# Overview of Blotting

Maria Cristina Negritto[1] and Glenn M. Manthey[2]

[1]Pomona College, Claremont, California
[2]City of Hope, Beckman Research Institute, Duarte, California

Blotting techniques are among the most common approaches used in a molecular biology laboratory. These techniques, Southern, northern, and immunoblotting, are applicable to a variety of macromolecules including DNA, RNA, and protein, respectively. Each of the techniques are dependent on the ability to resolve the individual macromolecules in a size-dependant manner, transfer the molecules to a solid support, and finally use a defined probe to detect the specific molecule of interest. The utilization of the blotting technology over the last 30 years has been instrumental to the elucidation of many fundamental biological processes. The continued use of blotting technology holds promise for even greater discovery over the next 30 years. © 2016 by John Wiley & Sons, Inc.

Keywords: Southern • Northern • Western • probe • hybridization • antibody • membrane • blotting • protein blot • DNA blot

**How to cite this article:**
Negritto, M.C. and Manthey, G.M. 2016. Overview of blotting. *Curr. Protoc. Essential Lab. Tech.* 13:8.1.1-8.1.22.
doi: 10.1002/cpet.5

## GENERAL OVERVIEW

Blotting is a technique by which a macromolecule such as DNA, RNA, or protein is resolved in a gel matrix, transferred to a solid support, and detected with a specific probe. These powerful techniques allow the researcher to identify and characterize specific molecules in a complex mixture of related molecules. Some of the more common techniques include Southern (DNA) blotting, northern (RNA) blotting, and immunoblotting (for protein; also known as western blotting). In this unit, a brief introduction to the main concepts used in blotting techniques will be discussed.

Blotting techniques such as Southern blotting or immunoblotting share some common steps that are described first in general terms and then more specifically. To access specific protocols on blotting methods please refer to *UNIT 8.2* (Hoopes, 2012) and *UNIT 8.3* (Ni et al., 2016).

The blotting procedures can be divided into six main steps, as illustrated in Figure 8.1.1.

### Electrophoresis

Typically, the molecule of interest is present in a complex mixture of molecules. To detect the protein or nucleic acid sequence of interest, the mixture must be resolved, usually on the basis of size. This is achieved by separating the molecules by gel electrophoresis on either an agarose or polyacrylamide gel. See *UNIT 7.2* (Armstrong and Schultz, 2015) for DNA and RNA electrophoresis and *UNIT 7.3* (Gallagher, 2012) for protein electrophoresis.

### Transfer

Following separation, the molecules are transferred to a solid support such as a nylon, nitrocellulose, or polyvinylidene fluoride (PVDF) membrane. The transfer results in a



*Current Protocols Essential Laboratory Techniques* 8.1.1-8.1.22, November 2016
Published online November 2016 in Wiley Online Library (wileyonlinelibrary.com).
doi: 10.1002/cpet.5

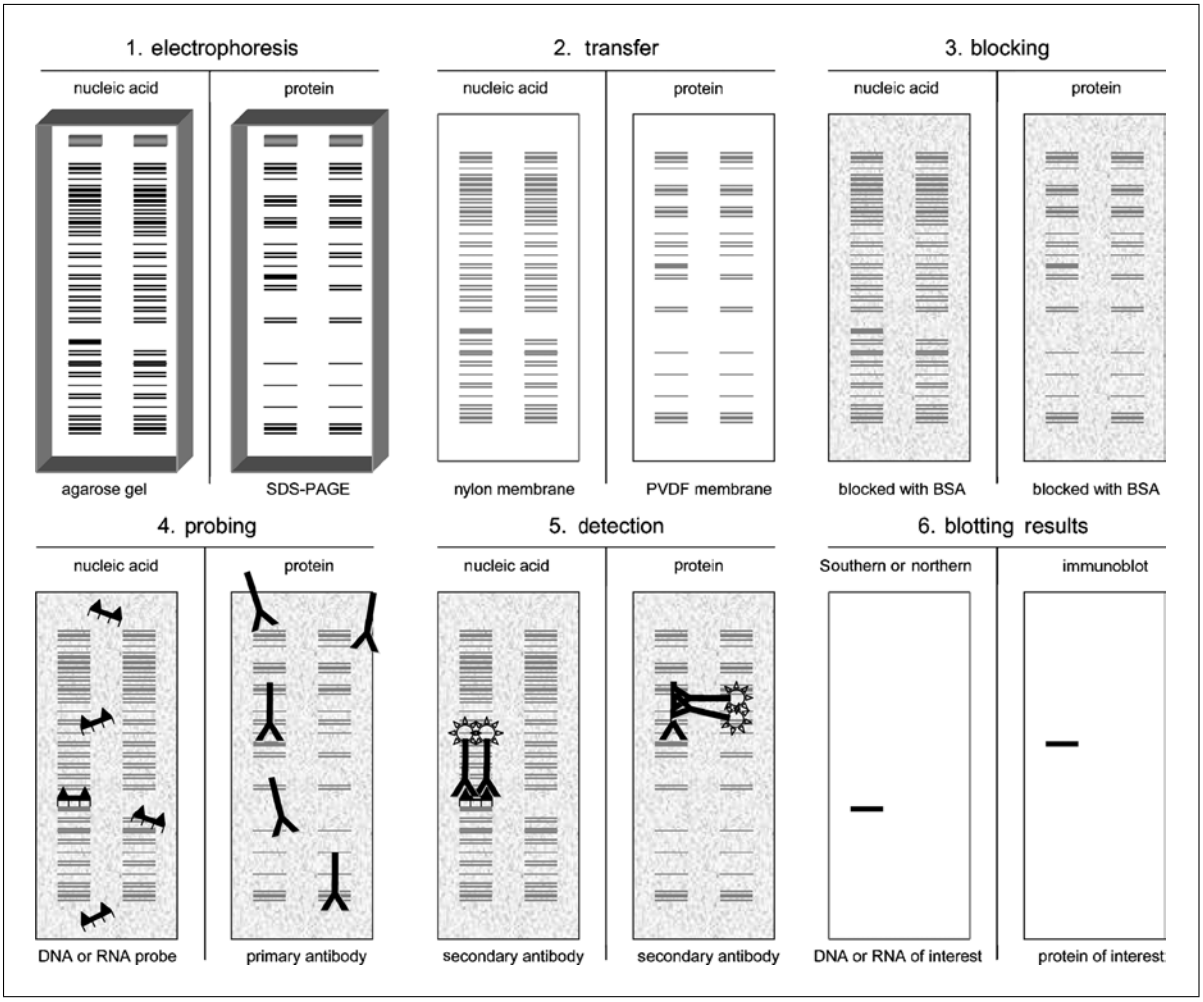


**Figure 8.1.1** Blotting Procedure. General overview of the steps involved in a blotting procedure that are common to nucleic acid blotting (Southern and northern) and protein blotting (western or immunoblotting). (1) Separation of the molecules by gel electrophoresis on either an agarose (DNA or RNA) or a SDS-polyacrylamide gel (protein). (2) Resolved molecules are transferred to a membrane maintaining the same pattern of separation they had on the gel. (3) The blot is treated with blocking agents, such as proteins (BSA) or detergents that bind to unoccupied sites on the membrane. This is depicted as a gray background. (4) A specific probe that binds to the protein or nucleic acid sequence of interest is incubated with the blot. In the case of a Southern or northern blot the probe consists of a complimentary DNA or RNA sequence. For an immunoblot, the probe consists of a primary antibody that recognizes a particular protein or epitope. (5) Detection step. When using a radioactively labeled probe, the signal is detected by X-ray film or phosphorimager, resulting in the banding pattern depicted in step 6. Nonradioactive probes can utilize a reporter enzyme directly conjugated to the probe or a labeling moiety that is then detected by a specific antibody conjugated to a reporter enzyme. (6) The reporter enzymes are then presented with colorimetric, fluorogenic, or chemiluminescent substrates that produce signals, which can be detected as a colored product (analyzed visually), as a fluorescent precipitate (detected with a camera after excitation), or as a compound that emits light during its decomposition (detected with X-ray film or a cooled CCD camera).

replica of the molecules that were present in the gel and that are now immobilized on a membrane. The most common transfer techniques include capillary blotting, for use with Southerns or northerns, and electroblotting for immunoblots. These techniques are discussed in more detail in *UNIT 8.2* (Hoopes, 2012) and *UNIT 8.3* (Ni et al., 2016).

## Blocking

Before detection of the target sample immobilized on the membrane, care needs to be taken to avoid nonspecific binding of the probe to the remaining binding sites on the membrane. Prior to the addition of the probe, the membrane is treated with general

blocking agents such as proteins or detergent to reduce the nonspecific association of the probe molecule with the membrane: this is referred to as the blocking step.

### Probing

Once the membrane has been blocked, it is incubated with a specific probe that binds to the protein or nucleic acid sequence of interest. In the case of a Southern or northern blot, the probe consists of complementary DNA or RNA sequences that will anneal to the target (see *UNIT 8.4*; Haushalter, 2008). The nucleic acid is labeled radioactively or enzymatically to allow for detection. By comparison, the probe used for an immunoblot is an antibody that recognizes a particular protein or epitope. A secondary antibody, conjugated to a reagent that allows for its detection, is incubated with the blot. A secondary antibody will bind to the primary antibody with high affinity to facilitate the generation of a specific signal. Following a period of incubation, the unbound probe or nonspecifically bound probe is removed by sequentially washing the membrane with increasingly stringent wash buffers.

### Detection

The last step in a blotting experiment involves a detection step to visualize the bound probe. The method of detection will be determined by the nature of the probe. If a radioactive probe is used, exposure of the blot to X-ray film or a phosphorimaging device will allow for detection and quantitation of the bound probe. [Refer to *APPENDIX 1A* (Lunn and Strober, 2008) for information on the safe use of radiation.] If chemical- or enzyme-based detection systems are used, the appropriate substrates are added to the blot and the resulting signal is developed and can be documented by colorimetric, fluorescent, or chemiluminescent imaging (see *UNIT 7.5*; Moomaw et al., 2014).

