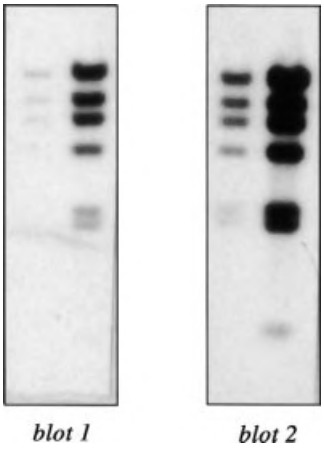

Figure 14.3 Lambda Hind III Southern blot (1 ng and 100 pg loadings) hybridized with a lambda DNA probe using ECL direct. Exposed to Hyperfilm™ ECL for 30 minutes. Blot 1 Hybond™—C pure; Blot 2 Hybond™—N+. Published by kind permission of Amersham Pharmacia Biotech, UK Limited.

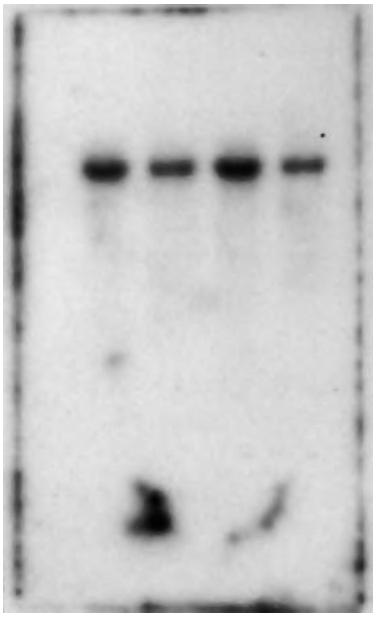

Figure 14.4 Human genomic Southern blot hybridized with the proto-oncogene *N*-ras DNA probe (1.5 kb), labeled using [alpha-^{32}P] dCTP and Megaprime™ labeling (random primer-based) system. Exposed to Hyperfilm™ MP for 6 hours. Membrane damage at the cut edges has caused the probe to bind; subsequent stringency washes are unable to remove the probe. Similar results are obtained with non-radioactive labeling and detection systems. Membranes should be prepared using a clean, sharp cutting edge. Published by kind permission of Amersham Pharmacia Biotech, UK Limited.

