

Appendices

Appendix 1. A primer on making solutions.

1. Always use the purest water to make solutions. There are three sources of water in the laboratory. The crudest water is common *tap water*.

A purer form of water is **deionized water** provided campus-wide by UA Facilities Management. Deionized water has had particles and most of the ions removed. It is available at the sinks in the lab from the gray, plastic taps labeled **DW**. Although deionized water can be used in making culture media and as a final rinse when washing glassware, you should not use water from these taps to make your stock solutions.

The purest water, *reagent grade water*, is produced when deionized water is processed through a filtration system consisting of a series of cartridges containing organic resins and activated charcoal. A final pass through a 0.22 μm filter removes most biological agents. This ultra-filtration system removes most of the remaining ions from deionized water. A popular cartridge filtration system in research labs is made by the Millipore company and is called the milli-Q system. For this reason, reagent grade water often is referred to as "milli-Q water". In this course we use a *Photronic™ Reagent Grade Water System* to provide reagent grade water. It is referred to as **RGW** throughout the lab manual.

In summary, **always use reagent grade water to make solutions**. Reagent grade water is provided to you in 20 L carboys, and in plastic squeeze bottles. *This water is not sterile.*

To completely sterilize RGW requires high pressure heating in an autoclave. The high pressure in an autoclave allows the temperature to climb well above the normal boiling point, to 121°C. Depending on the volume of liquid being autoclaved, exposure at this temperature for 20 to 45 minutes is required to effect sterilization. If high temperature is harmful to a solution, then the solution must be passed through a sterile filter element to remove biological agents.

2. Make sure that the form of each component is known.

For example: You will be using **Tris** as a buffer in many of the solutions. Tris can be obtained in many crystalline forms: Tris-base, Tris-HCl, Tris-acetate, *etc.* Each has a different formula weight. In this class you will use the Tris-base form which has a formula weight of 121.1 g/mole.

Also, note from the list of reagents provided for an exercise whether a reagent is in dry form or in the form of a stock solution to be diluted.

3. Calculate the amounts of each reagent required to make the solutions you need. The *formula weight (FW)* of a compound will always be written on the reagent jar from

which you take the compound. The formula weight is simply the number of grams in one mole (**mol**) of the compound: **FW = g/mol**.

The FW of a compound may differ from expectations based on the weights of the component atoms because the compound may be *hydrated* with one or more water molecules per molecule of reagent.

The concentration of a reagent in a solution is often stated in *molar (M)* units. Molar concentration is simply the number of moles of a compound *per liter of total volume of solution (mol/L)*. Pay close attention to the units of measurement of concentration in recipes. For example, in many cases the recipe for a solution will state concentrations both in **M** and **mM** (1 M = 1000 mM), and perhaps as weight/volume percentages also.

To calculate the amount of reagent (in grams) needed when concentration is stated in molar units, recall:

a. **M = mol/L**

b. **g/FW = mol**

c. From a. and b. you get **M = (g/FW)/L** (liters of total solution; not just solvent)

d. Which can be rewritten as: **g = (M)(FW)(L)**

Example: To make 100 ml of 0.5M EDTA solution, calculate the number of grams of Na₂EDTA·2H₂O needed in 100ml of the solution:

$$\text{FW of Na}_2\text{EDTA}\cdot 2\text{H}_2\text{O} = 372.24 \text{ g/mol}$$

$$\text{Concentration} = 0.5\text{M} = 0.5 \text{ mol/L}$$

$$\text{Volume of solution} = 100 \text{ ml} = 0.1 \text{ L}$$

$$\text{Formula: } (\mathbf{M})(\mathbf{FW})(\mathbf{L}) = \mathbf{g} \text{ (of dry reagent required for solution)}$$

$$(0.5 \text{ mole/L})(372.24 \text{ g/mol})(0.1 \text{ L}) = 18.61 \text{ g}$$

To make 100 ml of 0.5M EDTA, 18.61 gm of EDTA is added to about 80 ml of RGW, stirred to dissolve, then the final volume brought up to 100 ml.

Preparation note: EDTA can be dissolved faster if the pH of the solution is adjusted to 8.0 after the reagent is added and before the final volume is adjusted. Then, after the solution is brought to the final volume, the pH should be rechecked.

Note: 18.61 gm of EDTA is not added to 100 ml of water! If this is done, the concentration will be less than 0.5M because the final volume will be greater than 100 ml.

Dilutions: Often, rather than adding a reagent in dry form to a solution, you will dilute an existing *stock solution* of the reagent. Also, the preparation of a solution from a dry reagents may be stated in a recipe as a dilution, rather than a molarity.

Below are described three different ways to specify dilutions: weight/weight, volume/volume and weight/volume. Which type of dilution is required usually will be stated in the recipe.

A dilution may be expressed as a ratio, for example: 1:10. In general a 1:10 ratio means that the solute comprises 1 unit in a **total** of 10 units of solution. If the dilution involves two solutions, then the 1:10 ratio indicates that 1 volume unit of the more concentrated solution is added to 9 units of solvent (to make 10 units of the total solution).

• *Volume/volume dilution.* This type of dilution is done frequently in molecular biology. Typically, a stock solution is diluted to a lower concentration as part of a solution you are preparing. When diluting a stock solution to a lower concentration you may find the following equation useful:

$$C_1V_1 = C_2V_2 ,$$

where **C₁** is the initial concentration of the stock solution and **C₂** is the final (target) concentration; **V₂** is the final volume that you want and **V₁** is the amount of the stock solution that you would need to add. You would be solving this equation for the value of **V₁**. *It is essential that the units for concentration and volume be the same on both sides of the equation* (or use the appropriate conversion factors).

