7

Making Reagents and Buffers

MUCH OF THE TIME spent in an experiment is not found in the experiment itself, but in preparation of the reagents and tools needed to do and analyze the experiment. The first thing a newcomer to the lab generally needs to do is to make a supply of buffers, so that the shelves above the lab bench won't look so newcomer-ishly empty, and you can start doing experiments. Buffer- and reagent-making is an activity that will continue throughout your time in the lab: Your experiments will depend on the quality of your reagents.

A *buffer* is a solution that does not change pH readily. Since each enzymatic reaction, each cell, and each extraction has a pH optimum, buffers have countless uses. They are used to wash cells, to cut DNA, to perform electrophoresis—any activity in which the structure and/or activity of biological material must be maintained. Some salt solutions, such as sodium chloride, do not need to be buffered, usually because they become part of a complex solution that will be buffered.

DETERMINING WHAT YOU NEED

If you are in any lab doing biomedical research, you will need certain reagents, no matter what your particular experiments will be. These are the first reagents you will make, and you can find out from other lab members exactly which ones to make before you start on experiments. Most of these chemicals and reagents will be stocked in the lab. Some of these reagents are listed in Chapter 3, and the recipes for some common reagents are listed later in this chapter.

After discussions with the P.I. and other lab members, and after your own readings and research, you will be given or will devise a protocol, a game plan detailing the logistics of the experiment. At least a day or so before the experiment you must ensure that all the buffers and reagents needed for the experiment will be made and autoclaved and ready for use. For more detail on following protocols, see Chapter 4.

Always work to a protocol!

1. **Go through the protocol for your experiment, and write down all the solutions that are used.** Record the name, the concentration (molarity or percentage), the pH, and the volume used in the experiment.
2. **Determine how much of each solution you should make.** If you know you won't do the experiment more than a couple of times (unlikely—most experiments are done multiple times), make the reasonable *minimum* volume. If the protocol calls for 10 ml of 1 M NaCl, plan on making 100 ml. And if 1–50 ml are used at a time, and you will do the experiment routinely, make 500 ml or a liter. However, if the material is expensive or unstable, make only what you require for one experiment.
3. **Decide whether you should make a "straight" solution or a concentrated solution.** Many complex buffers are made at a 5, 10, 20, or 50X concentration and are diluted to the desired concentration at the time of the experiment.

> For example, Tris/glycine SDS buffer (Laemmli running buffer), commonly used for protein gel electrophoresis, is often made at a **10X concentration.** Since the working concentration of 1X is 25 mM Tris, 192 mM glycine, and 0.1% SDS, 10X would be 250 mM Tris, 1.920 M glycine, and 1% SDS. At the time of the experiment, the 10X would be diluted 1:10 in water.

The limit in concentrated buffer stocks is the solubility of the buffer, for the salts will come out of solution if they are too concentrated. This isn't a disaster, but you aren't exactly saving time if you have to warm and mix the buffer until it is back in solution. Check with someone in the lab or with the manufacturer if you aren't sure how concentrated you can make a particular buffer, but you are probably safe with 5X. Concentrated buffers are usually stored at room temperature to avoid precipitation.

Other buffers commonly stored as concentrated stocks are **PBS** (10X), **SSC** (10 or 20X), and **TAE** (10X). Many companies also offer premade concentrated buffers, so check into the lab custom before you fire up the buffer factory. These are expensive, but they make life very easy.

Keep one or two bottles of autoclaved double-distilled water in the refrigerator, to be used to make buffers from concentrated stocks.

4. **Find out if the chemicals you need are available in the lab.** Okay, let's say that your protocol calls for a 1 M NaCl solution. Before you get all geared up, find out if the lab has the NaCl, or if it will have to be borrowed or ordered. Commonly used materials such as NaCl are usually in stock in the lab, and generally, they are available for common use. *But actually open the bottle and check it first,* before getting out the glassware—someone may have left only half a gram, and neglected to order more ("But I didn't use it up! There was some left in the bottle!").

5. **Get what you need if it isn't in stock.** Check with the person who orders, or with the purchasing department or company, to see if the replacement has been ordered. If you are in a huge hurry, the NaCl is really gone, and the newly ordered NaCl will take 3 days to get to you, you have several options. You can go down the hall and borrow some NaCl, with the promise to return what you took when your delivery comes in. Politics will determine whether you actually should return it, and whether, in fact, you should borrow it from that particular lab in the first place!

