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Electrophoresis

ELECTROPHORESIS, THE SEPARATION of charged molecules in an electrical field, is an essential technique in any lab. With electrophoresis, molecules in a mixture are separated from each other on the basis of size, shape, or charge. It is the first step for dozens of procedures, such as DNA sequencing and Western blots. Electrophoresis is automated in some labs, but in most labs, electrophoretic separation of DNA, RNA, and protein is done manually. Pouring a gel (the matrix through which the molecules are separated) will probably become routine.

Whether your sample is a plasmid mini-prep or a purified protein, the steps for performing electrophoresis will be similar. Once you have run one gel, all other gels will be familiar.

BASIC RULES

- **Turn the power OFF** before manipulating a gel box.
- **Make sure the gel has solidified** before loading and running it.
- **Record what you loaded immediately.** As you load the wells, keep the tubes in the rack in the order in which they were loaded: Don't throw them away as you are working, only move the used tubes to another row. Use this order to check against your protocol or to write down the contents of each lane.

- **Wear gloves** if you are touching gels or gel buffer. The buffer may contain ethidium bromide (EtBr), a strong mutagen, and there might be traces of acrylamide powder around.
- Don't melt agarose with EtBr (or any lab stuff, actually) in a microwave used for food: **be sure the microwave you use has been okayed for EtBr use.**
- **Don't leave your gel to dry up in the gel box.** As soon as you have finished with the gel, dispose of the buffer and rinse out the gel box with distilled water.
- **Put the leads on correctly.** Black is –, the anode. Red is +, the cathode. Check the leads into the power supply, and the leads on the gel box, and check again. Unbelievable as it sounds, everyone makes this mistake, just once, and the samples migrate upward until halted.

Watch the samples for a minute or two after you turn on the power supply to the gel, to be sure they are migrating in the proper direction.

GENERALITIES

Sample preparation

- The sample must be actually dissolved in the sample buffer (also known as loading buffer), or it will not move through the gel. Too concentrated a sample can lead to artifacts.
- Sample buffers contain salts needed to maintain the sample, glycerol to add the weight needed to sink the sample in the well, and a tracking dye that allows you to monitor the progress of the electrophoresis.
- Sample buffers can be frozen in aliquots.
- Samples are loaded after buffer has been added to the gel.
- Tracking dyes in the sample buffer are used to indicate when the run should be terminated. The two most commonly used dyes are bromophenol blue (BPB) and xylene cyanol FF.

Standards/markers

- Molecular weight standards should be run. These can be used to monitor the progress of the run and to analyze the results.

- Use standards appropriate to the size of the molecular species you are interested in.

- Hang a Polaroid or a picture of a gel with the molecular weight markers you often use. Label the bands with the sizes. You will refer to these constantly.

- Standards are available unlabeled or labeled. Label can be fluorescence, luminescence, or radioactivity. If labeled markers aren't available, the stained gel can be compared with the labeled blot afterward.

- Load the standards into the same lane on every gel. It is easiest to always load your markers into the first lane. Knowing your standards are always in a particular lane gives you a point from which you can always orient yourself, should you drop the gel or just become confused.

- Whenever possible, positive and negative controls should be run.

Format

- Agarose gels are usually run horizontally, acrylamide gels are run vertically. Submarine gels are a type of horizontal gel in which the gel lies flush on the bottom of the chamber.

- Gels can be made in a variety of sizes. Sequencing gels do need to be large, but for most screening and transfers, even for 2-D gels, mini sizes will work fine as long as you are not trying to distinguish between bands of similar molecular weights.

- Capillary electrophoresis uses narrow-bore capillaries to perform automated and high-efficiency separations of DNA, proteins, or other small molecules. Separation is coupled to detection and analysis, in a similar way to chromatography instrumentation. This is only found in specialty labs.

The gel

- Acrylamide versus agarose. Although gels can be run on paper, cellulose acetate, starch, or other matrixes, acrylamide and agarose gels are the only ones most investigators run. Both are porous gels, acting as molecular sieves (the higher the percentage of acrylamide or agarose, the smaller the pores), and theoretically, either could be used to separate DNA, RNA, or

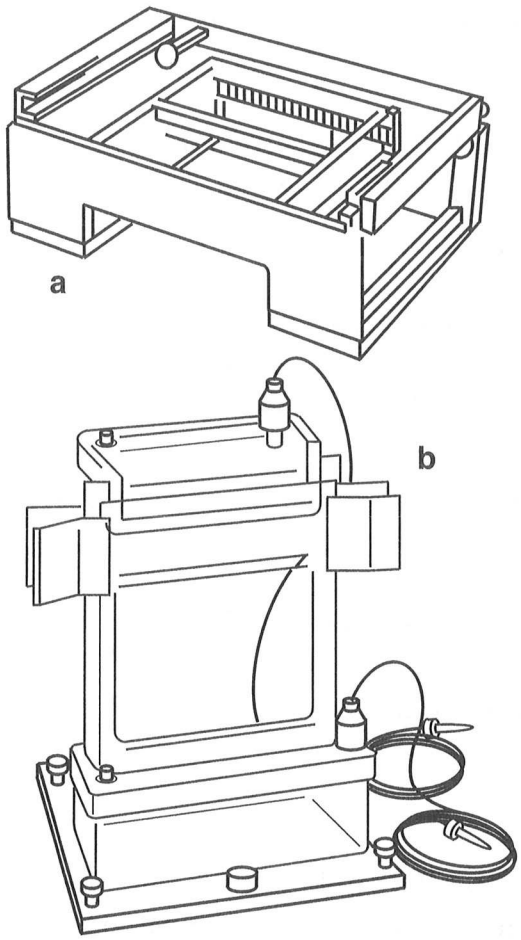

FIGURE 1.

Gel box formats. Horizontal boxes (*a*) are used for agarose gels; vertical boxes (*b*) are used for acrylamide gels.

protein. But acrylamide, at low percentages, is very floppy and difficult to handle: It is usually used only at high percentages to analyze proteins and small oligonucleotides.

- Low percentage agarose gels are relatively rigid and easy to handle, and are used to separate large molecules such as DNA and RNA, and very large proteins and protein complexes.

- The concentration of the gel should be appropriate to the size of the fragment of interest.

- Precast gels are available from many companies. They are expensive, costing a few dollars per gel, and can be stored at 4°C for months. But if you run gradient gels, or run gels infrequently, it is well worth it. There are also multi-gel casting systems, used to make up to 10 gels at a time.

- Cut off the same corner of the gel every time. This gives you an orientation point in case the gel is dropped or turned, and for setting up the gel for transfers.