Example: To make 500 ml of 10mM Tris, pH 8.0 from a stock solution of 1M Tris, pH 8.0, the following calculations must be performed:

$$C_1 = 1\text{M Tris (stock)}$$

$$V_1 = ?$$

$$C_2 = 10\text{mM Tris} = 0.01\text{M Tris}$$

$$V_2 = 500 \text{ ml} = 0.5 \text{ L}$$

$$C_1V_1 = C_2V_2$$

$$(1\text{M})V_1 = (0.01\text{M Tris})(0.5 \text{ L})$$

$$V_1 = 0.005 \text{ L} = 5 \text{ ml}$$

Add 5 ml of 1M Tris, pH 8.0 to 495 ml of RGW to make 500 ml of 10 mM Tris, pH 8.0.

• *Weight/volume dilution.* This type of dilution commonly is performed by biologists. Water is usually the solvent. The correct amount of dry reagent is added to the solvent, and the volume is brought up to that stated in the recipe. Weight/volume dilutions are often expressed as percentage dilutions. For example, a 1:5 (w/v) dilution is also a 20% (w/v) dilution. This makes it easier to calculate the dilution and to label the solution.

General expression:

$$\text{g of reagent} = (\% \text{ dilution}/100)(\text{volume in ml})(1 \text{ g/ml})$$

Example: To make 100 mls of 20% (w/v) SDS solution would require the following calculation:

$$\text{grams of SDS} = (0.2)(100 \text{ ml})(1 \text{ g/ml}) = 20 \text{ g}$$

Add 20 g of SDS to about 75 ml of RGW in a beaker. Stir do dissolve the SDS crystals. Then bring the volume up to 100 ml in a graduated cylinder with RGW.

- *Weight/weight dilution.* In this case the final weight is critical in the calculations. If you wanted a 1:10 dilution (w/w) of NaCl into water, 1 gm of NaCl would be added to 9 g of water or 9 ml of water (*1 ml = 1 g of water at 20°C.*). The total weight would be 10 g. This dilution is rarely performed by biologists.

4. Make a **reasonable** quantity of each solution. You would not want to make a liter of a solution for which there is only need for 100 ml during the semester. In this lab manual, in most cases your needs for the semester have been anticipated.

5. Have all the lab equipment you will need at hand. *Rinse all labware with deionized water, then RGW, prior to use to remove accumulated dust.*

- Beakers and stir bars for mixing and dissolving reagents.
- Graduated cylinders and pipets for measuring volumes of solutions. Use the smallest graduated cylinder possible to ensure accurate measurement of volume. Remember to read the volume along the *bottom* of the meniscus.
- Bottles for the new solutions. Use the smallest bottles possible to save space. For example, don't use a 1L bottle if you're making only 200 ml of solution; use a 250 ml bottle.

Check the cap of your bottle to see if it has a good cap liner. If it does not, get another cap. The cap liner is important in sealing the bottle. This will both prevent spills and maintain the correct pH and concentration of reagents.

For solutions of 250 ml or less, always use *milk dilution bottles* unless directed otherwise. These bottles are available in two sizes: 150 ml and 250 ml. They are easy to recognize by their square shape. The class has large numbers of these bottles available for student use. They have the advantage, because of their shape, of packing tightly into small spaces.

If you have time and the inclination to assist the prep staff, you can wash labware and reuse it. First, wash the items with a bottle brush or sponge and some cleaning solution. Then, thoroughly rinse out the item, first with deionized water, followed by

RGW. Shake water from the item into the sink. You do not need to dry it. Reshelve the items if you no longer need them.

6. Measure the correct amount of reagent.

Dry reagents

Weight out the correct amount on one of the electronic balances. *Don't forget to tare (subtract the weight of) the weighing paper or tray.*

All the reagents used in the laboratory are analytical grade (relatively pure). Do not contaminate these reagents by using a dirty spatula or your hands.

It is not always necessary to use a spatula to weigh reagents. If the reagents are crystalline in form, like Tris-base, you may simply pour a small amount into the cap. Since the reagent bottle is relatively cumbersome to handle, it is easier to use the cap of the reagent bottle to gently tap crystals onto the weighing paper. Also, if you make a mistake, you will not have poured out (and wasted) the entire jar of reagent.

If the amount of reagent required exceeds the capacity of the weighing paper, use one of the disposable weighing trays. There are three sizes of weighing trays available for use. Do not use the largest size if a smaller one will do. If only a small amount of a reagent is needed, use the weighing paper rather than a tray — it is much less expensive.

In general, you should dispose of a weighing tray after a single use to avoid contaminating solutions. However, it is acceptable to use the same weighing tray for different dry reagents, but *only if they all are going into the same solution.*

If you have poured out too much reagent, use a *clean* spatula to remove the excess weight. Throw it away, not back into the reagent jar. Because this is expensive and wasteful, be very careful when you are weighing reagents.

Liquid reagents (stock solutions)

It is more accurate to use a pipet with safety bulb than small graduated cylinders to measure volumes of 25 ml or less. Larger volumes will require the use of graduated cylinders (or multiple pipet transfers). When measuring volumes, keep the following points in mind:

- Graduations are designed for solutions at room temperature (20-25°C). If the solutions you are measuring are not in this range, allow them to equilibrate to room temperature.
- Make sure you are reading the bottom of the meniscus.