   An easier solution is to get some concentrated stock from a coworker. Researchers often have a bottle of 5 M NaCl on their shelf. NaCl is a component of many complex buffers used in molecular biology, and 5 M can be easily diluted to the molarity needed. Figure out the minimum you need to get by until you can make your own stock, and *ask* to "borrow" that amount. You probably won't need to return the 20 ml—most people won't even want to take a buffer from a new lab member. But make the offer.

Safety

Before you make up any reagent or buffer, *you* must check to see what, if any, hazards are associated with handling the material. No one else will do this for you. Some hazards are well known and well advertised: For example, no one in a lab would let you be too casual with ethidium bromide. **But because no one says anything to you, you cannot assume a material is safe to handle freely.**

- **Know the composition and associated hazards of every material you work with.**

  1. Check the Materials Safety Data Sheet (MSDS). The MSDS must be included with every chemical purchased and should be kept on file in the lab. It is a

description of the composition and properties of the chemical, and it lists the hazards of the chemical and the ways in which the hazard should be dealt with.

2. Look for a hazardous material classification label. These labels (which follow DOT and NFPA recommendations and are approved by OSHA) describe the health hazard, fire hazard, reactivity, and specific hazards of the material.
3. Read the bottle carefully for warnings. Not all materials will have the hazardous materials classification label, but may still carry warnings, sometimes in small print.
4. If you see no warnings, look the material up in the Merck Index.
5. If the label is in another language, or you can't find a listing for it, consult the manufacturer or your EHS Office.

- **Follow the recommendations for handling** that you find. Follow the precautions needed for the particular material you will be working with. Your lab should have a chart or a list of the safety precautions needed for classes of dangerous reagents. If not, call EHS.

1. **Gloves.** Be sure you are wearing the correct gloves for the job. Latex or polyvinylchloride gloves should always be worn when making up any reagent, and these are good protection against most powdered reagents. But these offer no protection against, for example, phenol. For organic solvents you will need chemical-resistant rubber or neoprene gloves. If you have an allergy to latex, speak with EHS.

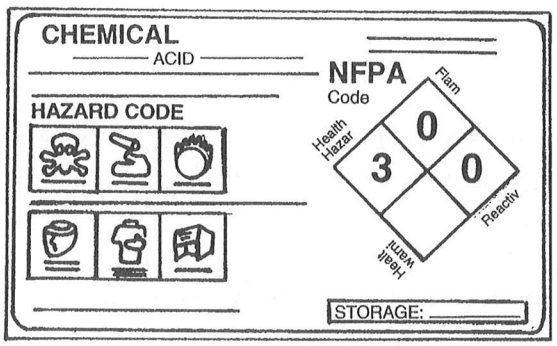


FIGURE 1.

Hazardous materials classification label. The specific hazard is listed in the lower left quadrant. Reactivity, health, and fire hazards are rated with increasing numbers, with 0 posing no hazard and 4 being very dangerous.

Some people have an allergy to the chemicals used to manufacture latex. This allergy usually shows itself as chronic dermatitis, and gets worse over time. If you develop a rash on your hands that is linked to the use of latex gloves, stop using latex gloves. Consult with EHS about a substitute kind of glove. The use of powder-free latex gloves may help prevent latex allergy.

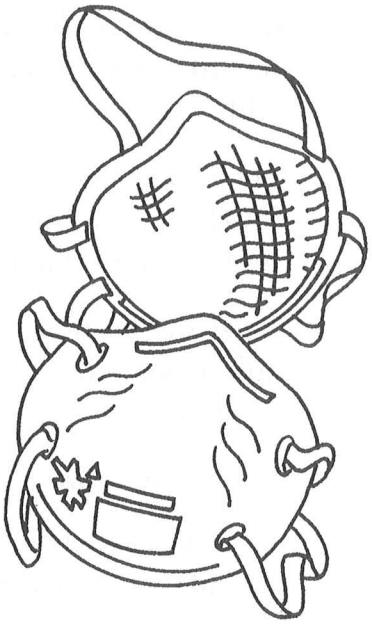


FIGURE 2.
Dust-mist mask.