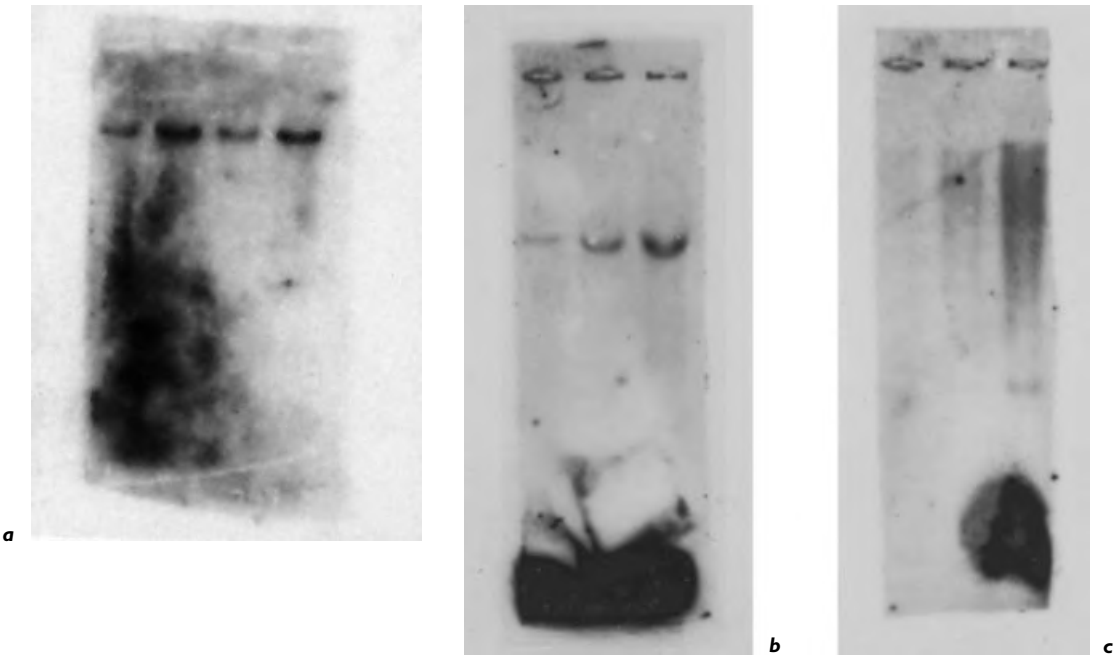

Figure 14.5*a* Human genomic Southern blot hybridized with the proto-oncogene *N*-ras DNA probe (1.5 kb) labeled using [alpha-^{32}P] dCTP and Megaprime™ labeling (random primer-based) system. Exposed to Hyperfilm™ MP for 6 hours. Labeled probe has been added directly onto the blot to cause this effect. Labeled probe should be added to the hybridization buffer away from the blot or mixed with 0.5 to 1.0 ml of hybridization buffer before addition. **Figure 14.5*b*, 5*c*** Human genomic Southern blot hybridized with *N*-ras insert labeled via ECL™ Direct labeling system. Exposed to Hyperfilm ECL for 1 hour. These probes were also directly added to the membrane, rather than first added to hybridization buffer. Published by kind permission of Amersham Pharmacia Biotech, UK Limited.

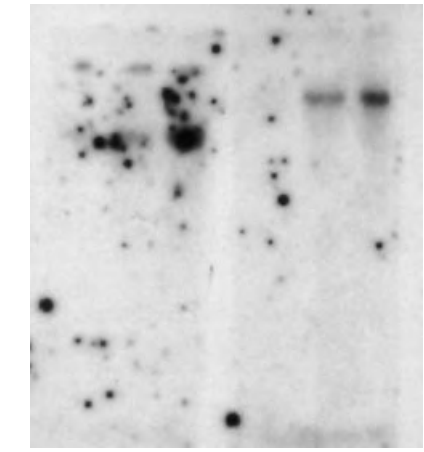

Figure 14.6 Human genomic Southern blot hybridized with the proto-oncogene *N*-ras DNA probe (1.5 kb) labeled using [alpha-^{32}P] dCTP and Megaprime™ labeling (random primer-based) system. Exposed to Hyperfilm™ MP for 6 hours. There are two probable causes of this "spotted" background: (1) Excess unincorporated labeled nucleotide in the probe solution. Always check the incorporation of the radioactive label before using the probe and purify as required. (2) Particulate matter present in the hybridization buffer. Ensure that all buffer components are fully dissolved before used. Published by kind permission of Amersham Pharmacia Biotech, UK Limited.