Buffers

- Most running buffers are made or purchased as concentrated stock, and are diluted at the time of the run.

- Each buffer will have a characteristic voltage at a particular amperage. Get to recognize the characteristics of each buffer, so you will recognize when something is wrong.

Power

- Power output can be in several modes: constant voltage (mV), constant current (measured in amperage, or amps), or constant power (watts, or W). Many models allow you to program a run for automatic crossover between modes, so you can use the optimum voltage (which can change during the run) without exceeding the power capacity.

- Not all power supplies are the same, and you cannot merely plug your electrophoresis apparatus into any available power supply. Know what amperage or voltage you need, and identify the power supply that will provide that.

- Most labs have at least one power supply for sequencing gels (which require high wattage), electrophoretic transfers (which require high current), and one for agarose and acrylamide gel electrophoresis (which use a wide range of voltages). There are few power supplies that can satisfy all three needs.

- Samples in denaturing protein gels and DNA and RNA gels will run from the cathode (negative) to the anode (positive). Use the red lead for the anode (+), and the black for the cathode (–).

- You can run multiple gels on the same power supply, but don't do it without asking the other user, as electrophoretic conditions may be changed.

- If the power supply has a timer and will terminate a run automatically, use it; otherwise, set a timer to remind yourself to check the gel. Especially if

TABLE 1. Effects of Currents

Current (mA)		
AC	DC	Effects
≤1	5	Causes no sensation
1–8		Sensation of shock, not painful
8–15		Painful shock; individual can releaase grasp
15–20	75	Muscular control lost; cannot release grasp
20–50		Muscular contractions; hard to breathe
50–100	300–500	Possible ventricular fibrillation
100–200		Certain ventricular fibrillation
≥200		Severe burns; muscular contractions so severe that heart may stop

Current is not entirely dependent on the voltage, but on the resistance of the body. In general, the body's resistance to electrical shock is minimal, and voltages of 45 to 60 mA have proven fatal. (Reprinted, with permission, from Gershey et al. 1991.)

you are looking at a molecule of low MW, it is easy to run the samples off the gel and into the buffer.

- Electrophoresis units are built very safely, and there is little to worry about if you obey the Big Rule: Make sure power is OFF to electrophoresis apparatus before you touch anything! Don't "just add a little more buffer" to your mini-gel, or shift the box an inch over, or quickly load one more well, or ever put the lid on the unit, *until the power is off.* Period.

Fixing

- Whether or not a gel needs to be fixed depends on the application. Gels that will be stained must usually be fixed, whereas gels that are used for transfers are not fixed.

Drying

- By removing the water from a gel, the matrix is made thinner. Dried gels give a sharper band after autoradiography.

- Drying a gel will take less than an hour in a gel-dryer. The gel-dryer can be heated, making the drying process more rapid.

Staining

- DNA and RNA gels are stained by the addition of dye to the sample or buffer before the electrophoresis run. They can be stained afterward, as well, but it is less convenient.

- Protein gels are stained after electrophoresis.

Documentation

- Polaroid pictures of gels stained with EtBr and 35 mm photographs of protein gels are the most common documentation.
- Digital documentation and analysis systems are growing in presence. No film is used, and the data can even be set up for presentations.
- Documentation of transfers will depend on the system and experiment. For example, autoradiography and chemiluminescence results can be recorded on X-ray film, and the signal quantitated with a densitometer.

Determining molecular weight. The molecular weight of proteins can be determined by SDS-PAGE, and of DNA and RNA by agarose gel electrophoresis. There is a linear relationship between the logarithm of the molecular weight of a molecule and its R_f (effectively, the distance traveled). A standard curve of the distance migrated against the $\log_{10}$ MW of the standards can be plotted and the R_f of the sample—and hence, the molecular weight—can be extrapolated from the graph.

1. Make a gel of a concentration that will best resolve molecules of the approximate size. Always run molecular weight standards, of a range encompassing the approximate size of the molecule in question.
2. Run the gel so the dye front almost runs off the end of the gel.
3. Stain the gel, and take a picture. If samples are radiolabeled, you can either use radiolabeled standards or compare the gel with the autoradiography film.
4. Measure the distance from the well to each band of standard. Take the $\log_{10}$ of each number, and plot it on regular (non-log) graph paper, on the y axis. The x axis is the distance traveled (cm are probably easiest to translate.) You should have a straight line for much of the distance of the standards.
5. Plot the distance migrated for the sample. Extrapolate to the standard line and determine the molecular weight.

Computer programs available on the WWW (and many calculators) can be used to extrapolate the molecular weight of an unknown from a standard curve. The advantage to plotting the curve manually is that you can be sure if the value of your protein falls into the linear (and valid) part of the standard curve.

SPECIFICS

DNA Gels

DNA gels are run to separate, identify, or purify DNA fragments. Sequencing gels, used to run and analyze DNA sequencing reactions, are not described here. There are as many ways to pour a sequencing gel as there are lab members, and all of them work. Get someone to demonstrate one method, and stick with it until you can get it to work.

Sample preparation

- 6X loading buffer is typical: 30% (v/v) glycerol, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, in distilled water. Store at –20°C.

Electrophoresis is usually halted when the BPB dye front is 3/4 of the way to the bottom for agarose gels.

- Sample buffer and DNA are heated at 60°C for 5 minutes.

Standards/markers

- In agarose gels, marker dye BPB will co-migrate with DNA molecules of approximately 600 bp, and xylene cyanol with DNA molecules of approximately 4000 bp. The exact migration is dependent on the quality and concentration of agarose.

TABLE 2. Migration of Marker Dyes

	% Polyacrylamide	Bromophenol blue[a]	Xylene cyanol[a]
In polyacrylamide gels			
	3.5	100	460
	5.0	65	260
	8.0	45	160
	12.0	20	70
	20.0	12	45
In denaturing polyacrylamide gels			
	5.0	35	130
	6.0	26	106
	8.0	19	70–80
	10.0	12	55
	20.0	8	28

(Adapted, with permission, from Maniatis et al. 1982.)

[a]The numbers are the approximate sizes of fragments of DNA (in nucleotide pairs) with which the dyes would comigrate.

- BPB and xylene cyanol can also be used for marker dyes in DNA acrylamide gels. See Table 2.

- There is an endless supply of DNA standards available. Most of them are pieces generated from the enzymatic digestion of a known piece of DNA. These standards yield fragments of nonuniform sizes, making it easier to quickly estimate the size of an unknown, and provide a control for gel-to-gel variability. You can make your own, but it isn't usually worth the trouble.