7. Once you combine your reagents, stir the solution to dissolve dry reagents and mix ingredients thoroughly. Use a magnetic stir plate and stir bar. It is best to use a glass

beaker with a capacity greater than the final volume for this step: using the graduations on the side of the beaker as a guide, bring the volume of the solvent (usually RGW) to within about 10% of the final volume. This will expedite the dissolving of reagents without the risk of going over-volume.

8. Adjust pH if necessary at this point. This is especially important for those solutions that require large volumes of pH-adjusting reagents. Be careful not to damage the electrode with the stir bar. If you are not sure how to measure and adjust pH, read the information in *Appendix 2.2*. If you are not certain which acid (HCl or Acetic acid) should be used to lower the pH of a solution, ask your instructors.
9. Adjust the final volume. When all the reagents are dissolved, decant into a graduated cylinder and carefully bring the solution to the correct volume. Remember that graduated cylinders are calibrated for solutions at 20-25°C, so if the solution either warmed or cooled during preparation, allow it to come to room temperature before measuring the final volume.

Use the RGW in a squeeze bottle to "fine tune" the volume in the graduated cylinder.

Beware: if you go beyond the correct volume you either must start over or recalculate reagents for a larger volume and augment your solution.

10. Check the final pH of the solution and make adjustments if necessary.
11. Decant the solution into a labeled bottle and seal tightly with a cap. Never write directly on bottles or other labware with a marking pen.

Include the following on the label:

Solution name
Reagent concentrations
pH (where applicable)
Your group name
Date of preparation.

12. Store the solution under the appropriate conditions of temperature (frozen? refrigerated? room temperature?) and light.

Always store bottles upright to avoid possible leakage. If the solution can be stored at room temperature, it is best to put the bottle in one of the cabinets below the benchtop. (Label shelf space for use by your group.) This location is more desirable than the benchtop or the central shelving unit because it reduces clutter in the bench space of your group and those of other lab sections.

Appendix 2. Operation of the Orion analog pH meter.

The Orion analog pH meter

The pH of a solution is most accurately measured with a pH meter. The pH meter measures the H^+ concentration of the solution (remember $pH = -\log[H^+]$). You will adjust the pH of solutions by adding acids, such as HCl, or bases, such as NaOH. These add or remove free H^+ ions, respectively. These solutions also add Cl^- and Na^+ ions to the solution which are not measured by the pH meter. The pH is measured as the potential generated between two small electrodes enclosed within a *very fragile* glass bulb which is permeable to H^+ ions. The electrode resides at the end of a pen-shaped probe, attached to the pH meter by a wire. The electrode is a delicate and expensive instrument; treat it as such.

Important

It is absolutely crucial that the electrode not be allowed to dry out because of extended exposure to air. When you have completed use of the pH meter, always replace the protective cap on the probe.

Appendix 2.1. pH station setup.

The following items comprise the **pH station setup**:

Orion™ analog pH meter with probe (electrode)
pH buffer standards:
 4.00, 7.00, and 10.00 in bulk squeeze bottles and screw-cap vials
Stirring machine
Stirring bars
Pasteur pipets
Bulbs for pasteur pipets
Reagent grade water in squeeze bottle (for rinsing off probe)
250 ml beaker (to collect rinse water)
Support stand with clamp for probe
Reagents for adjusting pH:
 Concentrated HCl (approximately 10N)
 Glacial Acetic Acid (CH_3COOH ; approximately 17M)
 10N NaOH
Kimwipes

Appendix 2.2. Procedure for calibrating the Orion analog pH meter.

Two buffer standardization procedure:

1. Select the two pH standardization buffers that bracket the desired pH of your solution, that is, *4 and 7* or *7 and 10*.
2. Place the electrode in the pH 7 buffer, allow the reading to stabilize, then turn the CALIB control until the needle points to 7.00 on the dial.

3. Remove the electrode from the buffer, place it over a beaker and rinse the electrode with RGW.
4. Place the electrode in the second buffer (pH 4 or pH 10) and turn the TEMP control until the needle points to the correct value for the buffer (4.00 or 10.00). If you had to turn the TEMP control by more than 10°C from the actual temperature, inform your instructor. This indicates that the electrode requires maintenance.
5. Remove the electrode from the buffer and rinse with RGW.
6. The meter now is calibrated and ready to measure the pH of a solution.
7. When you complete your work with the pH meter, rinse the electrode with RGW, then replace the protective cap.

Appendix 2.3. Measuring and adjusting the pH of solutions.

If the pH meter is correctly calibrated, you can begin measuring the pH of your solutions and adjusting them when necessary.

1. Place the beaker with your solution and a stir bar on the magnetic stir plate and mix the solution thoroughly for a few seconds. Remove the protective cap from the probe and insert the electrode into the solution. Read the pH from the analog readout — wait a few seconds for the reading to stabilize.
2. If the pH needs adjusting, select the appropriate reagent (10N NaOH to raise pH; either HCl or Acetic acid to lower pH) and slowly add it to the solution with a new, clean pasteur pipet. Stir the solution as you add the reagent and monitor the change in pH.

You can use the clamp on the support stand to hold the probe in position as you add reagent.