### Results and Analysis

Once the blot is developed, the resulting banding pattern can be analyzed. Analysis involves determining the amount and apparent molecular weight or size of the molecules on the blot and comparing the results to the predicted pattern. To determine the molecular weight of the molecules of interest, a standard curve of size versus migration distance is derived from the molecular weight markers (see *UNIT 7.2*; Armstrong and Schultz, 2015). The standard curve is plotted on semilog paper or with a graphing and analysis program. The distance that each maker migrates from the origin is plotted on the *x*-axis and the corresponding size or weight of the marker is plotted on the *y*-axis. The resulting plot should produce a straight line that enables estimation of the size of the unknown. Automated analysis programs increase the accuracy and greatly simplify this process (see *UNIT 7.5*; Moomaw et al., 2014). In practice, the exact determinations of size are difficult with this type of approach because changes in a variety of parameters, including structure, salt concentration, and the speed at which the gel was run, can affect the resolution characteristics of a molecule. The resulting plot will allow for a reasonable determination of the molecule's size, allowing for a comparison of the predicted and observed outcomes.

Analysis of the blot can provide the researcher with a variety of details concerning the nature of the molecules being studied. In the case of a Southern blot, the structure of the gene of interest can be assessed. Initially, a restriction map of the gene was used to select restriction enzymes that would produce a distinct pattern of bands once the blot is developed. If the predicted banding pattern is observed, one can conclude that the structure of the gene of interest behaves as predicted. If large rearrangements such as insertions, deletions, or inversions have occurred, the banding pattern will deviate from the predicted pattern and the investigator can conclude that the gene of interest is altered in an unexpected fashion. Additionally, the relative dosage of a molecular species

can be determined from a blotting experiment. The key to such an analysis is including an independent loading control to which the signal of the molecule of interest can be compared. If done carefully, one can differentiate the signal derived from a single copy of the gene as compared to two or more copies. A Southern blot may allow the researcher to detect related sequences that share homology to the molecule of interest but reside in different locations in the genome. Northern blots and immunoblots can be used to assess different levels of expression from a particular gene. Northern blots are also used to detect post-transcriptional modifications to the RNA, such as splicing. Immunoblots can be used in a similar fashion to detect post-translational modifications such as phosphorylation.

## GENERAL CONSIDERATIONS

It is critical to remember that when running a blotting experiment only the molecules that your probe recognizes will be visualized. If a blotting experiment that is designed to detect a single species results in more than one band being detected, it may indicate one of several problems. The appearance of extra bands could be due to cross-reactivity of the probe with other molecules present in the original sample. This may occur if the antibody used in an immunoblot is polyclonal in nature and thus recognizes multiple proteins or antigens in the sample. Alternatively, if performing a Southern or northern blot, multiple bands may indicate that your probe sequence can associate with repetitive elements, that there is more than one copy of the sequence of interest in the genome, or that multiple isoforms of the RNA are expressed in your sample. In each case, selecting a different antibody or redesigning your nucleic acid probe may reduce this problem. Additionally, increasing the stringency of the hybridization or washing procedures may reduce the cross-reactivity of your probe with off-target species. On the other hand, the appearance of bands of lower molecular weight could be due to degradation of your sample of interest. Higher molecular weight bands could be the result of incomplete digestion of your DNA sequences (Southern) or incomplete denaturation or heat-induced aggregation of your protein sample (immunoblot). This is why it is very important to include controls so that one can distinguish among these possibilities. Such controls include samples that are identical to your experimental sample but are missing the target that the probe is supposed to recognize. This is called a negative control. Negative controls are very useful in determining the existence of any background that can be due to cross-reactivity between the probe and your sample.

Conversely, a positive control is a sample that contains the protein or nucleic acid of interest. For example, a plasmid containing a sequence of interest or the parental strain bearing the wild-type locus can be used as a positive control in a Southern blot. Similarly, a protein carrying the epitope of interest, such as FLAG or MYC, can be used as a positive control in an immunoblot. When included in the experiment the positive control allows the investigator to confirm that the experiment was successfully executed. Thus, if the samples being tested fail to produce a signal it may indicate that the problem lies with the experimental samples and not with the procedure.

## SOUTHERN AND NORTHERN BLOTTING

The analysis of DNA and RNA through the use of Southern and northern blotting, respectively, allows the researcher to gain exquisite insight into basic biological processes. These techniques are among the most common applications in the molecular biology laboratory. Southern blotting is used to address a wide variety of basic biological problems, including defining the structure of a genomic locus, generating physical maps of a genome (Botstein et al., 1980), identifying genes involved in disease states (Gusella et al., 1983; Rommens et al., 1989), and analyzing replication and recombination intermediates. Similarly, northern blots have been instrumental in the elucidation of the transcriptional

control paradigm, defining post-transcriptional modification such as splicing and poly(A) addition, and, more recently, defining processes involving siRNA and miRNA.

In 1975 Edwin M. Southern published a paper describing the technique, which became known as Southern blotting (Southern, 1975; Southern, 2000). Briefly, this technique allows the researcher to resolve DNA fragments of defined size on an agarose gel and transfer them to a membrane, which is subsequently hybridized with a probe generated from unique sequences. The result of this approach allows the researcher to visualize DNA sequences as discrete bands among a complex mixture of other DNA molecules (Fig. 8.1.1). Southern blotting was the result of a convergence of several independent technologies. The utilization of restriction enzymes to cleave the DNA into fragments of defined size allowed for the generation of discrete banding patterns for a particular digest (Kelly and Smith, 1970; Nathans and Smith, 1975). The development of slab gel electrophoresis systems to resolve the DNA fragments (McDonell et al., 1977) was a significant advance over the tube gels used previously. Prior to slab gel development, tube gels were the method of choice for resolving nucleic acids and proteins. Tube gels were difficult to prepare and process. Analysis of the resolved products was difficult, involving slicing the gel into discs, which were individually examined for the molecule of interest. By comparison, slab gels are easy to cast and multiple samples can be resolved simultaneously. Following resolution, the processing and subsequent analysis are much less laborious and more reproducible.

Finally, the development of membranes to serve as solid supports to which the resolved DNA fragments could be transferred and analyzed made it possible to carry out the Southern blotting technique. Shortly after Southern's ground breaking paper, the adaptation of a similar approach was used to analyze RNA resulting in the northern blot technique (Alwine et al., 1977; Alwine et al., 1979). The name northern was chosen to reflect Southern's contribution, and, henceforth, other blotting techniques such as western, southwestern, northwestern continued the tradition. Together, the Southern and northern blotting techniques opened avenues of investigation in the burgeoning field of molecular biology that were previously unassailable.

The following is a brief discussion of the issues that must be considered when designing a Southern or northern blot experiment. The specific details will be covered in greater depth elsewhere as noted. As an investigator designing a blotting experiment one must consider four distinct issues: (1) resolution and denaturation of the molecules, (2) membrane selection, (3) transfer methodology, and (4) hybridization methodology.

## Resolution

The first issue to consider when designing a blotting experiment is how to resolve your molecules into a distinct pattern. When dealing with RNA, the molecules are of a defined size as determined by the transcriptional unit, i.e., mRNA, tRNA, etc. Because of its single-stranded nature, RNA molecules have the potential to form secondary structures by forming typical Watson-Crick base pairs between complementary bases that are part of the same strand, which may affect the resolution. To address this problem, the RNA should be treated with denaturants such as glyoxal or formamide prior to resolving the molecules in the gel [Thomas, 1980; also see *UNIT 7.2* (Armstrong and Schultz, 2015)]. Alternatively, treatment of the RNA and resolution of the molecules in the presence of formaldehyde will also prevent the formation of secondary structures (Lehrach et al., 1977). As a result, the RNA will migrate as a function of its size rather than its structure.

In resolving DNA molecules, the size of a fragment will be determined by the particular restriction enzyme and the frequency of cutting within the interval of interest. Typically, the DNA is digested and then resolved on a gel. Many of the model systems used today

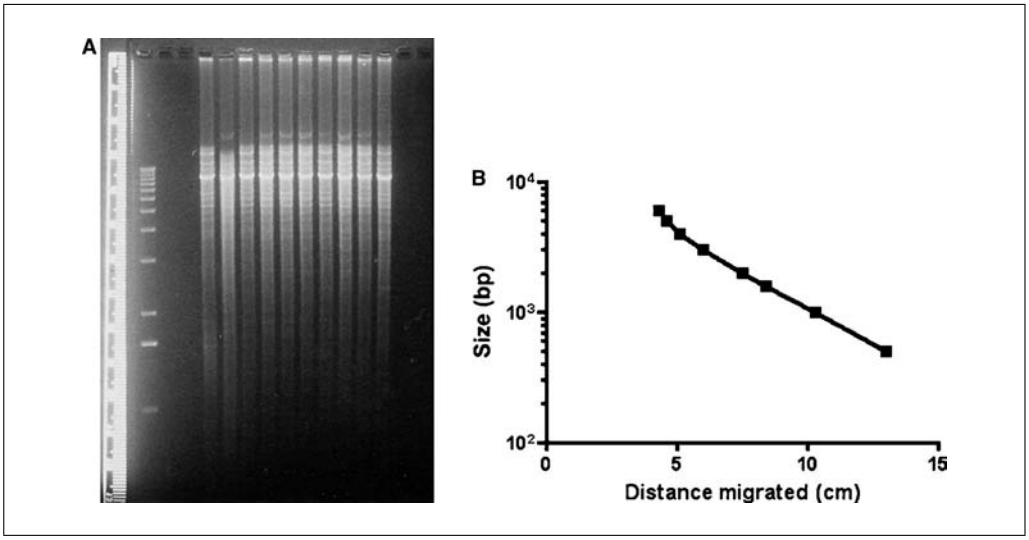


**Figure 8.1.2** Gel documentation and standard curve. (**A**) Photograph of a 14 × 14–cm 0.8% agarose gel with samples of restricted yeast genomic DNA, stained with ethidium bromide and aligned with a fluorescent ruler. (**B**) Standard curve of the migration distances of the molecular weight standards resolved on the agarose gel and plotted as a function of size (bp) versus distance migrated from the origin (cm).

have had their genomes sequenced; thus, it is possible to identify the interval of interest and select enzymes that will produce a defined pattern of fragments of discrete size to assay with the Southern blot.