- Molecular rulers are DNA ladders, with sizes of regular intervals. These are best for precise measurement of the molecular weight of the sample DNA. Most contain a visually distinct reference band.

- Have your own supply of the DNA standards you use most often. Two useful sets of DNA size markers in bp are:

  Lambda cut with *Hin*dIII: 23,130, 9416, 6557, 4361, 2322, 2027, 564, 125
  ϕX174 cut with *Hae*III: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72

Format

- *Medium gel boxes versus mini-gels.* Mini-gels can be used to monitor the progress of restriction digestions, or to check the quality of plasmid preps, two of the most common uses of DNA gels. The main advantages of mini-gels over medium gels is that the gel is finished running much sooner (less than an hour, compared to 3–4 hours) and they require less DNA. Southern blots are better done as larger size gels, since the signal may be weak and more DNA can be run in a larger format.

- *Preparative versus analytical gels.* An analytical gel is run to gather information. In a preparative gel, the DNA of interest is removed from the gel: Preparative gels are often larger, to accommodate a larger amount of sample. A preparative gel may have only one huge well at the top of the gel.

Resolving agent

- Agarose or acrylamide: resolving power versus range of separation!

- Agarose (good range of separation) is used standardly, good for 200 bp to 50 kb, and is run as a horizontal gel. DNAs up to 10,000 kb may be run in pulsed-field gel electrophoresis.

- Use polyacrylamide (good resolving power) for separating small fragments of DNA from 5 to 500 bp. It is set up as a vertical gel. Alkaline agarose gels are used to hydrolyze DNA and analyze the individual DNA strands. A common reason to run an alkaline agarose gel is to check the size of first and second DNA strands synthesized by reverse transcription in the first step in making cDNA. The addition of base to hot agarose would hydrolyze the agarose, so the gel is prepared in a neutral solution and is equilibrated in freshly made alkaline electrophoresis buffer before running.

- Low-melt agarose has been chemically modified to gel and melt at lower temperatures (it gels at 30°C and melts at 65°C), and resolves better than normal agarose but not as well as acrylamide. Perform the electrophoresis run in the cold room to prevent melting of the agarose. Low-melt agarose is useful for preparative gels, since there are a number of ways to recover DNA from low-melt agarose.

- Use the appropriate concentration of agarose. See Table 3.

TABLE 3. Concentration of Gels Used for Electrophoresis of DNA

A	Agarose (%)	Effective range of separation of linear DNA molecules (kb)
	0.3	60–5
	0.6	20–1
	0.7	10–0.8
	0.9	7–0.5
	1.2	6–0.4
	1.5	4–0.2
	2.0	3–0.1

B	Acrylamide (%)	Effective range of separation (nucleotides)
	3.5	100–1000
	5.0	80–500
	8.0	60–400
	12.0	40–200
	20.0	10–100

(Modified, with permission, from Maniatis et al. 1982.)
(A) Choose the concentration of agarose that gives good separation of the size of DNA molecules you are analyzing.
(B) If the DNAs you are interested in are smaller than 1 kb, an acrylamide gel should be used.

Buffer

- The same buffer is used to make the gel as to actually run the gel.
- If you mix the agarose in water instead of buffer by mistake (and this is one of the most common mistakes made), there is little electrical conductance and the DNA will move slightly or not at all. Discard the gel and make a new one.
- The most common buffers for DNA are TAE (Tris-acetate-EDTA) and TBE (Tris-borate-EDTA). TPE is also used less frequently.
- Gels prepared and run with different buffers look different. For example, double-stranded linear DNA fragments migrate approximately 15% faster through TAE than through TBE or TPE. Resolution of supercoiled DNA is better in TAE.

TABLE 4. Commonly Used Electrophoresis Buffers

Buffer	Working solution		Concentrated stock solution (per liter)	
Tris-acetate (TAE)	1X:	0.04 M Tris-acetate 0.001 M EDTA	50 X	242 g Tris base 57.1 ml glacial acetic acid 100 ml 0.5 M EDTA (pH 8.0)
Tris-phosphate (TPE)	1X:	0.09 M Tris-phosphate 0.002 M EDTA	10X:	108 g Tris base 15.5 ml 85% phosphoric acid (1.679 g/ml) 40 ml 0.5 M EDTA (pH 8.0)
Tris-borate[a] (TBE)	0.5X:	0.045 M Tris-borate 0.001 M EDTA	5X:	54 g Tris base 27.5 g boric acid 20 ml 0.5 M EDTA (pH 8.0)
Alkaline[b]	1X:	50 mN NaOH 1 mM EDTA	1X:	5 ml 10 N NaOH 2 ml 0.5 M EDTA (pH 8.0)

(Reprinted, with permission, from Sambrook et al. 1989.)

[a]A precipitate forms when concentrated solutions of TBE are stored for long periods of time. To avoid problems, store the 5X solution in glass bottles at room temperature and discard any batches that develop a precipitate. TBE was originally used at a working strength of 1X (i.e., a 1:5 dilution of the concentrated stock) for agarose gel electrophoresis. However, a working solution of 0.5X provides more than enough buffering power, and almost all agarose gel electrophoresis is now carried out with a 1:10 dilution of the concentrated stock. TBE is used at a working strength of 1X for polyacrylamide gel electrophoresis, twice the strength usually used for agarose gel electrophoresis. The buffer reservoirs of the vertical tanks used for polyacrylamide gel electrophoresis are fairly small, and the amount of electric current passed through them is often considerable. 1X TBE is required to provide adequate buffering power.

[b]Alkaline electrophoresis buffer should be freshly made.

- *Recommendation:* Use TBE. It has the highest buffering capacity.
- If EtBr was mistakenly omitted from the gel, it should be added to the running buffer.

Power

- Applied voltage. The effective range of separation in agarose gels decreases as the voltage is increased. To achieve maximum resolution of a DNA fragment greater than 2 kb, run gels at 5 V/cm or less (for cm, approximate the distance between electrodes). 5–10 V/cm is good for most gels. 100 V will be approximately 50 mA for some buffers.
- If you want the gel to run overnight, the total voltage should be 20–25 V. You can turn it higher in the morning.
- Running a gel at constant power rather than constant voltage or current will prevent large voltage spikes or excessive heating from occurring.
- Run low-melt agarose gels more slowly than a regular gel, to avoid generating heat.

Staining

- Add EtBr to the gel mixture after it is melted. Add 1 µl of 10 mg/ml EtBr to every 10 ml of agarose solution.
- It is not usually necessary to add EtBr to the gel buffer, or to stain the gel after electrophoresis: EtBr in the gel mixture is enough to see most bands.