2. **Eye protection.** For any potential splatters, aerosols, combustible, or breaking glass, wear plastic glasses or goggles.
3. **Hood.** Volatile substances should always be made up in a chemical hood. Be sure that the hood is certified for use.
4. **Mask.** Some powders, such as SDS, can cause damage to the nasal passages. A simple dust-mist mask or a surgical mask will protect you. But other substances, particularly if volatile, may require the use of respirator masks. Don't feel you are going overboard because no one else is taking precautions!
5. **Labels.** Label your bottle with the hazard associated with it. You may not remember, or it may be necessary for someone else to use your reagents.

Hazardous reagents

The following common reagents are potentially dangerous, and should be handled with more than the usual care. Wear gloves. Follow the cautions carefully—don't cut corners here!

Acrylamide. This neurotoxin is used for protein and sequencing gels. Wear a mask.

Ethidium bromide. EtBr, a mutagen, intercalates in DNA and is used to label nucleic acid. It is toxic, and the solid is irritating to skin and mucous membranes.

Phenol. Phenol is highly corrosive and can burn skin. Use a fume hood when doing extractions with or preparing phenol.

Phenylmethyl-sulfonate fluoride (PMSF). Used to inhibit proteinases during protein isolations, PMSF can be fatal if swallowed or absorbed through the skin.

Sodium dodecyl sulfate (SDS). This detergent is a burning powder and is extremely irritating, especially to nasal passages. It is light and fluffy; measure gently, and wear a mask.

- **Know how to operate the fume hood.**

  1. *Turn the fume hood on,* and be sure it is exhausting; you will be able to hear and feel it. No air conditioners or fans should be working near the fume hood, as this will interfere with the air currents within the hood.
  2. *The sash must be below chin level*! Lower the sash to the level indicated on the front of the hood. This level is determined by EHS when the hood is certified. The lower the sash, the more containment there is: If you are working with high-speed aerosols, the sash must be all the way down.
  3. *Work at least 6 inches within the hood.*
  4. *Secure papers and other lightweight materials.* They could become trapped in the exhaust system.
  5. *Do not block the rear exhaust slot* or the space between the tapered metal front lip and the work surface.

Which Water to Use?

Water is an important component of solutions; it is not merely something to dissolve things in. Water quality, composition, and pH can drastically affect experiments, and everyone knows stories of experiments that stopped working after the water source was changed.

> *Assume the solvent is water, unless it is stated otherwise.*

There are several broad classifications of water quality, in order of increasing purity:

1. **Tap water.** Tap water quality varies wildly, its mineral and impurity composition dependent on the geographical location, the pipes, or the amount of rainfall that spring. Some minerals can inhibit enzyme reactions, or prevent cell growth: At the very least, tap water is a nonreproducible variable in the experimental equation and should not be used. It is used only for washing glassware and other laboratory equipment.
2. **Laboratory grade water.** Laboratory grade water has been pretreated by reverse osmosis or distillation. It is adequate for making buffers.
3. **Reagent grade water.** Through distillation or deionization, laboratory grade water is further purified, and can be used for cell culture and biochemical reagents, as well as for buffers.

4. **Ultrapure reagent grade water.** Some labs have particular and stringent water requirements. For example, water treated by ultrafiltration to be sure it is endotoxin-free (endotoxin is a ubiquitous bacterial cell wall component) may be required for certain cell cultures.

Distillation is still the most commonly used method of water purification in labs. Reagent grade water is also made with a combination of units capable of *reverse osmosis* (the water is pushed through a semipermeable membrane through which impurities can't pass) and *deionization* (the removal of ions by passage through anion and/or cation removal resins). *Ultrafiltration, adsorption, and ultraviolet oxidation* are used in some facilities to further purify the water. Ultrapure reagent grade water can also be purchased from scientific and hospital vendors.

Many institutions have "house-distilled" water, which is distilled at a central location and is dispensed through a tap near the sink. This is laboratory grade water, and can be used for buffers: However, it should not be used for cell culture or buffers that will be used in enzymatic reactions. Its most common use is rinsing glassware after washing with tap water.

Always keep ready a liter or two of autoclaved, reagent grade water, to use for making diluted buffers from concentrated stock buffers.

If reagent grade water is plentiful, use it for *all* of your buffers.

Plasticware and Glassware

To make reagents, you will need **graduated cylinders, beakers, volumetric and Erlenmeyer flasks.** These are reusable laboratory supplies and will be stored in a closed or sheltered cabinet to keep dust off.

Some labs use glass, some plastic; most use a mix. Glass is clear, but breaks during washing. Plastic is hardy, but some types, particularly after being autoclaved, become tinted or opaque, and no meniscus forms in some plastic graduated cylinders. Use whatever is there, as it all works.