Once the sizes of the molecules have been defined, the gel conditions to optimize resolution can be chosen as described in *UNIT 7.2* (Armstrong and Schultz, 2015). In most cases an agarose slab gel will be sufficient to resolve the molecules. Because it is possible to adjust the concentration of agarose over a wide range, which will in turn affect the size of the pores in the gel, one can easily resolve fragments from very small (hundreds of base pairs) to very large sequences (thousands or tens of thousands of base pairs). It is even possible to resolve entire chromosomes for certain organisms using a CHEF (clamped homogeneous electrical field) apparatus (Chu et al., 1986; *CP Molecular Biology Unit 2.5B*, Finney, 2000). Once the size of the molecules of interest drop below 100 bp, it might be advisable to use polyacrylamide as the gel matrix instead of agarose. Because polyacrylamide can be used at much higher concentrations, the pore sizes associated with the gel matrix are much smaller. Thus, it is possible to achieve nucleotide resolution under the appropriate conditions. These gels may be prepared as denaturing or nondenaturing depending on the exact application, see *CP Molecular Biology Unit 2.12* (Ellington and Pollard, 1998) and Sambrook et al. (1989). Finally, optimizing the running conditions for the gel is primarily an empirical process. Using a standard laboratory electrophoresis unit (14 × 14–cm), a gel run at 50 to 100 V, or 1.9 V/cm$^2$, in TBE buffer for 4 to 7 hr will resolve the nucleic acids sufficiently for analysis. It is usually advisable to use a larger gel (14 × 14–cm) as compared to a mini-gel (7 × 10–cm), when running a blotting experiment. A larger gel allows for greater separation of the molecules of interest, as a result of the increase in distance migrated from the origin. This in turn will result in better resolution which will aid in the subsequent analysis of the blot. After running the gel, it is important to examine it by staining with ethidium bromide. This will allow the researcher to visualize the nucleic acids and the size markers on a UV transilluminator to assess the resolution of the gel and the appearance of the cut genomic DNA or RNA. It is important to note that your band of interest will not be able to be distinguished: instead a smear will be observed that corresponds to the entire genome cut into fragments of different sizes (Fig. 8.1.2A) or all the RNA molecules present in your experimental

sample. At this point it is advisable to take a picture of the ethidium bromide stained gel aligned with a ruler (Fig. 8.1.2A). This will document the gel for later reference because the molecular weight standards will not show up in the blot since your probe will not anneal to them. This allows the investigator to compare the distances migrated by the molecular weight standards in the gel and the bands of interest on the blot during the analysis step. In order to determine the size of the observed bands on the blot, a standard curve can be generated by plotting the distance migrated by the standards versus their molecular weight (Fig. 8.1.2B). The investigator can measure how far the bands on the blot traveled from the origin and correlate this to the standard curve to determine the sizes of the bands. Alternatively, there are commercially available molecular weight standards that can be visualized with the detection system used, such that the molecular weight markers and the bands of interest will be simultaneously developed on your blot.

### Denaturation

As discussed above, the RNA used for northern blot experiments is denatured with formaldehyde or glyoxal prior to running the gel to disrupt secondary structures that may affect the resolution of the molecules. In the case of a Southern blot, the DNA is resolved as double-stranded DNA fragments of defined size. If the expected fragments are >10 to 15 kb in size, an additional step involving depurination or UV radiation can be used to break the DNA into smaller, more readily transferable fragments. Depurination involves treating the gel, following resolution, with a solution of 0.1 to 0.25 M HCl for 15 min. This treatment results in the formation of abasic sites throughout the DNA. Subsequent incubation in a solution of 0.5 M NaOH leads to strand scission at the abasic sites. Excessive depurination can fragment the DNA into such small fragments that its ability to hybridize with a probe later in the procedure will be reduced. Treating the resolved DNA, which is intercalated with ethidium bromide, with short-wave, 240-nm UV will also generate strand breaks. As above, excessive irradiation can shear the DNA or lead to cross-linking, which will inhibit its ability to hybridize with the probe fragment. If done appropriately the resulting smaller DNA fragments should transfer efficiently. This step is discussed in *UNIT 8.2* (Hoopes, 2012). Once the fragments have been resolved and fragmented, the DNA needs to be denatured to allow for subsequent hybridization with the probe. There are two basic approaches to achieve this. The first involves incubating the gel in a denaturation buffer containing sodium hydroxide (NaOH). Alkaline denaturation results in the deprotonation of the atoms that form the hydrogen bonds between the base pairs of the duplex DNA. This reaction destabilizes the interaction between the DNA strands, generating single-stranded species and water by association of the extracted proton and the hydroxyl ion (Ageno et al., 1969). Following denaturation, the gel is incubated in a neutralization buffer containing 1 M Tris·Cl, pH 7.4. The DNA in the gel remains single stranded following the neutralization step. The single-stranded nature of the DNA is likely to be maintained for a variety of reasons. First, alternative interactions between the DNA bases (see Figure 8.1.2B) and the sugar moieties of the agarose, and later the amide moieties of the membrane, will limit reannealing. Whereas some limited reassociation may occur, it will probably result in mispaired sequences that will have significant amounts of single-stranded DNA available to hybridize with the probe sequence later in the hybridization step experiment. Alternatively, one can use an alkaline transfer buffer, as discussed below, allowing for simultaneous denaturation, transfer, and cross-linking.

### Membrane Selection

The membrane used in a blotting experiment serves as a solid support to transfer the nucleic acids and subsequently hybridize them with a probe derived from a unique sequence. When blotting technologies were first developed, there were several types of solid supports, including DBZ paper (Wahl, 1979) and nitrocellulose (Southern, 1975).

**Table 8.1.1** General Properties of Blotting Membranes

| | Type of membrane | | | | |
|---|---|---|---|---|---|
| | Nitrocellulose | Supported nitrocellulose | Nylon | Charged nylon | PVDF |
| Application | Western | Western | Southern | Southern | Western |
| | Southern | Southern | Northern | Northern | — |
| | Northern | Northern | — | — | — |
| Binding capacity | 80 to 150 μg/cm$^2$ | 75 to 90 μg/cm$^2$ | >400 μg/cm$^2$ | >600 μg/cm$^2$ | >200 μg/cm$^2$ |
| Transfer methods | Capillary blotting | Capillary blotting | Capillary blotting | Capillary blotting | Electroblotting |
| | Vacuum blotting | Vacuum blotting | Vacuum blotting | Vacuum blotting | — |
| | Electroblotting | Electroblotting | Electroblotting | Electroblotting | — |
| | — | — | Alkaline blotting | Alkaline blotting | — |
| Immobilization | UV-cross-linking | UV-cross-linking | UV-cross-linking | UV-cross-linking | Air drying |
| | Baking (80°C) | Baking (80°C) | — | — | — |
| Detection methods | Isotopic | Isotopic | Isotopic | Isotopic | Isotopic |
| | Chemi-luminescent | Chemi-luminescent | Chemi-luminescent | Chemi-luminescent | Chemi-luminescent |
| Reprobing | ± | + | + | + | + |

Initially, nitrocellulose became the membrane of choice because of its relatively low cost and ease of handling. However, today a variety of membranes are available for blotting experiments (Table 8.1.1). Nylon membranes are the preferred substrate for most Southern and northern blotting experiments. Nylon has the advantages of being relatively cost effective, very stable, and having improved mechanical strength as compared to nitrocellulose. Thus, the blot can be stripped and rehybridized multiple times. Following hybridization, the blot can be either treated with an alkaline buffer or boiled in the presence of SDS to dissociate the bound probe from the blot. This process, termed "stripping," allows the investigator to hybridize the same blot many times with different probes. The primary consideration in selecting a membrane that will be stripped and probed multiple times is mechanical strength. Nylon is preferable to nitrocellulose for this type of experiment. When using commercially available detection kits, make sure to follow the stripping protocol suggested in the user's manual.

Additionally, nylon membranes can be either charged or neutral, which facilitates the use of a variety of transfer protocols. The selection of a charged or neutral membrane may be determined by the nature of the fragments being transferred. If very small fragments (50 to 200 bp) are being blotted, a charged membrane that exhibits a higher degree of retention may be advantageous. Alternatively, a neutral membrane might be better for larger fragments due to a reduced background problem. Nylon membranes are commercially available from a number of companies including Hybond N and N$^+$ (GE healthcare), and Zetaprobe (Bio-Rad).

### Transfer

Once the RNA or DNA is resolved and a membrane selected, the next step in the blotting process involves transferring the nucleic acid from the gel to the membrane. Over the years, numerous variations on the basic protocol have been developed. Most laboratories

**Table 8.1.2** Transfer Methods

| | Capillary blotting | Electroblotting | Vacuum blotting |
|---|---|---|---|
| Technology requirements | Low tech | Requires a transfer chamber | Requires a vacuum blotting chamber |
| Speed | 2 to 24 hr | 1 to 2 hr | 5 to 30 min |
| Applications | Nucleic acids | Protein | Nucleic acids |
| | — | Nucleic acids | — |
| Transfer efficiency | Good | Excellent | Excellent |

will have a basic protocol in place. It is well worth the investigators time to experiment with different conditions to arrive at an optimal protocol for their particular application.