Analysis

- DNA in a gel is visualized on an UV transilluminator.
- Lay the gel (use gloves) on a piece of plastic wrap and carry the gel to the UV transilluminator. You may carry the gel/plastic wrap on the base or tray, but put only the gel/plastic wrap on the UV box: The tray will absorb too much light, and you may not see weakly stained bands.
- In gels with DNA not treated with RNase, tRNAs will often be seen as diffuse bands on the bottom of the gel.

- Why are there so many DNA bands? Even uncut plasmid DNA may appear on a gel as more than one band. Superhelical circular (uncut), nicked circular (partially cut), and linear (completely cut) DNA migrate through agarose gels at different rates: One prep of incompletely digested DNA may show three different bands.

- It is difficult to predict which band is which (since agarose concentration, current strength, and buffer type are some of the influences), but usually, superhelical DNA moves like a compact bullet through the gel and runs the fastest, with linear and then nicked DNA following.

Making gels

Agarose gels

- DNA gels are made just before the gel will be run. A prepared gel can be either stored in running buffer or wrapped in plastic wrap overnight in the refrigerator.

- Use diluted 10X stock buffer and agarose to prepare the gel. Either make just what you need, in an Erlenmeyer flask, or make enough gel for several gels in a glass bottle. The agarose will solidify, but the contents of the bottle can be melted again in a microwave. Store agarose/buffer "solutions" at room temperature.

- Agarose comes in various grades: the purer (and more expensive), the fewer contaminants of proteins, salts, and other polysaccharides you will find. It is also available for the isolation of differently sized nucleic acids. If you must recover the DNA, use the purest agarose you can afford, as contamination can inhibit enzyme reactions and is a major impediment to successful cloning. FMC BioProducts is a source of high-quality agarose.

The microwave

Hazards: EtBr, a teratogen, is often included in agarose mixtures (although it should be added after melting) and may be splattered on the inside of the microwave. Protective gloves should always be worn. Overheated agarose mixtures may suddenly boil over, so always remove agarose with tongs and/or heat-protective gloves. A wad of paper towels should not be used.
Remarks: No metal (No stir bars!). No food in any but a dedicated food microwave, no reagents in a food microwave. Put paper towels under anything you microwave, to make a boil-over easier to clean.
Alternatives: Heater stir plate.

To microwave

1. Put the top very loosely on the bottle. Air must be able to escape, or the bottle could explode. If you are using a flask, use a large one to reduce the chance of boiling over. You can leave off the top, or cover the top loosely with plastic wrap (not aluminum foil!).
2. Put the microwave on high. Freshly made agarose mixture will take longer to heat than solidified agarose. 100-ml volumes will take 3–5 minutes, larger volumes will take approximately 6–10 minutes.
3. After a minute, stop the microwave, grab the bottle with a gloved hand, and rotate to swirl the contents. A homogeneous mixture will heat more quickly and evenly.
4. Put the bottle back in the microwave. Stop it again after a minute, and swirl.
5. Replace the bottle in the microwave and heat until it just starts to boil.
6. Using tongs or heat-resistant gloves, remove the bottle.
7. Let the agar cool until the bottle can almost be touched. Swirl well before pouring. If you see that part of the agarose mixture has started to solidify, microwave it briefly again.

Yes, you can reuse gels. Some investigators have been known to keep a pet gel for checking reactions, running the current samples out of the gel before reloading. The gel must be stained with every new run, and using an old gel doesn't really save much time or money.

Acrylamide gels

- Polyacrylamide gels must be carefully polymerized by the mixing of monomeric units of acrylamide, an initiator of polymerization, and a catalyst, as well as the appropriate salts or buffer.

The percentages of acrylamide and BIS are different in sequencing gels and protein gels. If you used prepared acrylamide:BIS solutions, be sure you have grabbed the correct one.

- *Acrylamide and bis* (N,N′-methylenebisacrylamide) are the monomeric units that form the gel matrix. Buy the premade solutions!
- *Ammonium persulfate* initiates gel polymerization. Gel recipes call for a 10% ammonium persulfate solution, which should be made in water. Most protocols tell you to make it up fresh, every time. However, a 10%

solution can be stored for weeks at 4°C without a noticeable loss of activity. Make 10 ml of maximum and discard it either when a gel won't polymerize or whenever it makes you feel better.

- *TEMED* (N,N,N′,N′-tetramethylethylenediamine) is the catalyst. It comes in a brown bottle, and is stored in a brown bottle, usually in the cold. It is always added last, just before the gel is poured.

Unpolymerized acrylamide is a neurotoxin. Wear gloves, even when handling polymerized acrylamide, as there may be monomers in the area.

- In addition, polyacrylamide sequencing gels have gel buffer (TBE) and urea added. The urea is a denaturant and makes the formation of hairpin loops in the DNA reactions less likely.

- The glass plates for acrylamide electrophoresis should be washed before and after every electrophoresis run. After a run, wash the plate in warm, soapy water, using a soft brush or rag that won't scratch the glass. Rinse well in distilled water, and place upright to dry.

- Water or dust can lead to patchy polymerization. Before a run, use Windex or another glass cleaner to wash the plate. Use a soft brush to scrub. Rinse well in distilled water, and dry the plate fairly well with a paper towel and completely with a Kimwipe. A last rinse with 70% ethanol before using a paper towel can help clean and speed up the drying. Add ingredients in order: Acrylamide:BIS, water, buffer, APS, TEMED. Mix well by swirling, and pour immediately.

- It is not necessary to degas the acrylamide solution before polymerization. (Acrylamide solutions used to be placed under a vacuum, to remove air bubbles: Polymerization is inhibited by O_2.)

Loading tips

Remember that the samples have glycerol in them, and will sink into the wells.

Set up the gel box

- Place a black piece of paper under the gel box. A dark background makes the wells more visible.

- Fill the tank with enough buffer that the gel is just covered.

- If there is an overhead light, turn it on and direct the light onto the gel.

Load the pipettor with the sample

- Use an automatic pipettor.
- The tips that fit on the 10–200-µl pipettors are fine for most wells. For very small (under 10 µl) wells, the long tips used for sequencing gels can make the job easier.
- With just the tip in the sample, aspirate the sample slowly and deliberately into the tip. The sample may be viscous because of the glycerol, and rapid aspiration will leave sample in the tube and air bubbles in the tip.
- After loading the tip, touch the tip gently on the edge of the tube or with a Kimwipe to remove drops on the outside of the tip. Don't allow capillary action to drain the sample.