Glassware to use for mixing

Beaker. Use for most solid-to-solvent mixing. Plastic or glass is fine.
Erlenmeyer flask. Use if a gas will be generated.
Test tube. Use for volumes less than 10 ml.
Volumetric flask. Use for two liquids, but mix by swirling, not with a stir bar.

Glassware not to use for mixing

Graduated cylinder. Resist the temptation to dump the solids directly into the cylinder. The base of the cylinder is usually too high for a magnetic stir plate to work, and covering the top with parafilm to shake the cylinder can turn into an extremely messy and potentially dangerous procedure.

Plasticware and glassware (both sometimes referred to as glassware) are usually **autoclaved** before being placed back on the shelves after washing: Foil closing may be the only indication that the item is sterile.

Don't hoard glassware at your bench. If the lab starts to run out of graduated cylinders in the afternoon, order more.

There is probably a motley selection of bottles for **reagent and buffer storage.** Some were bottles purchased just to hold buffers, but many are recycled bottles that had been purchased for their contents.

Glassware to use for buffer storage

Glass bottles with lined caps. Good for media.

Cell culture medium bottles. Premade culture medium and buffers are purchased in these bottles. They can be reused indefinitely.

Narrow mouth bottles. Excellent for buffers.

Glassware not to use for buffer storage

Wide-mouthed jars. The contents get contaminated easily.

Amber containers. Use these only if you have a light-sensitive reagent.

Poorly threaded capped bottles. Contamination will be a problem. Also, a bottle without a good-fitting cap probably wasn't made to last.

Any container that looks chipped, scratched, or damaged in any way. Damaged glass is much more susceptible to breakage during autoclaving or even changes in temperature.

CALCULATING WHAT YOU NEED

The protocol or recipe will describe each solution either as a molar solution or a percent solution.

Molar (M) Solutions

To make a solution of a particular molarity from scratch, you need to know what volume you require and the formula molecular weight of the substance.

To determine the formula molecular weight

- If the name is given, and you don't know the formula, check the *Merck Index* or one of the on-line chemistry dictionaries.
- You could determine the molecular weight (MW) from the periodic table (for example, for sodium chloride, Na is 22.98, Cl is 35.45, so the formula molecular weight of the substance is 58.43).

The Merck Index also contains information on solubility and stability.

- The best way to get the molecular weight is from the bottle from which you will take the substance. This way, you can be sure that the substance is exactly what you need. If the molecular weight is obscured on the label, check the catalog or call the technical services of the company that made the material.

  Read the label carefully and be sure you are getting the formula weight of the substance you need. Be sure to look at the formula, not just the name, as there are differences that could be missed, and will make a difference. In particular, check for:

  Salt or acid/base form. The major problem in using a salt vs the acid is in the difference in pH. For example, Tris, a common buffer, comes as Tris HCl, or as Tris base, and a 1 M solution will vary in pH by several units.

  Anhydrous or hydrated. Extra water in the material won't usually be a problem, as long as the water is calculated as part of the formula weight.

To calculate molarity

There is only one calculation you need

Gram molecular weight of known is to 1 M as unknown grams is to desired molarity.

MW: molarity as *x* : desired molarity

Multiply the MW by the desired molarity, and divide by the molarity of the known. This presumes a desired volume of 1000 ml. (Always think of 1 liter as 1000 ml.)

Example: 1 liter (1000 ml) of 1 molar NaCl
1 molar (or 1 M) is easy, since 1 mole equals the gram molecular weight of the substance. Thus, since the formula weight of NaCl is 58.43, you need 58.43 g in one liter of water. No calculations.

Example: 1 liter (1000 ml) of 5 M NaCl
58.43:1 as *x* :5
58.43 X 5 /1 = 292.15 g

Example: 300 ml of 1 molar NaCl
If you only need 300 ml of 1 M NaCl, reduce the grams the same proportion as you reduce the volume of water.
58.43 g :1000 ml as *x*: 300. (58.43 g is to 1000 ml as the unknown g are to 300 ml) Multiply 58.43 by 300, and divide the product by 1000. The answer, 17.5 g, should be dissolved in 300 ml to give 300 ml of 1 M NaCl.