There are three commonly used techniques to transfer nucleic acids from the gel to the membrane: capillary blotting, electroblotting, and vacuum blotting. The relative merits of each technique are outlined in Table 8.1.2.

### *Capillary blotting*

Capillary blotting was used in the original Southern protocol (Southern, 1975). This approach involves the construction of a stack consisting of a wick, several pieces of blotting paper cut to the size of the gel, the gel, the membrane cut to the size of the gel, an additional piece of blotting paper, and finally a stack of paper towels. When assembled, the stack is placed on a platform such that the wick is in contact with a pool of the transfer buffer. As the name implies, the buffer wicks through the blotting paper and gel and into the paper towels on top of the stack. As the buffer moves upward thru the gel, the DNA migrates with it through the pores of the gel until it encounters the membrane and becomes trapped.

To carry out a capillary blotting experiment, there are several issues to be considered. First, one must select a transfer buffer. Traditionally, people have used ionic solutions such as 1× SSC (0.15 M NaCl/0.15 M NaCitrate)—e.g., Southern used this buffer in his original experiments with nitrocellulose. In doing so, he showed that increasing the ionic strength of the buffer improved the retention of the DNA to the membrane (Southern, 1975). In contrast, later studies using nylon membranes showed that it was possible to transfer the DNA in pure deionized water (Reed and Mann, 1985). Thus, the ionic nature of the transfer buffer is less important when using nylon membranes; however, most protocols still recommend the use of a 5× to 10× solution of SSC. Alternatively, as mentioned above, an alkaline transfer buffer can be used (Reed and Mann, 1985). This approach allows the investigator to skip the denaturation step described above. When used in concert with a charged membrane such as Hybond $N^+$ (GE Healthcare), the conditions lead to a cross-linking of the DNA when it encounters the membrane. The decision to use neutral or alkaline transfer protocols is dependent on the individual investigator.

Finally, there is the issue of how long to blot the DNA to the membrane. Most protocols suggest 16 hr or overnight. There are reports that 1 to 2 hr is sufficient for the transfer of most nucleic acids species (Reed and Mann, 1985). The caveat is that larger molecules tend to take longer to migrate out of the gel and onto the membrane surface.

### *Electroblotting*

Electroblotting (Bittner et al., 1980) utilizes a tank transfer (see below) apparatus identical to that used for immunoblotting. Once the nucleic acid has been resolved in the gel, a sandwich of blotting paper, gel, membrane and blotting paper is built and placed in a cassette. The cassette is inserted into the buffer chamber filled with an ionic buffer such

as TBE, so that the membrane is proximal to the positive electrode. When voltage is applied to the chamber, the negatively charged nucleic acid molecules will move from the gel to the membrane. Because of the small pore sizes associated with polyacrylamide gels, the capillary blotting approach is less efficient than electroblotting (Sambrook et al., 1989). As mentioned above, a charged membrane may be preferable for this approach. The primary advantage of this approach is the rapid transfer time, usually 1 to 2 hr, and the high recovery of nucleic acids. This is the method of choice for blotting nucleic acids when using a polyacrylamide gel to resolve the molecules.

Another advantage of this system is that it allows for the efficient transfer of whole chromosomes resolved in agarose gels by CHEF (contour-clamped homogeneous electric field electrophoresis) without having to fragment the DNA (Genie blotter, Idea Scientific).

If electroblotting is used with an agarose gel, it is important to monitor the current during the transfer. High current may lead to a significant increase in the buffer temperature, which could, in turn, melt the agarose gel. Adjusting the salt concentration of the transfer buffer will limit this problem. Some systems come with a dedicated power supply that provides a low-voltage power source and ensures that specific voltages are applied (Genei power supplies, Idea Scientific). Polyacrylamide gels are much less sensitive to temperature fluctuations and are thus better suited for this approach. The only requirement for this technique is the availability of a transfer chamber large enough to accommodate the gel.

### *Vacuum blotting*

The third approach that is commonly used to transfer nucleic acids to a membrane support is vacuum blotting (Olszewska and Jones, 1988). This approach takes advantage of the application of a vacuum to a transfer system, which requires the transfer buffer to pass through the gel and membrane to reach a solution trap. As with electroblotting, the advantages of this system are the quick transfer time, ranging from 5 to 30 min, the tight resolution of the resulting bands, and the improved recovery of the nucleic acids as compared to capillary blotting. This approach is applicable to both Southern and northern blots. These transfer systems are commercially available. With all approaches, monitoring transfer efficiency is important. One simple strategy is to stain the agarose or acrylamide gel post transfer to evaluate if quantitative transfer of the nucleic acids has occurred. Typically, the higher molecular weight species will not transfer as well, with some remaining in the gel.

## Cross-Linking

Once the nucleic acids have been transferred to a membrane, they have to be linked to the membrane. This process is called cross-linking. The approach to cross-linking varies depending on the membrane. As mentioned above, an alkaline transfer with a charged membrane often leads to spontaneous cross-links being formed between the membrane and nucleic acids, obviating the need for additional treatment. If a neutral membrane is used, UV irradiation is required to covalently attach the nucleic acids to the membrane in preparation for hybridization. Application of UV radiation at a wavelength of 254 nm for 1 to 5 min results in the formation of a covalent bond between the amide groups on the nylon and the carbonyl groups found on the thymine and uracil bases. This is the same reaction that produces pyrimidine dimers in genomic DNA following exposure to UV radiation. The two most common sources of UV radiation are the UV transilluminator or a cross-linking instrument available from several suppliers (e.g., Stratagene, Bio-Rad, and UVP). These instruments are built specifically for the purpose of applying a defined amount of UV radiation to a blot. If a transilluminator is used, the membrane needs to be exposed to 1.5 kJ/$m^2$ (Church and Gilbert, 1984).

Alternatively, the investigator can bake the membrane at 80°C for 2 hr. Baking leads to the dehydration of the nucleic acids on the blot, resulting in the generation of stable hydrophobic interactions between the nucleic acid and the membrane. Because water has been excluded during this process it is very difficult to disrupt these interactions, even when the membrane is rehydrated and processed. This approach leads to stable associations between the nucleic acids and membranes, allowing for multiple rounds of rehybridization (Nierzwicki-Bauer et al., 1990). Thus, baking is a very good method to immobilize nucleic acids to a solid support. If nitrocellulose is used as the solid support, the baking must occur under vacuum; otherwise the nitrocellulose will combust. Additionally, baking for longer than 2 hr can cause the nitrocellulose to become very brittle and hard to handle.

## Hybridization

Once a blot has been generated, the next step involves applying a probe that will anneal to the sequence of interest and allow for the visualization and/or quantitation of the desired sequence. To accomplish this, the blot is processed in three successive steps: prehybridization, hybridization (probing), and washing.

### *Prehybridization*

The prehybridization step is used to "block" the blot so as to limit nonspecific interactions of the probe fragment with the membrane. There are a wide variety of prehybridization solutions, of which the most popular are Denhardt's (Denhardt, 1966), Blotto (Johnson et al., 1984), and Church's buffer (Church and Gilbert, 1984). Although all three of these buffers are inexpensive and readily prepared with reagents commonly found in most molecular biology laboratories, Denhardt's will be used as an example for the remainder of the section. A 50× solution of Denhardt's consists of 1% BSA, 1% Ficoll, and 1% polyvinylpyrrolidone. The prehybridization solution consists of 5× Denhardt's, 6× SSC, 0.5% SDS, and 100 µg/ml single-stranded salmon sperm DNA. The components of a prehybridization solution typically consist of polar and nonpolar molecules that associate in a nonspecific manner with the polar and nonpolar moieties available on the membrane. The transferred nucleic acids only occupy a limited amount of the surface area of the membrane. The molecules in the prehybridization solution coat the rest of the membrane. In the absence of such a treatment, the probe would associate with the unoccupied sites on the membrane, resulting in very high background and a very low signal-to-noise ratio. To carry out the prehybridization step, the blot is placed in a container to which the prehybridization buffer is added. The blot is incubated at the desired temperature, typically between 50° to 65°C, for 3 to 24 hr. Once the membrane is blocked, the probe can be synthesized and added to the blot for hybridization.

### *Probing*

To detect the desired sequence on the blot, a probe that anneals to the sequence must be synthesized, as discussed in *UNIT 8.4* (Haushalter, 2008). The probe will have two properties. First, it will anneal specifically with the sequence of interest. Second, it will be modified in such a way as to allow for the detection of the annealed sequences. To address the first condition, a unique sequence must be available to serve as a template for the probe. This sequence can be an oligonucleotide or a DNA fragment. Depending on the nature of the template sequence, the hybridization and washing steps will be modified to accommodate the probe. If an oligonucleotide is used as the probe, it will be end labeled with $^{32}$P-γ-ATP so that the 5′ phosphate of the molecule is now radioactive and ready to use as a probe (*CP Molecular Biology Units* 3.4-3.15, Ausubel et al., 2007; Sambrook et al. 1989). Alternatively, oligonucleotide probes come in a wide variety of modifications that enable nonradioactive (e.g., fluorescent or chemiluminescent) detection. If a cloned sequence is used as a template, the DNA fragment must first be denatured. The

denatured DNA will be mixed with primers that will anneal to the template and prime DNA synthesis through the action of a DNA polymerase such as Klenow (Klenow and Henningsen, 1970). During this step, modified nucleotides are incorporated to generate the probe that can be detected after hybridizing with the target DNA. The nucleotides can be radioactive, such as $^{32}$P-α-dCTP, and thus be readily detectable with a Geiger counter or as an autoradiogram. When synthesizing a radioactive probe, it is important to assess the quality of the resulting reaction. To assay the resulting probe for efficiency of incorporation of the radioactive nucleotides, a TCA (trichloroacetic acid) precipitation is utilized. This approach allows for the quantitation of incorporation of the labeled nucleotide into the newly synthesized probe. An incorporation of 40% to 70% of the total radioactive nucleotide is usually acceptable for generating a probe to use in a Southern or northern blot experiment. Alternatively, one can utilize nucleotides containing a moiety that is recognized by an antibody or protein, which is conjugated to an enzyme (see Table 8.1.3 for examples of detection systems used). The enzyme will react with a substrate reagent to generate a detectable signal. There are a variety of commercially available kits that utilize either the radioactive or nonradioactive detection systems providing the investigator with flexibility in deciding how to generate and apply the probe to their blots. Among the labeling methods available that result in the most sensitive probes are random primed and labeling by PCR methods (for a detailed description of different DNA labeling methods see *UNIT 8.4* (Haushalter, 2008).