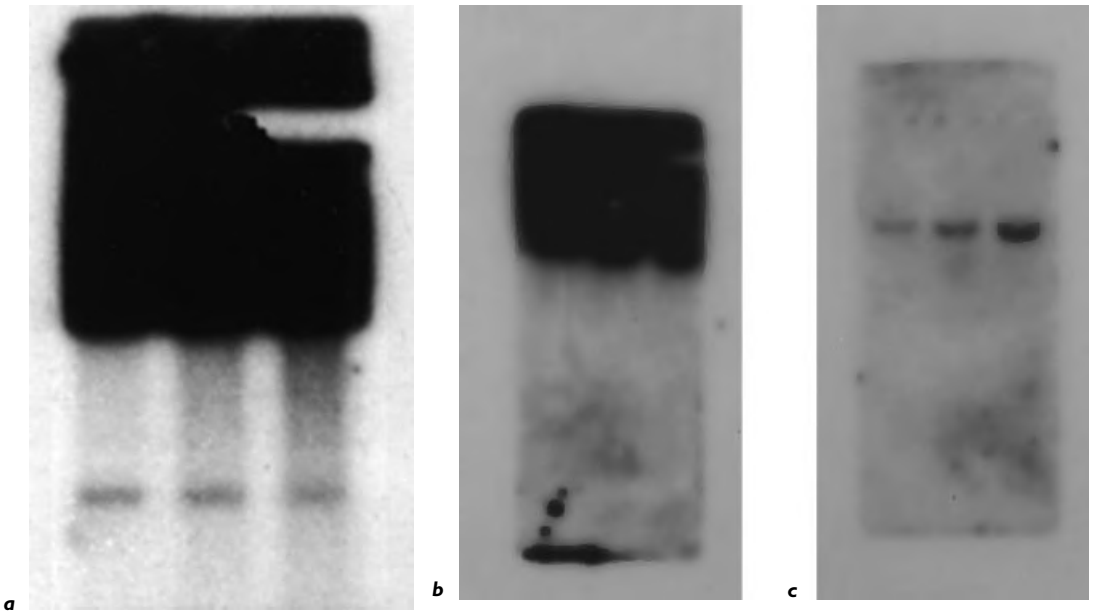

Figure 14.7*a* Human genomic DNA probe (0.8 kb), labeled using the ECL™ Direct system. Exposed to Hyperfilm™ ECL for 30 minutes. The heavy blot background nearest to the cathode has two possible causes: dirty electrophoresis equipment or electrophoresis buffer. Similar results are obtained with radioactive probes. Ensure that the electrophoresis tanks are rinsed in clean distilled water after use. Do not reuse electrophoresis buffers. **Figure 14.7*b*** Human genomic Southern blots on Hybond N^+ detected with ^{32}P labeled *N*-ras insert using [alpha-^{32}P] dCTP and Megaprime™ labeling (random primer-based) system. Exposed to Hyperfilm™ MP overnight. Electrophoresis was carried out in old TAE buffer. **Figure 14.7*c*** represents same samples as in Figure 14.7*b*, but after electrophoresis tank had been cleaned and filled with fresh TAE buffer. Published by kind permission of Amersham Pharmacia Biotech, UK Limited.

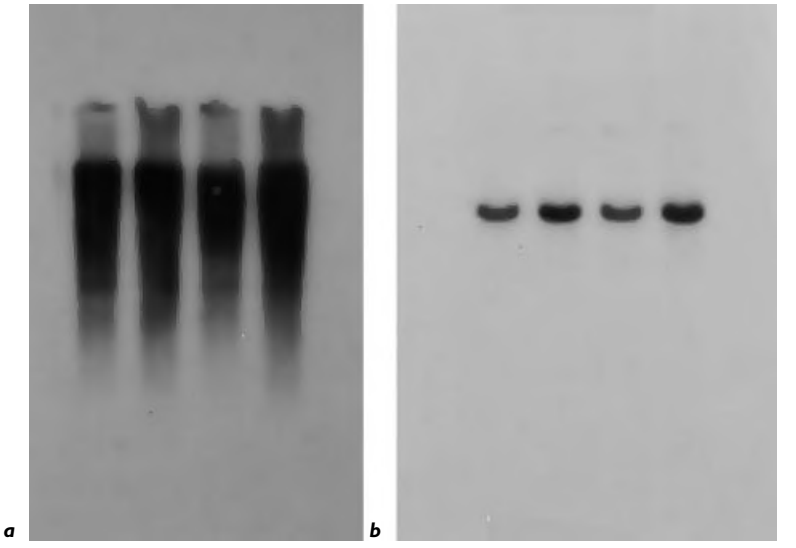

Figure 14.8 Human genomic Southern blots on Hybond N^+ detected with ^{32}P labeled *N*-ras insert using [alpha-^{32}P] dCTP and Megaprime™ labeling (random primer-based) system. **Figure 14.8*a*, 14.8*b*** Importance of controlling temperature during hybridization. (Figure 14.8*a*) The temperature of the water bath fell during an overnight hybridization, reducing the stringency and increasing the level of nonspecific hybridization. (Figure 14.8*b*) The temperature was properly controlled, and only specific homology is detected. Published by kind permission of Amersham Pharmacia Biotech, UK Limited.

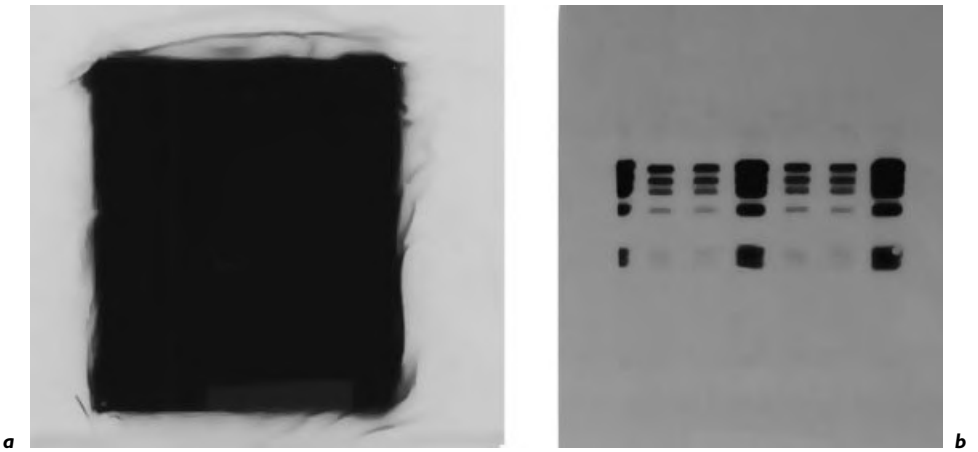

Figure 14.9 Hind III fragments of lambda DNA were blotted onto Hybond™ ECL, and probed with lambda DNA labeled via the ECL™ Detection system. **Figure 14.9 (*a*)** Blocking agent excluded from hybridization buffer. **(*b*)** Blocking agent present in hybridization buffer. Published by kind permission of Amersham Pharmacia Biotech, UK Limited.

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