Do not allow the probe to fall into the beaker; the electrode could be damaged by the stirring bar.

3. When the correct pH value is reached, wait a few seconds to be sure it is stable. Then bring the solution to full volume in a graduated cylinder. See **Appendix 1** *A primer on making solutions*. Check the pH again to be sure it is correct.

If the solution is not at room temperature, you will have to wait until it equilibrates to obtain both a correct volume and a correct pH.

4. When you are satisfied that the pH of the completed solution is correct, clean up the pH station.
 - a. Rinse the electrode with RGW, replace the protective cap on the probe and put it back on the support bar of the meter.
 - b. Put used pasteur pipets in the GLASS WASTE receptacle. Use the RGW to rinse out pipet bulbs contaminated with acid or base.

- c. Empty the waste water beaker into the sink and throw KimWipes in the PAPER WASTE receptacle.
6. Decant the solution you made into a clean, labeled bottle and cap tightly. Be certain the cap liner is in good condition; the pH of the solution can change if air gets in. Store under the appropriate conditions. If you are finished making solutions, rinse out your labware and place in the gray basins to be washed.

Appendix 3 Operation of the Sorvall™ centrifuges.

The class has two Sorvall RC2-B centrifuges available for use, one in CBS 541 and the other in CBS 540. These centrifuges are capable of spinning at very high rotational velocities (to 20,000 rpm) while maintaining samples at a temperature of 4°C. In this class the Sorvall centrifuges are used to pellet *E. coli* cells.

You will use only two types of tubes with the Sorvall centrifuges, and one type of rotor, the SS34. Oakridge tubes are plastic (polypropylene) and have a capacity of about 35 ml. Oakridge tubes fit the SS34 rotor and will be used to pellet cells. You also have an exercise in which glass 25 ml Corex® tubes are used. Corex tubes require an adapter to fit the SS34 rotor.

Severe damage can result if pairs of tubes placed in the Sorvall centrifuge are not balanced to within about 0.1 g of one another. An acceptable way to balance tubes is to place them on a double pan balance and add liquid to the lighter tube to balance the heavier one. There are two double pan balances available for this purpose.

If you use this method, do not assume the central marking on the scale of the balance is the balance point. Instead, note the balance point on the scale (the place on the scale indicated by the pointer when nothing is on the pans) before you place your tubes on it.

It also is possible to use one of the electronic balances to balance two tubes. Simply record the weight of the heavier tube and add liquid to the lighter tube to equal this value. You must place the tubes in a beaker on the balance to prevent spilling the contents of the tubes.

Whenever you are balancing tubes destined for the Sorvall centrifuge, always remember to include the caps, and in the case of the Corex tubes, include the adapters.

Students should wait for an instructor to start the Sorvall centrifuge when it is ready.

Appendix 2.1. Operation and care of horizontal gel electrophoresis apparatus.

Basic operation of horizontal gel electrophoresis apparatus. In this course you will use two different types of horizontal gel electrophoresis apparatus: the Hoefer Scientific Instruments (HSI) minigel device, and the FisherBiotech Large Horizontal Gel apparatus. Despite their obvious dissimilarities, the two devices are fundamentally the same, differing primarily in size. In both cases a gel (composed of agarose dissolved in running buffer) is formed in a special casting tray with a comb attached to form wells in the gel. The solidified gel is then placed on a platform in the electrophoresis box. A special buffer

solution — called "running buffer" — is then poured into the apparatus, filling both lateral buffer chambers and completely covering the gel to a depth of 1-2 mm.

Next the wells of the gel are loaded with samples of the DNA fragments you want to separate by size, mixed with a loading buffer containing dyes and glycerol. Most commonly you will load the products of a restriction enzyme digestion. The presence of glycerol in the loading buffer causes the sample to be denser than the running buffer, so that it falls into the well, rather than dispersing into the running buffer. After the gel is loaded, electrical leads are attached to the device and an electrical current is applied. The electrical current is conducted through the running buffer and the gel (which also is mostly running buffer) via the thin platinum wires that lay on the bottom of the buffer chambers and the presence of ionic compounds in the running buffer. Both types of devices have transparent covers which allow you to monitor the movement of loading buffer dyes through the gel. [See *Appendix 9 DNA -Lambda molecular weight markers (HindIII digest)*, for more information about the dyes in the loading buffer.]

Because DNA has a net negative charge, it is repelled from the cathode (the negative electrode) and attracted to the anode (the positive electrode). However, the movement of DNA is greatly retarded in the agarose gel. The higher the proportion of agarose in the gel, the slower is the rate of migration of the DNA fragments through the gel. Fragments separate from one another by size because larger fragments move more slowly through the gel than do smaller fragments. For fragments less than about 9000 bp in length, the relationship between size and distance migrated is rectilinear. (Refer to Sambrook, *et al.*, 1989, page 6.4.)

The electrodes are connected to a power source with red and black electrical leads terminating in jacks that plug into the power supply unit. By convention, the black lead connects to the the negative electrode and the red lead to the positive. If you follow this convention and are careful to match the lead color to that on the power supply, then the black lead should be on the side of the gel with the wells. Be careful not to get the leads switched or your DNA will migrate backward off the gel.