Random primed labeling utilizes a denatured DNA template that will be copied by a DNA polymerase in the presence of a mixture of normal and modified dNTPs (radioactive, or conjugated to a moiety). As the name implies, DNA synthesis is primed by the presence of a mixture of random hexanucleotides (single-stranded DNA oligos consisting of 6 nucleotides, in which the sequence was generated randomly); this means that it does not matter what the sequence or length of your template is. As long as it is greater than 200 bp in length, there will be hexanucleotides that will anneal and prime DNA synthesis. The polymerase used is the Klenow fragment, because it lacks 5′ to 3′ exonuclease activity, which prevents the polymerase from degrading any primers it encounters that are annealed to the template and are already extended. The resulting probe is very specific and of great sensitivity, and consists of a mixture of labeled DNA fragments of different lengths. This method is recommended when running a Southern blot with the objective of trying to detect a single-copy gene in a complex genome or to detect rare mRNA's in a northern blot. A disadvantage of this method is that a considerable amount, 0.3 to 1 μg, of highly purified template DNA is required.

The PCR labeling method relies on amplifying a DNA sequence by PCR in the presence of a mixture of normal and modified dNTPs (radioactive or conjugated to a moiety) such that the PCR product consists of uniformly labeled, double-stranded DNA. Because the probe is generated by exponential amplification of a template DNA sequence, you need very little template DNA and its quality is not as crucial as with the random primed method. When making a new probe specific to a new target using this method, new primer sets should be designed. This method requires use of a thermostable polymerase and likely some optimization for the reaction to work.

Traditionally, radioactive labeling methods were the method of choice because they were more sensitive, but nonradioactive methods of detection have gained great popularity as they are safer and have achieved the same level of detection. Additionally, nonradioactive methods are now of comparable cost relative to the radioactive methods. In the long term, nonradioactive methods may be more cost-effective because the waste products do not require special handling and disposal and are more environmentally friendly.

**Table 8.1.3** Chromogenic and Luminescent Visualization Systems[a,b]

| System | Reagent[c] | Reaction/detection | Comments[d] |
|---|---|---|---|
| ***Chemifluorescent*** | | | |
| Not enzyme-based, direct labeling of target protein | Antibody conjugated to fluorescent dye labels. Goat-IgG[a]-Cy3 ($\lambda_{max}$ 570 nm) Goat-anti-rabbit IgG[a]-Cy5 ($\lambda_{max}$ 670 nm) | Direct fluorescence detection using fluorescence scanner and CCD imagers. No need for enzyme-substrate amplification | Very sensitive (detects <1 pg protein). Reaction detected within a few seconds to 1 hr. Fluorescence can last for months. Two different proteins can be detected at the same time. Kits available from several different vendors. (e.g., GE Healthcare and Pierce) |
| ***Chemiluminescent*** | | | |
| HRPO[a]-based | Luminol/$H_2O_2$/ *p*-iodophenol | Oxidized luminol substrate gives off blue light. There are a variety of systems that use similar substrates with slight variations. Detection with film, CCD cameras or laser scanners. | Very convenient, reaction detected within a few seconds to 1 hr. Very sensitive (detects < 1 pg to 50 pg of protein). Kits available from several different vendors. |
| AP-based | Substituted 1,2-dioxetane-phosphates (e.g., AMPPD[a], CSPD[a], Lumigen-PPD[a], and Lumi-Phos 530[g]) | Dephosphorylated substrate gives off light. Detection with film, CCD cameras or laser scanners. | Reasonable sensitivity on all membrane types; reaction can last for >1 hr. Kits available from several different vendors. Consult reagent manufacturer for maximum sensitivity and minimum background. |
| ***Chromogenic*** | | | |
| AP[a]-based | BCIP[a]/NBT[a] | BCIP hydrolysis produces indigo precipitate after oxidation with NBT; reduced NBT precipitates; dark blue-gray stain results | More sensitive and reliable than other AP-precipitating substrates; note that phosphate inhibits AP activity |
| HRPO-based | *TMB*[a,f] | Forms dark purple stain | More stable, less toxic than DAB/$NiCl_2$; may be somewhat more sensitive[f]; can be used with all membrane types; kits available from several different vendors |
| | DAB[a]/$NiCl_2$[e] | Forms dark brown precipitate | More sensitive than 4CN[a] but potentially carcinogenic; resulting membrane easily scanned |
| | 4CN[a] | Oxidized products form purple precipitate | Not very sensitive (Tween 20 inhibits reaction); fades rapidly upon exposure to light |

[a]Abbreviations: AMPPD or Lumigen-PPD, disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-tricyclo[3.3.1.1$^{3,7}$] decan}-4-yl)phenyl phosphate; AP, alkaline phosphatase; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; 4CN, 4-chloro-1-napthol; CSPD, AMPPD with substituted chlorine moiety on adamantine ring; DAB, 3,3′-diaminobenzidine; HRPO, horseradish peroxidase; NBT, nitroblue tetrazolium; TMB, 3,3′,5,5′-tetramethylbenzidine. IgG, Immunoglobulin G.

[b]Table adapted from *CP Molecular Biology UNIT 10.8* (Gallagher et al., 2004).

[c]Recipes and suppliers are listed in *CP Molecular Biology UNIT 10.8* except for TMP, for which use of a kit is recommended.

[d]See Commentary for further details.

[e]DAB/$NiCl_2$ can be used without the nickel enhancement, but it is much less sensitive.

[f]McKimm-Breschkin (1990) reported that if nitrocellulose filters are first treated with 1% dextran sulfate for 10 min in 10 mM citrate-EDTA (pH 5.0), TMB precipitates onto the membrane with a sensitivity much greater than 4CN or DAB, and equal to or better than that of BCIP/NBT.

[g]Lumi-Phos 530 contains dioxetane phosphate, $MgCl_2$, CTAB, and fluorescent enhancer in a pH 9.6 buffer.

Once a probe is generated, it must be denatured and added to the blot for 1 to 24 hr. Determining how long to hybridize the blot depends on a variety of factors and must be determined empirically. It is often convenient to hybridize the blot overnight and thus be confident that the hybridization of the probe to the target molecule has been maximized. If an investigator is interested in accelerating the hybridization process, one can include reagents such as 10% dextran sulfate or polyethylene glycol molecular weight 8000 to the buffer (Wahl et al., 1979; Amasino, 1986). These reagents disrupt the water structure around the nucleic acids, thereby increasing the relative concentration of these species and improving the hybridization characteristics. Addition of these reagents can reduce the hybridization time to 1 to 2 hr. There are a variety of premade hybridization buffers commercially available for this purpose. Each probe will have specific characteristics that affect its ability to associate with the target. The most important characteristic for a hybridization experiment is the melting temperature of the probe sequence ($T_m$). The $T_m$ is the temperature at which 50% of the base pairs in a duplex DNA have been denatured and will be affected by the base composition of the probe. GC base pairs have three hydrogen bonds as compared to two hydrogen bonds in an AT base pair; thus, GC base pairs are more stable. Probes with a high GC content have a higher $T_m$ than probes with a high AT content. It is important to determine the temperature at which your probe will specifically interact with the target sequence, but at which other nonspecific interactions will be disrupted. To roughly calculate the $T_m$ of an oligonucleotide 14 to 20 nucleotides in size, the Wallace rule (Wallace et al., 1979; Suggs et al., 1982) can be used. The Wallace rule uses the following equation, $2 \times (\#A + \#T) + 4 \times (\#G + \#C)$. Each AT base pair contributes 2°C to the melting temperature and each GC base pair contributes 4°C. For example, the $T_m$ for the oligonucleotide AGTTGGCACTGGATTGCC can be expressed as $T_m = 2 \times (3 + 5) + 4 \times (6 + 4) = 56°C$.

Alternatively, the $T_m$ of sequences longer than 50 base pairs can be determined using the %GC method (Meinkoth and Wahl, 1984): $T_m = 81.5 + 16.6[\log(Na^+)] + 0.41(\%G+C) - 500/N - 0.61(\%formamide)$; where $N$ = length of the probe and the $Na^+$ refers to the concentration of monovalent cations in the buffer. Many buffers use sodium salts as one of the reagents. As an example, Denhardt's, described above, contains 6× SSC, which is made of a mixture of sodium chloride and sodium citrate. A 1× solution of SSC is 0.165 M $Na^+$. By changing the concentration of the SSC during the experiment, the $T_m$ of the probe fragment can be altered. The presence of mismatches between the probe and the target also reduce the $T_m$ by about 1°C/1% mismatching. Thus, probes associating with a sequence with which it shares 85% sequence homology would have a $T_m$ 15°C lower than an association between a probe and target sharing 100% homology. These differences in $T_m$ between mismatched and correctly paired probes allows the researcher to choose a washing temperature that will remove nonspecific binding while maintaining specific interactions. In addition to probe length and salt concentration, inclusion of reagents such as formamide can dramatically affect the $T_m$ of the probe and thus affect the choice of hybridization conditions. Formamide is a denaturant, which tends to destabilize duplex nucleic acids by disrupting the hydration skeleton around the molecule. Additionally, formamide can provide alternative hydrogen bonding partners for the nucleotide bases in a single stranded molecule. As such, the predicted $T_m$ of a probe is reduced substantially in the presence of formamide. This may be advantageous in situations where the investigator is trying to maintain stringent conditions at lower temperatures. This change in $T_m$ is accounted for by including the %formamide as a factor in the %GC method. Working at higher temperatures can be problematic because of the need for specialized equipment, problems with evaporation, and maintaining consistency. If you can derive the same results at a lower temperature, then a low-temperature protocol may be advantageous. Several commercially available prehybridization and hybridization solutions incorporate formamide or urea to reduce the $T_m$ of DNA probes (ULTRAhyb

Ultrasensitive Hybridization Buffer, Fisher Scientific; DIG Easy Hyb, Roche). These agents allow one to perform the hybridization step, for example, at temperatures between 37° and 42°C.