If sample is not limiting, it is helpful to follow the 10% plus rule: For each sample, make 10% more volume and content than you need. For example, if you intend to load 1.0 µg in 5 µl, make up 1.1 µg in 5.5 µl. Several microliters can be lost in pipetting, and this can be crucial if you are comparing amounts. Making a bit extra takes this worry away, and makes the pipetting job easier, as you don't have to suck up every last drop.

Load the well with the sample

- Maintain a bit of pressure on the pipettor, so the sample is slightly bulging out of the bottom of the tip.

Maintaining positive pressure on the sample while loading prevents bubbles or buffer from entering the tip as you position the pipet.

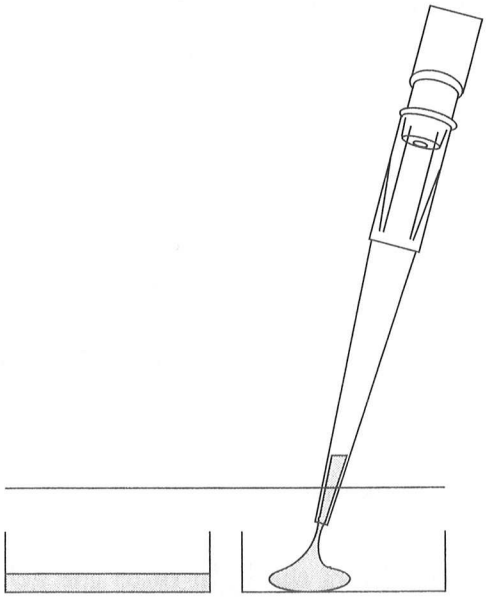

FIGURE 2.

Maintaining positive pressure on the sample while loading prevents bubbles or buffer from entering the tip as you position the pipettor over the well. Keep pressure in the tip while removing the pipettor as well.

- Put the tip into the buffer, just above the well, maintaining positive pressure. The tip of the tip (!) can be just into the well.

- Very slowly and steadily, push the sample out. If your tip is above the well, the sample will sink into the well. Fill by allowing the sample to sink, not by pushing into the well.

- As soon as the last drop has left the tip, push the pipettor to the second stop, slowly raising the pipettor tip straight up and out of the buffer as you do so.

Holding a last bit of air in the tip will prevent inadvertent suction from disturbing the sample.

How to load a vertical gel

- In a vertical gel, the wells are found between two glass plates. In a very thin gel, a pipettor tip will not even fit between the plates. Remember the glyc-

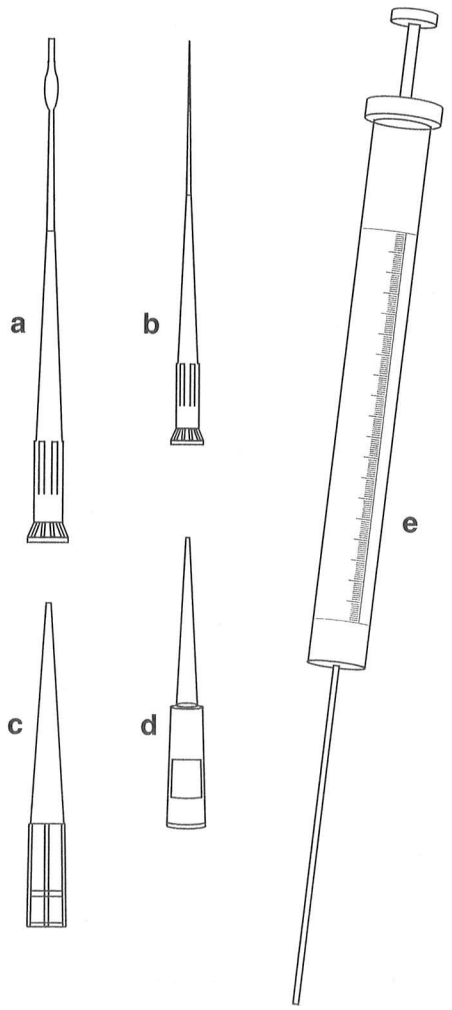

FIGURE 3.

Flat-tipped (*a*) and extended round tips (*b*) are very useful for loading vertical gels, as the tips can fit between the glass plates. Tips with a wide orifice (*c*) make it easier to load viscous DNA samples. Tips with an aerosol barrier (*d*) prevent carryover, and are used for radioactive samples. Microliter Hamilton syringes (*e*) are an easy way to load all gel samples: The syringe should be rinsed out with running buffer between samples. Of course, standard pipettor tips can be used for loading gels.

erol! If you position the tip above the well, the sample will sink into the well.

- Always flush out the wells of a vertical acrylamide gel before loading samples. This removes unpolymerized polyacrylamide and water that may be on the bottom of the well, and which effectively makes the well smaller. Use a 25- or 50-ml syringe, with an 18 gauge needle. Aspirate the running buffer, and flush the wells strongly but carefully to remove water.

- It can be hard to see the wells, but once you have loaded one well, the rest are easier to see. If you are having trouble and have an extra lane, load one well with sample buffer containing BPB.

RNA Gels

RNA gels are used to analyze RNA by Northern blotting. mRNAs comprise only about 5% of total RNAs, and are not visible on EtBr-stained gels: Therefore, a particular mRNA must be detected with a labeled probe.

Sample preparation

- RNA samples must be denatured before and during the electrophoresis run; otherwise, molecular weight cannot be accurately determined. This is done with formaldehyde and formamide. It can also be done with glyoxal and DMSO or methyl mercury (not recommended).

- Sample buffer for MOPS gels: 0.75 ml of deionized formamide, 0.15 ml of 10x MOPS, 0.24 ml of formaldehyde, 0.1 ml of deionized RNase-free water, 0.1 ml of glycerol, 0.08 ml 10% (w/v) of bromophenol blue. Store in small aliquots at –20°C or make fresh every time.

- Add 25 µl of sample buffer to 5 µl of RNA. You may have to concentrate your RNA.

- Samples in sample buffer are heated to 65°C for 15 minutes. Add 1 µl of 1 mg/ml EtBr to each sample and mix well. You do not need to add EtBr to the buffer.

- Load 5–20 µg of total RNA for a medium size gel, 1–5 µg in a mini-gel.

- You will usually get a cleaner and stronger signal with 3 μg of mRNA than with 5–20 μg of total RNA.

Standards/markers

- Yes, you do need standards for RNA gels.