Care of horizontal gel electrophoresis apparatus. The horizontal gel electrophoresis apparatus used in this class are easy to care for. When you finish with an electrophoresis run, simply remove your gel and place it on a sheet of Saran Wrap. Wear gloves and be careful not to break the gel; despite appearances, it is actually rather brittle and pieces break off easily. After you have completed your analysis of the gel, pour the running buffer carefully into the funnel of the waste bucket labeled TBE RUNNING BUFFER. Next, rinse the electrophoresis apparatus in the sink, first with tap water, then a final rinse with deionized water. Be sure to rinse all components: the cover, the box, and the running tray. If any of the TBE running buffer has crusted on the device, spend a few seconds rubbing it off with a sponge. You may use cleaning solution on the device, but never use abrasive cleansers.

If your casting tray and comb are still dirty, clean these now also. (Remember: EtBr contaminated gel material goes in the orange biohazard bag in the ETBR WASTE bucket.)

When everything is clean, shake off excess water into the sink, then place all components of the device on the north benchtop to dry. Place the components on an underpad or paper toweling. This is the draftiest location in the room, so there is a good chance

everything will dry before the end of the lab session. If so, return all equipment to its proper storage location.

Precautions

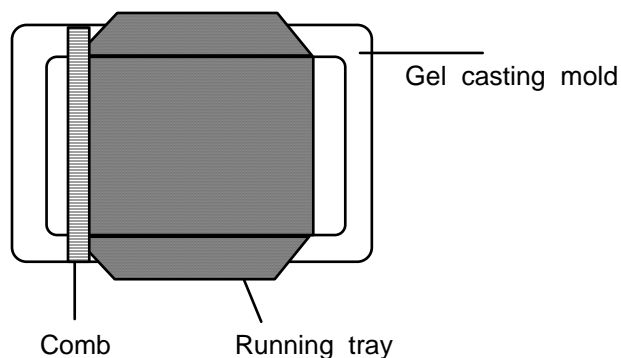
- Although plexiglass is a rugged material, take great care **not** to drop the electrophoresis devices. They may shatter or break at the joints.
- Be especially careful with the electrical leads into the devices. These are their weakest points. **Never separate the cover from the bottom of the HSI mini-gel devices by pulling on the electrical connectors.**
- **Never plug the electrical leads from the electrophoresis apparatus into the power supply unless the cover is on the device and you have completed preparations to run the gel.**

Because of the design of the two types of horizontal electrophoresis apparatus used in this course, it is not possible to apply an electrical current with the device open.

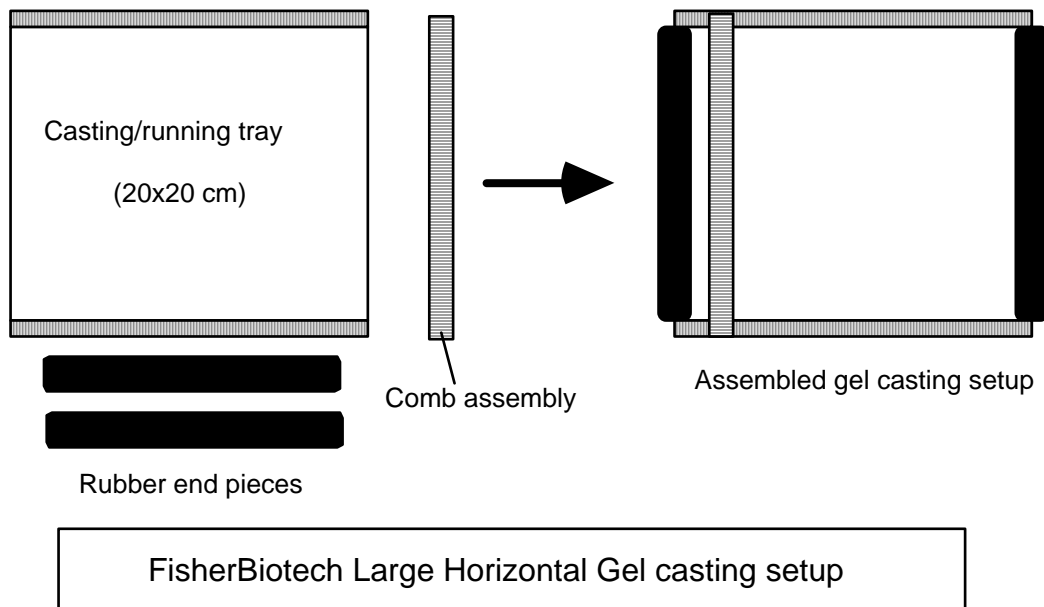
- **Once the run has begun (i.e., power is on), do not move the electrophoresis apparatus until you turn off the power and disconnect the electrical leads.**

Appendix 2.2. Directions for making a 0.8% Agarose-TBE-EtBr gel.

1. Assemble the gel casting setup. The illustrations below may be of assistance to you.



Top view of the HSI minigel casting setup



2. Add 0.8% (w/v) of agarose to the appropriate volume of 0.5X TBE buffer. At this concentration, the agarose will not change the final volume of the solution significantly, so there is no need to take a final volume reading.

A fundamental rule in electrophoresis: Always dissolve the agarose in the same solution that will be used as running buffer. If you do not, the gel will become distorted and migration of the samples aberrant when you run it.

3. Heat the mixture in a microwave oven until all the agarose is melted. There should be no crystals visible in the solution. Do not allow the solution to boil as this could affect the concentration of agarose in the gel.
4. Allow the agarose solution to cool below 60°C on the benchtop; this takes about 5 min for 100 ml of agarose-TBE solution. Cooling can be accelerated with a water bath when large volumes (>200 ml) are being made and time is in short supply. However, it is better to let the solution cool on the benchtop at room temperature to avoid inconsistencies in the gel.