### *Washing*

Following hybridization, the blot must be washed to remove unassociated and nonspecifically annealed probe from the blot. By altering the stringency of the washing conditions, one can affect the degree of specificity obtained by the probe. The term "stringency" refers to conditions that affect the association of two single-stranded nucleic acids. In a standard protocol, there are two variables that can affect the stringency of hybridization, temperature and salt concentration. The association of two single-stranded nucleic acids is dependent on their relative melting temperatures. Thus, as the temperature is increased, the likelihood that the strands will separate also increases. Conversely, the presence of salt in the solution tends to increase the $T_m$ for a given interaction. This effect can be explained by the interaction between cations such as sodium and the nucleic acids, which are negatively charged. When high concentrations of cations are present in the buffer there is a tendency to associate with the nucleic acids to neutralize their inherent negative charge. Thus, interactions between the probe fragment and the target are substantially stabilized. Therefore, as the salt concentration is decreased, the $T_m$ decreases, leading to greater disassociation between the annealed strands. By increasing the temperature and decreasing the salt concentration in the wash buffers the stringency of the wash is also increasing. A typical wash protocol involves a series of sequential washes with decreasing salt concentration. For example, the first wash uses a solution of 2× SSC, 0.1% SDS and is carried out at room temperature for 15 min. This is followed by a wash in 1× SSC, 0.1% SDS at 65°C for 15 min. Finally, the last wash is the most stringent, utilizing a buffer of 0.1× SSC/0.1%SDS at 65°C for 15 min. Over the course of the washes from the original hybridization solution containing 6× SSC to the most stringent wash of 0.1× SSC, the salt concentration decreases from almost 1 M $Na^+$ to 0.0165 M $Na^+$. As mentioned above, interactions between sequences with <100% homology are more likely to be disrupted by the wash protocol because of their inherently lower $T_m$ as compared to a perfectly base paired hybridization. By executing this wash protocol, one is selecting for highly specific associations between the probe fragment and the target sequences on the blot. It is possible to monitor the progress of the washing protocol, when using radioactive probes, with a Geiger counter (see *UNIT 2.3*; Meisenhelder and Bursik, 2008).

## Detection

Once the blot is washed, the next step is detection of the annealed probe as discussed in *UNIT 8.4* (Haushalter, 2008). If a nonradioactive probe kit is used, the detection protocol will be outlined by the manufacturer. If a radioactive probe is used, the blot is exposed to a sheet of film in a cassette. The film cassette will often have intensifying screens that, as the name implies, amplifies the signal generated by the probe. Following an exposure time ranging from hours to days, the film is developed and, hopefully, a pattern of bands is evident. Alternatively, an imaging device such as a phosphorimager can be used to visualize the banding pattern. This approach has the advantage of allowing the investigator to quantitate the intensity of the signal associated with a specific band. Such quantitation facilitates the determination of molecular half-life, relative abundance of a molecule compared to a loading control, etc. Such information can be invaluable to elucidate the mechanisms involved in a particular biological process.

A variety of digital systems are available for imaging blots developed with radioactive or nonradioactive probes. They have become more affordable and user friendly. As

mentioned above, it may be more cost-effective and environmentally friendly to switch from film to a digital system. Digital images are much more amenable to quantification. Digital systems can simultaneously capture bands that are very faint due to low amount of that particular target together with bands that are very bright corresponding to a very abundant target without the image being saturated. This is an important feature, since for quantification purposes it is important to compare the signal of your target of interest to that of a loading control: sometimes the abundance of these two are not within the same range. The digital systems all include software applications that have evolved over the years to become very user friendly and allow for an easy quantification process.

## IMMUNOBLOTTING

Immunoblotting, also known as "western blotting" when following the same humor used to derive the term "northern" (a play on the name "Southern"), is basically a method used to study protein expression, purification, and modification, and has many applications of great relevance in both the research and clinical setting. The term western blotting was first used by Burnette (1981) when referring to the method used to assay proteins immobilized on a membrane originally developed by Towbin et al. (1979).

One of the main differences between immunoblotting and Southern or northern blotting is the nature of the target and probe. As discussed above, Southern and northern blots are used to assay nucleic acids and the probe of choice is a complementary nucleic acid that anneals to the target with high affinity through base pairing interactions. In the case of an immunoblot, the molecule of interest is a protein and the preferred probe an antibody. Antibodies (Fig. 8.1.3) recognize specific antigens associated with a particular protein. Because the proteins are denatured during the separation step, the particular antigen recognized by the antibody may be disrupted. Thus the effectiveness of a given antibody to recognize a specific protein in an immunoblot experiment must be determined empirically. Antibodies are often characterized by the manufacturer as being effective for a particular application including western blotting, immunoprecipitations, etc. Such information is invaluable in selecting an antibody.

### Separation

The first step in an immunoblotting experiment requires the separation of a complex mixture of proteins into an ordered array based on the size of the proteins. This is accomplished with the separating power of a denaturing polyacrylamide gel electrophoresis (PAGE) system (Laemmli, 1970). As discussed in *UNIT 7.3* (Gallagher, 2012), this procedure requires the denaturation of the proteins in a mixture with sodium dodecylsulfate (SDS), also known as sodium lauryl sulfate. The denatured protein sample is loaded onto a discontinuous gel system, and resolved as described. The effective range of resolution on an SDS-PAGE depends on the concentration of acrylamide and bisacrylamide. As with agarose gels, the higher the concentration of the gel the higher the resolving capacity. *UNIT 7.3* (Gallagher, 2012) provides the detailed information necessary for determining the optimal gel percentage to resolve a protein of a particular molecular weight. Additionally, the thickness of the gel will affect the investigators ability to transfer the proteins from the gel to the membrane. Typically, gels between 1- and 0.4-mm are used for immunoblotting experiments. Thicker gels may impede the transfer step, while thinner gels are fragile and difficult to work with.

### Membrane Selection

The choice of membrane (Table 8.1.1) used for an immunoblot is crucial to the success of the experiment [see *UNIT 8.3* (Ni et al., 2016 Sambrook and Russell, 2001; Kurien and Scofield, 2006)]. The membrane serves as a solid support to which the proteins are transferred. The properties of the membrane will affect the retention of the protein, the ability

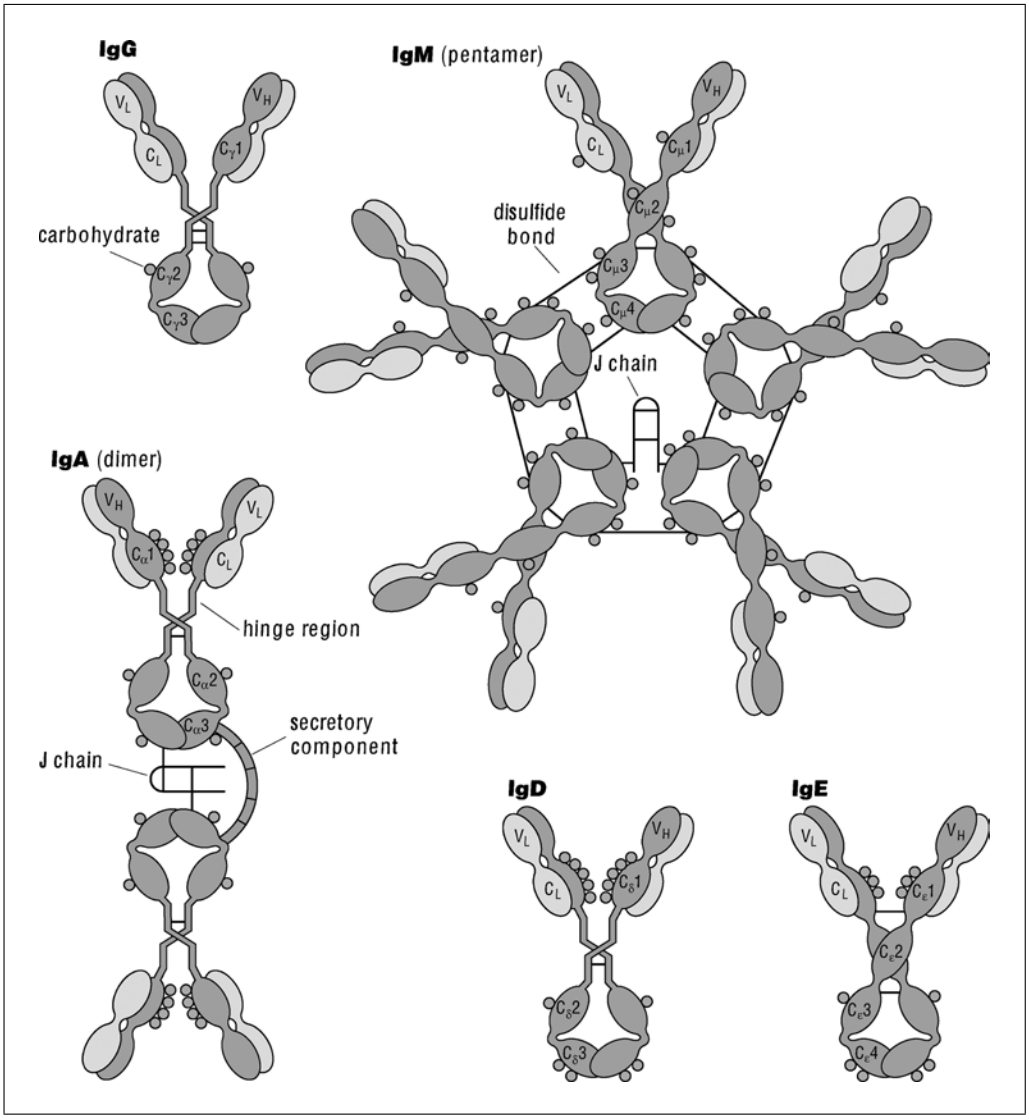


**Figure 8.1.3** Structures of the five major classes of secreted antibody. Light chains are shown in light gray; heavy chains are shown in dark gray. Circles denote areas of glycosylation. The polymeric IgM and IgA molecules contain a polypeptide known as the J chain. The dimeric IgA molecule shown includes the secretory component. Reproduced with kind permission from Coico et al. (2003).