- Many people use the ribosomal RNAs of the sample itself as a rough marker, and this is usually sufficient. Eukaryotic ribosomal RNAs are 28S and 18S (approximate sizes 5300 and 2000 bases): Prokaryotic ribosomal RNAs are 23S and 16S (approximate sizes 3566 and 1776 bases for *E. coli*).

- RNA standards are commercially available. DNA standards don't run well in formaldehyde gels, and shouldn't be used. Defined templates for in vitro RNA synthesis can be used to make defined-length RNA transcripts, needed if the size of an unknown RNA must be tightly defined.

- BPB and xylene cyanol can be used as tracking dyes. As in DNA electrophoresis, the exact position will depend on the agarose quality and concentration (see Table 5).

Format

- RNA gels are run just like DNA gels. *You do not need separate boxes and equipment to run RNA gels!* Just wash the gel box before and after use.

Buffer

- 10x MOPS/EDTA buffer contains 0.2 M MOPS [3-(N-morpholino) propanesulfonic acid], 50 mM sodium acetate, 10 mM EDTA adjusted to pH 7.0. Autoclave for 15 minutes. A slight yellow color appearing over time is normal. Use at 1x for electrophoresis.

- MOPS buffer (and other RNA buffers) are very low in ionic strength. During electrophoresis, a pH gradient may be generated along the length of the gel, resulting in hydrolysis of the gel. This may only be a problem during long electrophoretic runs and can be avoided by recirculating the buffer with a peristaltic pump or by occasionally pipetting buffer from one end to the other.

The gel

- RNA must be run under denaturing conditions. MOPS-formaldehyde gels are the safest and best bet.
- Formaldehyde gels must be poured and allowed to solidify in a fume hood. Hot formaldehyde vaporizes and is dangerous to breathe. Get directions before you do this.
- Agarose gels with formaldehyde are more fragile than standard agarose gels and must be manipulated carefully.
- 1% or 1.2% gels are generally good for most Northerns.

Power

- Power requirements are similar to DNA agarose gels. For a medium size gel, 100 V (approximately 50 mA) will take 3–4 hours to run.

Staining

- With the inclusion of EtBr in the sample buffer, further staining is not necessary.

Analysis

- The ribosomal subunits should be discrete. Unless the gel has been overloaded, the subunits should not be smeared, which would indicate degradation.
- mRNA, if visible at all on a gel, will look like a smear.

Protein Gels

Most protein gels run are sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels run in reducing conditions.

Sample preparation

- A reducing agent, either 2-mercaptoethanol or dithiothreitol, is included in the sample buffer for denaturing gels. This reduces disulfide bonds in

TABLE 5. Tracking Dye Mobilities for RNA Formaldehyde Gels

	Formaldehyde gels	
	xylene cyanol	bromophenol blue
SeaKem® Gold Agarose		
1.0%	6300	660
1.5%	2700	310
2.0%	1500	200
SeaKem GTG® and LE Agarose		
1.0%	4200	320
1.5%	1700	140
2.0%	820	60[a]
SeaPlaque® and SeaPlaque GTG Agarose		
1.0%	2400	240
1.5%	800	80[a]
2.0%	490	30[a]

[a]Nucleic equivalent for dye migration determined by extrapolation.

proteins, ensuring that they maintain the random-coil configuration necessary for the determination of molecular weight. Use stock reducing agents in a fume hood.

- Keep sample buffer at –20°C in small aliquots. The glycerol concentration is high enough to prevent freezing, so you don't have to worry about damage caused by freeze-thawing. When you can no longer smell the reducing agent in the tube, it is time to get a new tube of sample buffer.

- For SDS-PAGE: 2X buffer is 4% SDS, 20% glycerol, 10% 2-mercaptoethanol (or 100 mM dithiotheitol) 0.004% bromophenol blue, and 0.125 M Tris-HCl. pH should be approximately 6.8.

- Samples must be boiled (or heated at 95°C) in the sample buffer for 5 minutes just before being loaded. Use a floating tube rack if you heat the samples in a beaker or water bath: In a pinch, you could poke holes in a piece of styrofoam. Chill the tubes on ice after boiling. Hold each tube firmly when opening it, in case all the pressure hasn't yet dissipated, to prevent the lid from popping open.

- If you boil samples with the lids closed, the lids will pop open, and the entire tube can spectacularly explode into the air. This can be avoided in several ways. You could wait until the samples have just started to boil, and then gently open the tube to release the pressure. Or you could use a pin and poke a hole in the lid of each tube, but this shouldn't be done with radiolabeled samples. There are racks used to boil samples that clamp a lid on the microfuge tubes, preventing the caps from opening. The best alter-

native is the use of individual plastic clamps that slide onto the lids of microfuge tubes and prevent them from opening.

Standards/markers

- Standards are available in high, low, and broad ranges. They are available unstained or prestained: Prestained is more convenient. Store standards at –20°C, unless otherwise noted.

- Rainbow standards (Amersham) are very useful (and pretty), since each protein is stained a different color. They are expensive, but it is incredibly easy to monitor the progress of electrophoresis when the identity of each marker protein is clear.

- The covalent binding of dyes to the markers produces variation in the molecular weights of the proteins. For precise molecular weight determinations, use calibrated molecular weight standards.

- Biotinylated standards can be incorporated into horseradish peroxidase (HRP) or alkaline phosphatase detection procedures for immunoblots. Other markers are designed to be used for silver or other staining.

- Most markers are designed for SDS-PAGE gels. If you are running native (nondenaturing) gels, you must get markers designed for that.

Format

- Polyacrylamide gels are always poured between glass plates. The plates are held apart by spacers. Spacers come in different thicknesses, and are matched to a comb of the same thickness.

- Continuous vs. discontinuous. A continuous gel system has a resolving gel with the same buffer used at the anode and cathode. A discontinuous gel has two parts: a stacking gel (a large-pored gel) overlaid over the resolving gel. In a discontinuous system there is a different buffer used at the anode and cathode. Resolution is much better in a discontinuous system.