When pouring a large volume of agarose solution (>200 ml) use a thermometer to measure the temperature of the solution.

If you pour the solution while it is too hot, you will cause permanent damage to the gel casting assembly.

5. After the solution has cooled sufficiently, add 10 mg/ml EtBr stock solution to a final concentration of 0.5 µg/ml in the agarose solution.

For example, if you are making 70 ml of agarose-TBE solution, you will add 3.5 µl of the 10mg/ml EtBr stock solution.

6. Allow the gel to solidify completely before removing it from a mold. Wear gloves for this step.
 - a. Remove the comb first. Pull straight out while gently rocking the comb end to end. The wells should be smooth, rectangular depressions in the gel. The comb should not have penetrated the bottom of the gel.
 - b. To remove a minigel from its mold, pull gently on the handles of the running tray while holding the casting mold in place. remove any thin sheets of gel material from the both the mold and the running tray and place in an orange biohazard bag.

Leave the gel in the running tray. Both will be placed in the electrophoresis apparatus.
 - c. To get a FisherBiotech Large Horizontal Gel ready to run, after removing the comb, carefully remove the rubber end pieces from the casting/running tray. Rinse the rubber pieces with deionized water and set aside to dry. Leave the gel in the running tray. Both will be placed in the electrophoresis apparatus.
7. If the gel is not going to be run within 30 minutes, wrap the running tray/gel unit in Saran Wrap to protect from desiccation.

Appendix 2.3. Directions for assembling a horizontal gel apparatus.
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Wear gloves when performing this procedure.

1. When the gel is ready to run, place the running tray with the gel in one of the horizontal gel apparati. The running tray fits the central platform in the device.

For consistency, you may prefer to always position the apparatus in the same direction. For example, run your gels left-to-right, with the wells and the black electrical connectors on the left side.
2. Move the apparatus to a location near one of the power supplies where the gel will be run. It is much easier to place the device near the power supply now than after the gel is loaded.
3. Fill the gel apparatus with 0.5X TBE running buffer. Fill both lateral buffer chambers and continue pouring until the gel itself is submerged to a depth of 1-2 mm. Make sure that the wells also are filled with running buffer.
4. The gel is now ready for samples to be loaded.
5. After the samples are loaded (see *Appendix 2.4. Directions for loading horizontal agarose gels*), place the cover on the appararus.

HSI minigel apparatus: Place the cover vertically over the device, making sure that the electrical connectors are engaged properly. The black lead should be on the side of the device with the wells of the gel. This lead will be connected to the negative pole of the power supply.

When you disassemble the minigel apparatus after the gel has run, pull straight up on the cover. **Do not pull on the electrical leads.**

FisherBiotech Large Horizontal Gel apparatus: Slide the cover forward until you engage the electrical connectors. Make sure that the cover is moved fully into position and that the black electrical lead is on the side with the wells.

When you disassemble the Large Horizontal Gel apparatus after the gel has run, pull back on the plexiglass cover. There is a flange on the back edge to facilitate this operation. **Do not pull on the electrical leads.**

Appendix 2.4. Directions for loading horizontal agarose gels.

1. Have your samples ready to load before starting. Each sample should be in a labeled microfuge tube with the correct amount of DNA and loading buffer at (approximately) 1X concentration.

The DNA sample should sink to the bottom of the well because of the glycerol in the DNA loading buffer.

2. Depending on the volume of your samples and wells, use either a P20 or P200 pipetman to load the samples. Using your free hand, hold the shaft of the pipetman to add stability and to guide the tip over the well.

This sometimes is easier said than done; in some gels the wells are almost invisible. This is especially true of the gels made for the FisherBiotech Large Horizontal Gel apparatus. Be careful not to lose your sample.

3. Place the pipet tip just into the mouth of the well. When you are confident that the tip is positioned correctly, slowly release the contents into the well.

If you are not absolutely certain that the pipet tip is over the well, release 1-2 μl and note where the loading buffer dyes go. Even if it doesn't fall into the well directly, it should indicate the location of the well.

Do not stab into the gel material with the pipet tip. This almost certainly will lead to loss of the sample.

4. Once the DNA sample has left the pipet tip, do not attempt to repipet it.
5. If the volume of the sample is too great for the well, don't worry. You have loaded as much DNA as possible into the well, and the portion lost will not affect the running of the gel.
6. **Important:** Record the position of each sample in the gel; otherwise you will not be able to interpret your results correctly.
7. Once gel-loading has begun, do not move the electrophoresis apparatus until the samples have run a few mm into the gel. Failure to heed this advice may result in the loss of all or part of your samples into the running buffer.

Appendix 2.5. Operation of the electrophoresis power units.

MCB 473 uses Hoefer Scientific Instruments *PS 250* electrophoresis power units that supply constant current or voltage to as many as six devices simultaneously. The voltage range for the units is 0-250 V DC. The current range is 0-2.50 amps DC.

Arrayed along the lower right side of the control panel are output jacks for six electrophoresis units. The outlets are color-coded using standard conventions. Black represents the cathode (negative terminal), and red is the anode (positive terminal). Electrical leads from electrophoresis units should be inserted into the corresponding color of output jack on the power supply.