to strip and reprobe the membrane, and the detection methodology to use. In the event the immunoblot experiment is not producing high-quality results, it is worthwhile to test different membranes to optimize the conditions. The most commonly used membranes for immunoblotting are nitrocellulose, polyvinylidene difluoride (PVDF), and nylon.

Some of the main characteristics of each type of membrane are as follow.

### *Nitrocellulose*

Nitrocellulose is the standard membrane used for immunoblotting. It exhibits good binding capacity, between 80 μg/cm$^2$ and 250 μg/cm$^2$. Proteins interact with the membrane through hydrophobic interactions or possibly by hydrogen bonding between amino acid side chains and amino groups of the membrane. Nitrocellulose membranes tend to produce low background because they are readily blocked during the preincubation step. General blocking strategies are mentioned below and are explained in detail in *UNIT 8.3* (Ni et al., 2016). Blocking an immunoblot is analogous to the process of blocking a Southern or northern blot. By pretreating the blot with agents such as protein or detergent, the sites

on the membrane that are not occupied with protein following the transfer step are coated or "blocked," thus preventing antibodies from binding to these sites nonspecifically. The limitations of nitrocellulose include a relatively weak association of the proteins with the membrane. As a result, during the subsequent processing steps, following transfer, some of the protein may be lost, leading to a reduction in signal intensity. Additionally, the membrane is brittle and can easily crack or break, so it is not recommended for subsequent probing, making it difficult to assay for multiple proteins on a single blot.

### *Nylon*

Nylon membranes are very sturdy and can be probed multiple times with different antibodies. Additionally, the membrane has a high binding capacity, 150 to 200 μg/cm$^2$. Nylon membranes can be charged or display other chemical moieties that increase the association of the transferred proteins through electrostatic interactions in addition to hydrophobic interactions. However, one of the drawbacks of using a nylon membrane is that the unoccupied sites on the membrane are difficult to block. As a result, nylon membranes tend to have greater background problems. To address this issue, a more extensive blocking protocol or special blocking reagents can be considered.

### *Polyvinylidine difluoride (PVDF)*

PVDF membranes are perhaps the most commonly used membrane for immunoblotting experiments. PVDF membranes have a high binding capacity (170 μg/cm$^2$) and exhibit strong hydrophobic interactions with proteins, resulting in tight binding. As a consequence, less protein is lost during processing. PVDF membranes have good mechanical strength and chemical stability, and are compatible with most detection procedures. In addition, proteins blotted to this membrane can be excised from the blot and processed for subsequent analysis, including mass spectrometry and sequencing. Having all these nice features in one membrane usually comes with a drawback, which in this case is its high cost; PVDF membranes tend to be the most expensive choice.

When preparing a membrane for the transfer step it is important to prewet the membrane. This will produce an even interface between the gel and the membrane to facilitate maximal retention of protein during the blotting procedure. With nitrocellulose and nylon membranes, this can be achieved by immersing the membranes slowly into a container with water and then into transfer buffer. With PVDF membranes, due to their hydrophobic nature, it is necessary to prewet the membrane in methanol first, followed by equilibration in transfer buffer. The membrane is now ready for the transfer protocol.

## Transfer

Once your sample has been resolved by PAGE and a membrane has been chosen, the transfer can be carried out. This step can be achieved using different methods (Sambrook and Russell, 2001; Kurien and Scofield, 2006). One approach utilizes simple diffusion, which involves the same basic principles explained for the transfer of DNA or RNA onto a membrane when discussing Southern and northern blots. A membrane is placed in contact with the gel and a stack of dry filter papers is placed on top. The advantage of this technique is that several blots can be obtained from the same gel. For example, bidirectional transfer can be achieved if the gel is sandwiched between two membranes with filter papers on both sides. Because transfer efficiency is only between 20% to 50%, more than one membrane can be blotted consecutively. As a result, several blots of the gel are obtained and each membrane can then be assayed with a different antibody. Sufficient protein may be left behind in the gel to allow for staining with Coomasie blue. This will allow the investigator to ascertain where the protein of interest resolved in the original gel. By superimposing the immunoblot with the stained gel, the band of interest can be identified, excised, eluted from the gel, and identified by mass spectrometry or

sequencing. This transfer technique is not widely used when quantitative transfer of the protein is important for the analysis.

Electroblotting is the method of choice for the transfer of proteins from an acrylamide gel onto a membrane. It is much faster and far more efficient than capillary transfer, such that quantitative transfer of the proteins onto a membrane is possible. There are two main types of electrophoretic transfer, wet transfer and the semi-dry transfer. Wet transfer or tank transfer involves completely immersing the protein gel sandwiched by a membrane and filters into a chamber of transfer buffer. The second method, referred to as semi-dry transfer, consists of the protein gel sandwiched by a membrane and filters, but in this case the sandwich is in direct contact with the plate electrodes and the amount of buffer is limited to what the components of the sandwich retain. An electric current aligned perpendicular to the gel is applied so that the negatively charged proteins migrate towards the positive electrode and become trapped by the membrane that was placed on the side of the gel facing the anode. Alternatively, there is a method called eastern blotting which is modified so that the proteins are denatured with a detergent known as cetyltrimethylammonium bromide (CTAB), leading to a net positive charge compared to the negative charge achieved with SDS. As a result, proteins bound by CTAB will migrate to the cathode instead of the anode. This method is only used when working with highly charged proteins or glycoproteins that contain a high number of negatively charged carbohydrates that do not behave well under the standard electrophoresis and transfer conditions that make use of SDS (Buxbaum, 2003). Semidry methods of transfer have become very popular because of the speed with which you can accomplish a high-quality, complete transfer. In ~5 to 7 min the transfer is done, even with high-molecular-weight proteins that are difficult to transfer using a traditional tank transfer method (Bio-Rad's Transblot Turbo, Thermo Scientific's iblot, Pierce G2 Fast Blotter, etc.). The downside is the cost of the systems and the need to purchase consumables specific to the blotter used for the transfer process. These consumables can consist of a specific transfer buffer (Trans-Blot Turbo Transfer Packs or Transfer Kit, Bio-Rad; Pierce 1-Step Transfer Buffer, Thermo Scientific) or disposable stacks that contain the electrode, cathode, and appropriate buffer for the transfer process (iblot Transfer Stacks, Thermo Scientific). For labs in which western blots are a routine method, it is worthwhile to invest in a system that allows you to speed up the process without compromising quality.

### Blocking

Once the proteins have been transferred to a solid support, the transfer apparatus is disassembled and the membrane removed. Because the proteins are tightly associated with the membrane, a cross-linking step is not required. At this point, the membrane can be assayed immediately or stored. Prior to the addition of a primary antibody, the blot has to be blocked. Typically, this involves incubating the blot in a solution of protein, such as 5% low-fat dried milk or 5% BSA (bovine serum albumin), and detergents such as Tween 20. The most common blocking solution used is dried milk, which is the least expensive and compatible with most immunological detection systems used. As described in *UNIT 8.3* (Ni et al., 2016), the blocking solution uniformly coats the membrane and thereby reduces the level of nonspecific binding during the following step. If the blocking procedure is skipped or done inefficiently, the antibodies used in the detection step will associate with the unblocked sites on the membrane resulting in a high background. The blocking step is essential to reduce the level of background and to improve the signal to noise ratio.

### Detection

Once the blocking step has been completed, the first step of immunodetection involves adding an antibody (monoclonal or polyclonal) that reacts with the epitope of interest. This reagent is referred to as the primary antibody. After incubating the blot with the

primary antibody, the blot is thoroughly washed and a secondary antibody added. The secondary antibody recognizes epitopes associated with the primary antibody and not the proteins in the sample. If the primary antibody was raised against the target of interest in a mouse, the secondary antibody will likely be an anti-mouse antibody raised in another organism like goat, pig, or rabbit. Alternatively, one can use other factors such as Protein A, which will tightly associate with the primary antibody. Protein A is a cell wall component of *Staphylococcus aureus* that binds to the constant region of antibodies, mainly thru hydrophobic interactions. Because of the great affinity of Protein A for antibodies, it has been extensively used in a variety of immunological approaches. To facilitate detection of the protein of interest in an immunoblotting experiment, Protein A or the secondary antibody is conjugated to a compound that allows the investigator to visualize the location of the bound protein. The conjugated compound can include radioactive or enzyme moieties. An example of a radioactive conjugate is $^{131}$I and $^{125}$I. These compounds are readily detected by X-ray film or phosphorimaging. Examples of enzyme conjugates include horseradish peroxidase or alkaline phosphatase, which are less hazardous than radioactive conjugates and are very sensitive (Blake et al., 1984; Knecht and Dimond, 1984). Each of these enzymes, when presented with the necessary substrate, can facilitate a localized reaction to produce a visible band. Additionally, there are commercially available kits that utilize chemiluminescent reagents that luminesce when cleaved by the enzyme conjugates. This fluorescence luminesce can be detected with X-ray film, digital imaging, or phosphorimaging. Please refer to *UNIT 8.4* (Haushalter, 2008) and *UNIT 7.5* (Moomaw et al., 2014) for specific information on detection systems.