- 2-D (2-dimensional) gels are used to more precisely characterize proteins. The samples are run twice, called the first and second dimensions. The first dimension is an isoelectric focusing gel (IEF), usually run as a tube gel, to determine the pI of each protein. This tube gel will then be placed

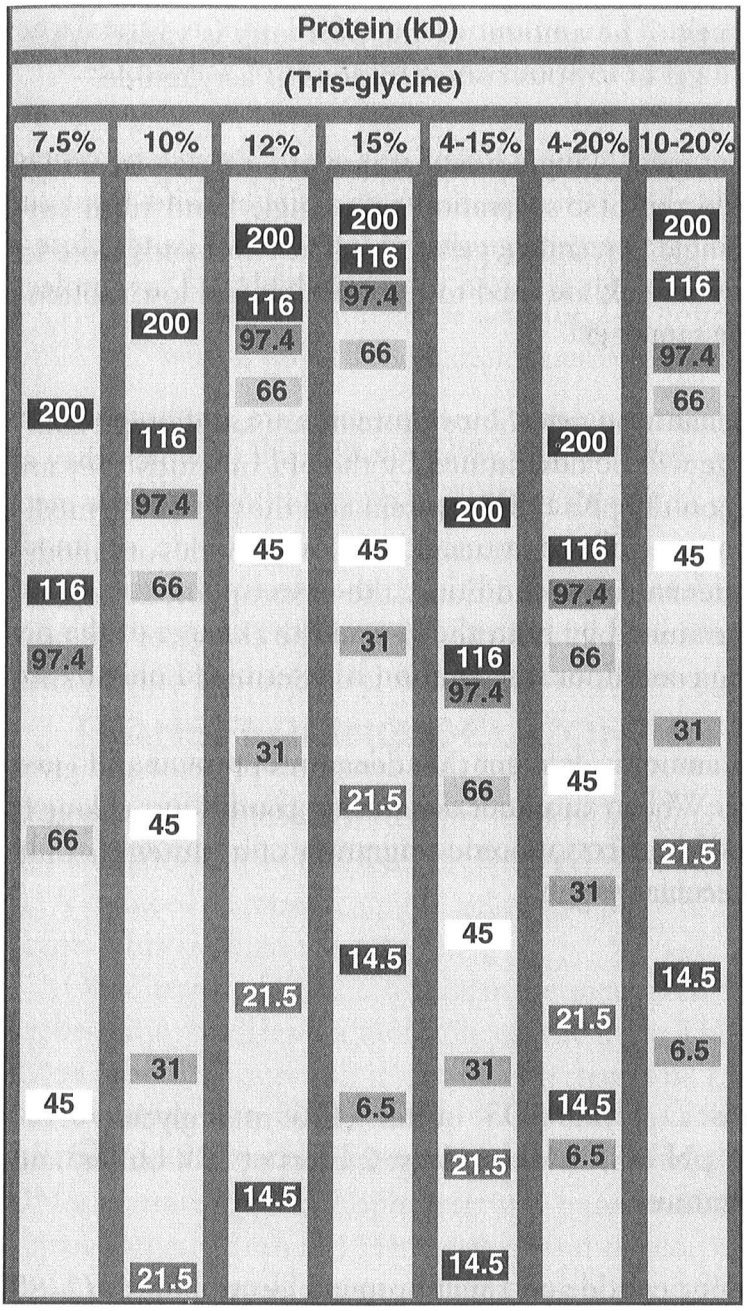

FIGURE 4.

Protein molecular weight markers. The positions of proteins of 200, 116, 97.4, 66, 45, 21.5, 14.5, and 6.5 kD are shown after electrophoresis through gels of different acrylamide concentrations, using Tris-glycine buffer. (Redrawn, with permission, from Bio-Rad Laboratories, Hercules, California.)

onto a slab gel for the second dimension, and the proteins are separated according to molecular weight.

- Tube gels versus slab gels. Tube gels were once used for general electrophoresis. They are now used only for the first dimension of 2-D gels.

The gel

- 38:1 w:w ratio of acrylamide to BIS is the usual ratio of stock solution

used to make protein gels. The amount of the 38:1 mix is varied when making the gel, to get a gel of various concentrations of acrylamide.

- Gradient or nongradient gels. Nongradient gels are of a single percentage acrylamide, and provide the best separation of a single band from surrounding bands. Use single percentage gels to look at two bands close in molecular weight. Gradient gels are used to resolve high and low molecular weight bands on the same gel.

- Denaturating or nondenaturing gels. Since proteins are amphoteric compounds, their net charge will be determined by the pH in which they are suspended: Depending on the pKa of the protein and the pH of the medium, a particular protein will be attracted to the cathode or anode. Therefore, under nondenaturing conditions, the electrophoretic separation of proteins is determined by both the size and the charge of the proteins. Under denaturing conditions, separation is determined only by size.

- SDS-PAGE. SDS is an anionic detergent that denatures proteins and gives them a negative charge. When run under denaturing conditions, charge is no longer a factor and the electrophoretic migration of a protein is only dependent on the molecular weight.

Buffers

- Most protein gels use a glycine-SDS buffer (196 mM glycine/0.1% SDS/50 mM Tris-HCl, pH 8.3). Make at least 2 liters of 10X buffer, and store it at room temperature.

- Tricine buffer is used for peptide and small protein electrophoresis (2–80 kD).

Power

- Run at 25–30 mA (200 V). Electrophoresis is usually halted when the dye front reaches the bottom of the gel.

- Run proteins more slowly through the stacking gel, at below 50 V. Once your samples are at the stacking gel/resolving gel interface, you can up the voltage to 200 V.

Staining

- Gels are stained after electrophoresis by incubation with staining solutions, followed by multiple washes to remove excess stain.
- The most common stain is 0.2% Coomassie Brilliant Blue, in 45:45:10% methanol:water:acetic acid for 2–3 hours at 37°C, with agitation. Destain with 25:65:10 methanol:water:acetic acid.
- Silver staining is a more sensitive stain and is used for proteins that stain weakly or not at all with Coomassie. It is also more complicated to use.

TRANSFERRING GEL CONTENTS TO MEMBRANES

The gel is a thick but fragile matrix, making it difficult to subject to handling or incubations. If hybridization of the gel contents is to be done, the material in the gel is induced to move from the gel onto a piece of nitrocellulose filter or nylon membrane. This is called the transfer.

Once transferred, the filter can be incubated with a probe, to see whether the probe can hybridize to a molecule on the filter. The probe is labeled with radioactivity or other indicators so it can be detected. The hybridization of the filter with the probe is referred to as blotting.

The first use of blotting, which was to detect specific nucleotide sequences in DNA separated by electrophoresis and transferred to nitrocellulose, was described by a man named Southern. Hence, this kind of blot was called a Southern blot. As different kinds of templates and probes were used, they were lightheartedly given other directional names.

Southern blot: DNA on the filter is probed with DNA.
Northern blot: RNA on the filter is probed with DNA or RNA.
Western blot: Protein on a filter is probed with antibody.
Southwestern blot: DNA on a filter is probed with protein.
Middle eastern blot: Poly(U)-derivatized paper is probed with mRNA.