The units can be set to run either at constant voltage or constant current. Because the protocols in the lab manual state voltage specifications, directions are provided below for running the power units in the *constant voltage mode*. The voltage reading is the same across all six sets of jacks, which are connected in parallel. Once the voltage is set and power established, the current for each output may be read separately, or the sum of the individual outputs may be displayed. Note that the knob at the output jacks does not need to be set to "current sum" for all outputs to be functioning.

Directions for operation of the PS 250 power supply at *constant voltage*

1. Up to six minigel electrophoresis units can be connected to the power supply. Black is the negative terminal, red is the positive terminal.

Make sure you are running your DNA in the correct direction!

2. Turn the *Voltage Set* knob all the way counterclockwise to the zero position.
3. Turn the *Current Set* knob all the way clockwise to the full scale position.
4. Turn on power to the output jacks. Either set the *Timer* to *ON-HOLD*, or set it to a specific time for the electrophoresis units to run. If you choose to use the timer, the power will shut off at the end of the period of time selected.

Usually you will set the power unit to *ON-HOLD* and monitor the progress of your dyes in the gel, then turn off the power when the dyes reach the correct position.

The light in the lower left corner of the control panel indicates that the power supply is on.

5. Turn the *Voltage Set* knob to the desired setting on the *Read Voltage* display. This value often will be indicated in the lab protocol. If it is not, a setting of 100 V usually will be appropriate for the minigel units.

The voltage will remain constant across each of the loads, with the current changing in response to changes in resistance in the electrophoresis units.

Important: Do not connect or disconnect electrophoresis units from the power supply without first turning the power off.

Appendix 2.6. Operation of the UV transilluminators.

The UV transilluminator boxes are used to visualize bands of DNA in agarose-EtBr gels. There are two types of UV transilluminators used in MCB 473. One is an HSI device with a mask designed for use with the HSI Photoman camera hood. Because the plexiglass shield of this UV transilluminator is open on the sides, it should be used only with the minigels and the Photoman camera hood because the mask forms a light-tight seal with the camera hood.

The other UV transilluminator — a large Fotodyne box — is of more general use. An aluminum foil mask cut to fit the gel size used during the session should always be attached to this box. Because the plexiglass cover for the Fotodyne box fully encloses the light source, it should be used when larger gels are being viewed.

Rules for use of the UV transilluminators

1. Limit your exposure to UV light. UV light is harmful to tissue and can cause genetic damage.
 - a. View the gel only long enough to determine whether or not it is worth photographing.
 - b. Turn off the UV light, set up for a photograph, then turn on the UV light only long enough to expose your photograph. (Make sure that the light is on, so you don't waste an exposure of film.)
2. *All members of a group must wear faceshields when using a UV transilluminator.*
3. Before turning on the UV light, make absolutely certain that the plexiglass shield of the box is in place.
4. To avoid unnecessary contamination of the UV transilluminator boxes with EtBr and buffer, always transfer your gel to and from the boxes on a sheet of Saran Wrap. The Saran Wrap does not interfere with viewing or photographing the gel.

Appendix 2.7. Operation of the BioDoc-it Gel Documentation System

The TAs will operate the gel documentation system.

Camera maintenance. The cameras require minimal maintenance. The surface should be wiped clean regularly to remove TBE and EtBr residues that inevitably accumulate. Squirt some ethanol on the transilluminator and wipe it down with Kim Wipes to dry.

Appendix 2.8. Handling enzymes.

The enzymes used in MCB 473 are commercial products, purchased from several different companies. In this class you mostly will use enzymes from Boehringer Mannheim because of the company's reputation for superior and reliable products that have yielded good results in 473 over the past few years.

In MCB 473 three basic classes of enzymes are used: restriction endonucleases (*aka* restriction enzymes) that cleave double-stranded DNA at sites with specific base sequences, ligases that reconnect the covalent bonds between DNA fragments with complementary ends, and DNA polymerases that synthesize a strand of DNA complementary to a template strand. A complete list of all enzymes used in this class is listed below.

Restriction enzymes	DNA polymerases	Ligases
<i>HindIII</i> <i>EcoRI</i> <i>PstI</i> <i>SpeI</i> <i>BamHI</i>	Klenow fragment (of DNA polymerase I) AmpliTaq® (used in PCR protocol) Sequenase™ (used in sequencing reactions)	T4 DNA Ligase
	Other	

Each enzyme has a particular reaction buffer that optimizes its activity. You can rest assured that the correct buffer will be provided to you, typically at a 10X concentration. Nonetheless, you should become familiar with the specifications for the various enzyme buffers you will be using. To this end, consult the product catalogs of the companies that provide enzymes to the scientific and medical communities, such as Boehringer Mannheim, Promega, Stratagene, and New England Biolabs (to name only a few).

Enzymes are proteins, and therefore are susceptible to inactivation (loss of activity) if they are not stored and handled properly. Enzymes are shipped on dry ice to maintain a temperature below -20°C and are kept at that temperature in the lab. Enzymes are usually stored in a buffer composed of 50% glycerol. Enzymes will be provided for your use in 0.5 ml microfuge tubes in small aliquots from the original tube, and kept within a chilled box called a *Cryo-Safe box*.

Enzymes suffer dramatic loss of activity when left at room temperature. For this reason, remove an aliquot of enzyme from cold storage only long enough to transfer the correct volume of enzyme solution to your reaction tube.