## LITERATURE CITED


Ageno, M., Dore, E., and Frontali, C. 1969. The alkaline denaturation of DNA. *Biophys. J.* 9:1281-1311.

Alwine, J.C., Kemp, D.J., and Stark, G.R. 1977. Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. *Proc. Natl. Acad. Sci. U.S.A.* 74:5350-5354.

Alwine, J.C., Kemp, D.J., Parker, B.A., Reiser, J., Renart, J., Stark, G.R., and Wahl, G.M. 1979. Detection of specific RNAs or specific fragments of DNA by fractionation in gels and transfer to diazobenzyloxymethyl paper. *Methods Enzymol.* 68:220-242.

Amasino, R.M. 1986. Acceleration of nucleic acid hybridization rate by polyethylene glycol. *Anal. Biochem.* 152:304-307.

Armstrong, J.A. and Schulz, J.R. 2015. Agarose gel electrophoresis. *Curr. Protoc. Essen. Lab. Tech.* 10:7.2.1-7.2.22. doi: 10.1002/9780470089941.et0702s10.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (eds.) 2007. Current Protocols in Molecular Biology. John Wiley & Sons, Hoboken, N.J.

Bittner, M., Kupferer, P., and Morris, C.F. 1980. Electrophoretic transfer of proteins and nucleic acids from slab gels to diazobenzyloxymethyl cellulose or nitrocellulose sheets. *Anal. Biochem.* 102:459-471.

Blake, M.S., Johnston, K.H., Russell-Jones, G.J., and Gotschlich, E.C. 1984. A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on Western blots. *Anal. Biochem* 136:175-179.

Botstein, D., White, R.L., Skolnick, M., and Davis, R.W. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *Am. J. Hum Genet.* 32:314-331.

Burnette, W.N. 1981. "Western blotting": Electrophoretic transfer of proteins from sodium dodecyl sulfate - polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* 112:195-203.

Buxbaum, E. 2003. Cationic electrophoresis and electrotransfer of membrane glycoproteins. *Anal. Biochem.* 314:70-76.

Chu, G., Vollrath, D., and Davis, R.W. 1986. Separation of large DNA molecules by contour-clamped homogeneous electric fields. *Science* 234:1582-1585.

Church, G.M. and Gilbert, W. 1984. Genomic sequencing. *Proc. Natl. Acad. Sci. U.S.A.* 81:1991-1995.

Coico, R., Sunshine, G., and Benjamini, I. 2003. Immunology: A short course. John Wiley & Sons, New York.

Denhardt, D.T. 1966. A membrane–filter technique for the detection of complementary DNA. *Biochem. Biophys. Res. Commun.* 23:641-646.

Ellington, A. and Pollard, J.D. 1998. Purification of oligonucleotides using denaturing polyacrylamide gel electrophoresis. *Curr. Protoc. Mol. Biol.* 42:2.12.1-2.12.7.

Finney, M. 2000. Pulsed-field gel electrophoresis. *Curr. Protoc. Mol. Biol.* 51:2.5B.1-2.5B.9.

Gallagher, S., Winston, S.E., Fuller, S.A., and Hurrell, G.R. 2004. Immunoblotting and Immunodetection. *Curr. Protoc. Mol. Biol.* 66:10.8.1-10.8.24.

Gallagher, S.R. 2012. SDS-polyacrylamide gel electrophoresis (SDS-PAGE). *Curr. Protoc. Essen. Lab.Tech.* 6:7.3.1-7.3.28.

Gusella, J.F., Wexler, N.S., Conneally, P.M., Naylor, S.L., Anderson, M.A., Tanzi, R.E., Watkins, P.C., Otinna, K., Wallace, M.R., Sakaguchi, A.Y., Young, A.B., Shoulson, I., Bonilla, E., and Martin, J.B. 1983. A polymorphic DNA marker genetically linked to Huntington's disease. *Nature* 306:234-238.

Haushalter, K.A. 2008. Labeling DNA and preparing probes. *Curr. Protoc. Essen. Lab. Tech.* 00:8.4:8.4.1-8.4.22.

Hoopes, L.L.M. 2012. Nucleic acid blotting: Southern and northern. *Curr. Protoc. Essen. Lab.Tech.* 6:8.2.1-8.2.26.

Johnson, D.A., Gautsch, J.W., Sportsman, J.R., and Elder, J.H. 1984. Improved technique utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. *Gene Anal. Tech.* 1:3-12.

Kelly, T.J. Jr. and Smith, H.O. 1970. A restriction enzyme from *Hemophilus influenzae*. II. *J. Mol. Biol*. 51:393-409.

Klenow, H. and Henningsen, I. 1970. Selective elimination of the exonuclease activity of the deoxyribonucleic acid polymerase from *Escherichia coli* B by limited proteolysis. *Proc. Natl. Acad. Sci. U.S.A.* 65:168-175.

Knecht, D.A. and Dimond, R.L. 1984. Visualization of antigenic proteins on western blots. *Anal. Biochem.* 136:180-184.

Kurien, B.T. and Scofield, R.H. 2006. Western blotting. *Methods* 38:283-293.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.

Lehrach, H., Diamond, D., Wozney, J.M., and Boedtker, H. 1977. RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. *Biochemistry* 16:4743-4551.

Lunn, G. and Strober, W. 2008. General laboratory safety and working with hazardous chemicals. *Curr. Protoc. Essen. Lab.Tech.* 00:A.1A.1-A.1A.12.

McDonell, M.W., Simon, M.N., and Studier, F.W. 1977. Analysis of restriction fragments of T7 DNA and determination of molecular weights by electrophoresis in neutral and alkaline gels. *J. Mol. Biol.* 110:119-146.

Meisenhelder, J. and Bursik, S. 2008. Radiation safety and measurement. *Curr. Protoc. Essen. Lab. Tech.* 00:2.3.1-2.3.20.

Moomaw, B., Medberry, S., and Gallagher, S.R. 2014. Overview of digital electrophoresis analysis. *Curr. Protoc. Essential Lab. Tech.* 9:7.5.1-7.5.31. doi: 10.1002/9780470089941.et0705s9.

McKimm-Breschkin, J.L. 1990. The use of tetramethylbenzidine for solid phase immunoassays. *J. Immunol. Methods* 135:277-280.

Meinkoth, J. and Wahl, G. 1984. Hybridization of nucleic acids immobilized on solid supports. *Anal. Biochem.* 138:267-284.

Nathans, D. and Smith H. 1975. Restriction endonucleases in the analysis and restructuring of DNA molecules. *Annu. Rev. Biochem.* 44:273-293.

Ni, D., Xu P., Sabanayagam D., and Gallagher S.R. 2016. Protein blotting: Immunoblotting. *Curr. Protoc. Essen. Lab. Tech.* 12:8.3.1-8.3.40. doi: 10.1002/9780470089941.et0803s12.

Nierzwicki-Bauer, S.A., Gebhardt, J.S., Linkkila, L., and Walsh, K. 1990. A comparison of UV cross-linking and vacuum baking for nucleic acid immobilization and retention. *Biotech.* 9:472-478.

Olszewska, E. and Jones, K. 1988. Vacuum blotting enhances nucleic acid transfer. *Trends in Genetics* 4:92-94.

Reed, K.C. and Mann, D.A. 1985. Rapid transfer of DNA from agarose gels to nylon membranes. *Nucl. Acids Res.* 13:7207-7221.

Rommens, J.M., Iannuzzi, M.C., Kerem, B., Drumm, M.L., Melmer, G., Dean, M., Rozmahel, R., Cole, J.L., Kennedy, D., Hidaka, N., Zsiga, M., Buchwald, M., Riordan, R.R., Tsui, L., and Collins, F.S. 1989. Identification of the cystic fibrosis gene: Chromosome walking and jumping. *Science* 245:1059-1065.

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. Molecular cloning: A Laboratory Manual, Second Edition. Cold Spring Harbor Press, Cold Spring Harbor, NY.

Sambrook, J. and Russell, D.W. 2001. Molecular cloning: A Laboratory Manual, Third Edition. Cold Spring Harbor laboratory Press, Cold Spring Harbor, NY.

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503-517.

Southern, E.M. 2000. Blotting at 25. *Trends Biochem. Sci.* 25:585-588.

Suggs, S.V., Hirose, T., Miyake, T., Kawashima, E.H., Johnson, M.J., Itakura, K., and Wallace, R.B. 1982. *In* Developmental Biology Using Purified Genes (D. Brown, ed.) pp. 683-693. Academic New York, NY.

Thomas, P.S. 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. U.S.A.* 77:5201-5205.

Towbin, H., Staehelin, T., and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.* 76:4350-4354.

Wahl, G.M., Stern, M., and Stark, G.R. 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. *Proc. Natl. Acad. Sci. U.S.A.* 76:3683-3687.

Wallace, R.B., Shaffer, J., Murphy, R.F., Bonner, J., Hirose, T., and Itakura, K. 1979. Hybridization of synthetic oligodeoxyribonucleotides to $\Phi\chi$ 174 DNA the effect of single base pair mismatch. *Nucl. Acids Res.* 6:3543-3547.