There are three ways in which biological material is transferred from the gel to a filter or membrane:

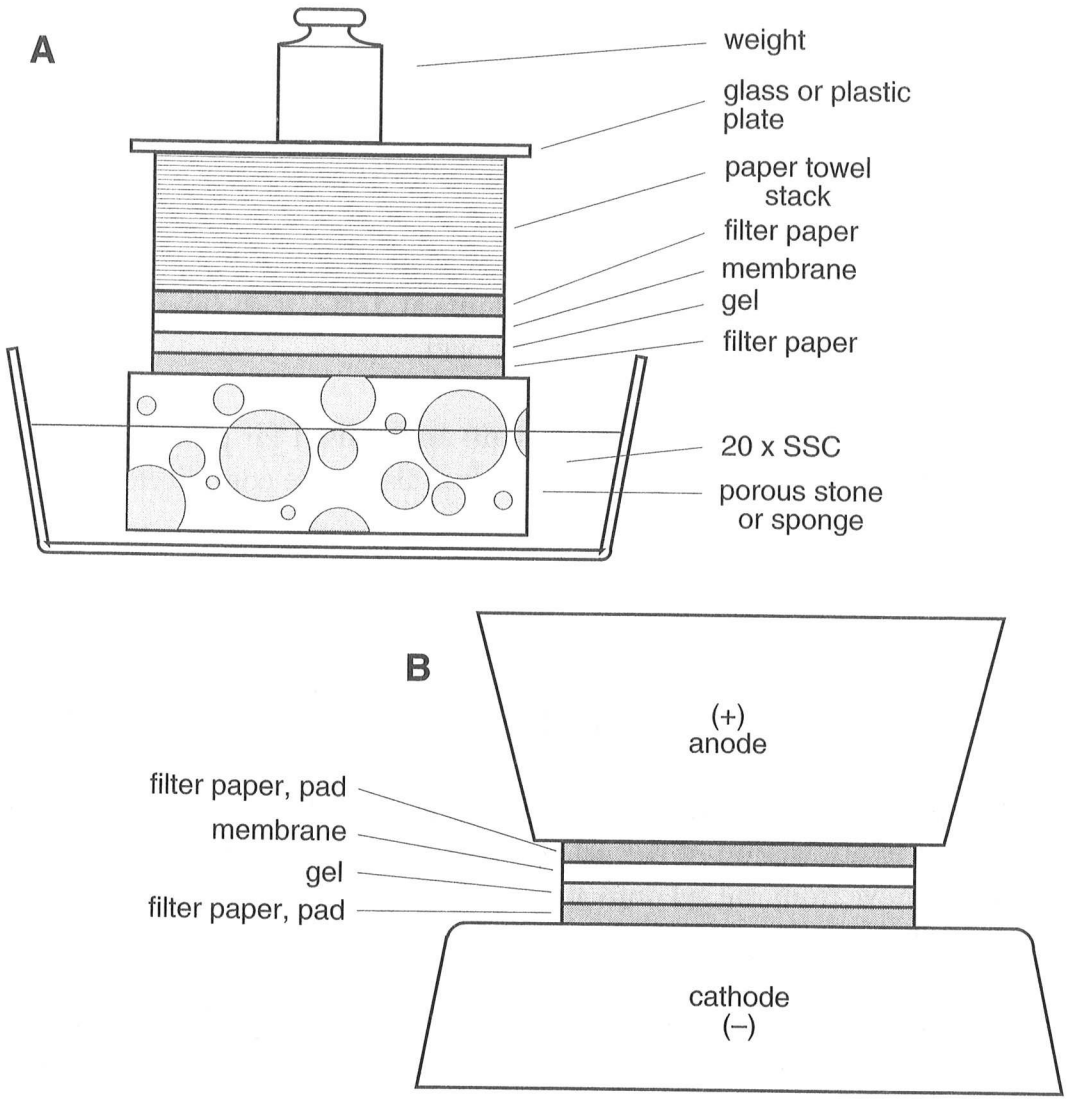

FIGURE 5.

(*A*) Capillary transfer of molecules relies on the movement of salt solution, which is pulled from the basin through the gel and membrane and into the wad of paper towels, and carries the DNA or RNA with it. Vacuum transfer is set up in the same way. (*B*) Electrophoretic transfer depends on the movement of negatively charged DNA, RNA, or protein from the gel to the membrane, in its attempt to reach the anode. In wet blotting, the entire system is immersed in one transfer buffer, whereas in semi-dry blotting, filters are wet with separate anode and cathode transfer buffers to facilitate the current.

- *Capillary transfer.* Used for DNA and RNA.
- *Vacuum transfer.* Used for DNA and RNA.
- *Electrophoretic transfer.* Used for DNA, RNA, and protein. There are two main apparatuses to do this, both of which use current to move molecules from the gel toward the anode, and onto a membrane. These are dry blotters or semi-dry blotters, in which the current is carried only through filter papers soaked in anode and cathode buffers, and wet blotters, in which the gel and membrane are immersed in a transfer buffer. This type of transfer requires a power source capable of very high amperage.

Membranes

- Membranes are available in rolls or in precut sizes. Unless everyone in the lab only runs one size gel, buy the roll.
- Wear gloves when you cut or handle any membrane. Grease or oil from your fingers can impede transfer.
- Pick up membranes with a clean, flat-edged forceps. Sharp or pointed tweezers can tear the membrane.
- Your choice for a transfer membrane is mainly between nylon (various kinds) and nitrocellulose (regular or strengthened with cellulose acetate for easier handling).
- Nylon is best for nucleic acids. It is durable, binds nucleic acid well, and can be stripped and reprobed multiple times. Nylon tends to have more background staining, and so requires a higher concentration of blocking agents during hybridization. Cationized nylon works best for nucleic acids, as binding does not depend on the ionic strength of the buffer. Nucleic acids cannot be electrophoretically transferred to nitrocellulose.

- Nitrocellulose is best for some proteins, nylon for others. Nylon has a higher binding capacity in general, but there are a few proteins that don't bind well at all. Essentially, both work, although lab workers will swear by one or the other. Use what is used in the lab for your particular molecule. If you must use nitrocellulose, use strengthened nitrocellulose. If you are starting anew, try a PVDF (polyvinylidene difluoride) nylon such as Immobilon-P (Millipore) for Western blotting.

- Protocols for nitrocellulose and nylon are very different: In fact, protocols are different for different types of nylon.

- You do not usually have to worry about pore sizes. 0.45 μm is the standard. Smaller sizes are available for specialized use.

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