Enzyme concentration. The concentration of enzymes is listed in *units/μl*. The definition of a *unit* is based on the type of activity expressed by the enzyme. For example, in the case of Boehringer Mannheim restriction enzymes, one unit is defined as the amount of enzyme required to digest completely one μg of DNA (usually a lambda-phage genome) in one hour under appropriate conditions of temperature and reaction buffer. This does not mean that the enzyme will become inactivated after one hour. Most enzymes will remain active for several hours. However, in some cases you will want to inactivate enzymes before continuing with a protocol.

Because the restriction enzymes provided to you will usually be at a concentration of 10 units/ μl , you will use 1 μl , and the amount of DNA you digest will not exceed 2 μg , all of your digestion reactions will involve the use of a considerable excess of enzyme over that actually needed.

Of course, unit values of other classes of enzymes must be determined differently. For example, the specification sheet for Boehringer Mannheim's Klenow enzyme defines a unit of its product as "...*the enzyme activity which incorporates 10nmol of total nucleotides into an acid-precipitable fraction in 30 min under assay conditions.*" You should consult the product catalogs of enzyme suppliers to see how the unit-activities of other enzymes are defined.

The prep staff of MCB 473 rarely dilutes enzyme, although this can be done if the appropriate buffers are used. The exception will be Perkin-Elmer *Sequenase*TM *Version 2.0*. This enzyme is an engineered version of wild-type T7 DNA polymerase, and is diluted with a special buffer that comes in the sequencing reagents kit.

A note concerning enzyme storage buffer. Enzyme storage buffer nearly always includes 50% glycerol. Unfortunately, a high concentration of glycerol (>10% v/v) in the reaction mixture may produce aberrant enzyme activity. Therefore, the maximum volume of an enzyme which may be added to a reaction is 1/5 of the total volume of the reaction. For example, a maximum of 2 μl undiluted enzyme can be added to a 10 μl total reaction volume.

General procedure for transferring enzymes to your reaction tubes.

1. The enzymes will be last thing you add to your reaction tubes. When you have assembled all other reaction components, bring your tubes to the common area where the Cryo-Safe box containing the enzyme is located.
2. Loosen the caps on your reaction tubes. Have your pipetman dialed to the correct volume. Attach a sterile tip securely.
3. Open the Cryo-Safe box and remove an aliquot of the enzyme needed in the reaction solution. Close the lid to the Cryo-Safe box. Open the enzyme tube and withdraw the correct volume for your reaction.
4. Return the tube with the enzyme to the Cryo-Safe box.

Because enzymes are stored in a viscous, 50% glycerol buffer, the enzyme solution tends to stick to the outside of pipet tips. To minimize the amount of enzyme accidentally removed in this manner, take care to place only the very end of the pipet tip into the enzyme solution.

5. Place the tip just into the reaction solution, transfer the enzyme, then flush all of the enzyme from the pipet tip by gently manipulating the plunger in and out. This procedure also helps mix the reaction solution. Do not create air bubbles, as they might cause the enzyme to denature.

IMPORTANT

- **Always minimize the amount of time that an aliquot of enzyme is out of cold storage.**
- **Never place an aliquot of enzyme unattended on the benchtop. Make your transfer, then immediately return the aliquot to cold storage.**

Appendix 2.9. DNA, Lambda Molecular Weight markers (*HindIII* digest)

0.5 µg/µl in TE buffer and 6X loading buffer

Preparation notes:

- If predigested Lambda phage DNA is purchased, the solution simply must be diluted appropriately with TE buffer and 6X loading buffer.
- If genomic Lambda DNA is purchased, it will have to be digested with *HindIII* restriction enzyme. The following reaction conditions have produced acceptable results:

200 µl Lambda DNA at 0.25 µg/µl in TE buffer (50 µg)
23 µl 10X *HindIII* restriction buffer
10 µl *HindIII* restriction enzyme (10 units/µl)

Incubate the digestion overnight at 37°C.

Analyze results in an agarose gel to confirm complete digestion of the DNA.

Dilute appropriately with 6X DNA loading buffer.

- Store in aliquots at 4°C.

- Complete digestion of Lambda-phage DNA with the *HindIII* restriction endonuclease results in eight fragments. These fragments are commonly run as size standards in an agarose gel with DNA of unknown size. The sizes of these eight restriction fragments are indicated below.

Fragment #	Number bp	log(bp)
1.....	23,130.....	4.364
2.....	9416.....	3.974
3.....	6557.....	3.817
4.....	4361.....	3.640
5.....	2322.....	3.366
6.....	2027.....	3.307
7.....	564.....	2.751
8.....	125.....	2.097

- Aliquots of lambda DNA MW marker solution with loading buffer are provided to you at a concentration of 0.1 $\mu\text{g}/\mu\text{l}$, ready to load in a gel. In those cases in which your unknown-length fragments are greater than 1000 bp in size, 1 μg of MW marker DNA should suffice to visualize clearly the useful size differences. If you believe that you may have smaller fragments, run up to 2 μg of MW marker to ensure your smallest fragments show brightly in your photograph.
- The loading buffer contains two dyes that run at predictable rates in an agarose gel submerged in 0.5X TBE running buffer. The *bromophenol blue* band runs at a rate equivalent to that of a 300 bp fragment of double-stranded DNA. This means that if the band runs to the end of the gel, then the smallest of the MW marker fragments has run off the gel. The *xylene cyanol FF* band runs at a rate equivalent to that of a 4000 bp fragment.