Example: 400 ml of a 0.25 molar solution of NaCl
For different volumes and different molarities, it might be easiest to do the cal-

culations in multiple steps, rather than trying to figure out the grand calculation. So, for 400 ml of a 0.25 M solution of NaCl:

1. Figure out how many grams you need for a liter of a 0.25 M solution.
   58.43 g : 1000 ml as *x* g : 250
   58.43 X 250 = 14607.5, divided by 1000 = 14.6.
   For 1000 ml of a 0.25 M solution, you need 14.6 g of NaCl.

2. Calculate the grams needed for 400 ml of 0.25 M.
   14.6 : 1000 ml as *x* g : 400 ml
   400 X 14.6 = 5840, 5840 divided by ~~400~~ 1000 = 5.84.
   For 400 ml of a 0.25 M solution, you need 5.84 g of NaCl.

Example: 10 liters of a 5.0 M solution of NaCl

1. Figure out how many grams you need for a liter of a 5.0 M solution.
   58.43 :1000 as *x* : 5000
   58.43 X 5000 = 292150, divided by 1000 = 292.15
   For 1 liter of a 5.0 M solution, you need 292.15 g of NaCl.

2. Calculate the grams needed for 10 liters of 5.0 M.
   292.15 g: 1 liter as *x* g : 10 liters 292.15 g X 10, divided by 1 = 2921.5

The calculations will become instinctive: For 10 liters of a 5.0 M solution of NaCl, it will be fairly obvious that you only need to multiply 58.43 X 50 to arrive at the grams needed. But work through the entire calculation until you have a feel for the numbers.

Record your calculations in your notebook. Even for experienced lab personnel, most mistakes in buffer making occur at this step, and it is much easier to track down the reasons for nonworking experiments if you record everything.

Normal (N) solutions

Molarity is defined as the number of gram molecular weights (moles) per liter of solvent. Normality is defined as *a gram molecular weight of a dissolved substance divided by the hydrogen equivalents per liter of solution.* Molarity sees the world as bases and their salts; normality sees the world in terms of acids.

What does this mean? For most chemicals, the value is the same for molarity and normality. 1 M HCl is the same as 1 N HCl. But when working with any divalent or trivalent species (sulfate, phosphate, carbonate, etc.), it will make a difference. For example, for concentrated sulfuric acid (H_2SO_4), there are two equivalents of H^+ per molecule: the concentration of sulfuric acid is 18 M but 36 N. If you multiply the molarity of a solution by the number of moles of that substance that occur in a chemical equation, you have the normality.

Percent Solutions

Percent solutions are based on 100 ml (or, occasionally, 100 grams). Almost all the percent solutions you will calculate will be (w/v) solutions, in which the weight in grams of the powder is mixed with the volume in milliliters of the water.

Three ways of expressing concentration in the form of a percent

- *Percent weight by volume (w/v). Grams of solute per 100 ml of solvent.* Generally, a percent solution is considered to be weight/volume (w/v) and w/v is assumed if not designated otherwise.

  Example: 20% NaCl. For a 20% NaCl solution, dissolve 20 g of NaCl in 70 ml of water, and bring the volume up to 100 ml.

- *Percent by volume (v/v). ml of solute per 100 ml of solution.* This is commonly used when diluting a concentrated stock.

  Example: 1% SDS solution. Everyone has a 10% SDS solution (w/v) on his or her shelf. Dilute 1:10 by adding 10 ml of 10% SDS to 90 ml of water, top up to 100 ml.

- *Percent by weight (w/w). Grams of solute per 100 g of solvent.* This is never used in making standard buffer or salt solutions, but is found in protocols for making gradient solutions.

  Example: 10% (w/w) sucrose solution. Weigh 10 g of sucrose, and add to 90 g of water. Theoretically, a ml of water equals a gram, so add 90 ml of water to the beaker in which you are weighing the water.

Dilution of Stock Buffers

Once you have really set your roots down, most of your working lab buffers will be made by simply diluting your stock buffers.

Do all manipulations as cleanly as possible. It is not necessary to flame the bottles, if they are not used for cell maintenance. Use sterile pipets, and replace caps immediately.

When you are diluting sterile buffers, use aseptic technique (Chapter 9). Use only sterile pipets, and replace all caps immediately.

To dilute the stock buffers:

$$C1 \times V1 = C2 \times V2$$

C1 = the concentration before dilution
V1 = the volume before dilution
C2 = the concentration after dilution
V2 = the volume after dilution

For example, if you need 100 ml of 1.0 M and have a 5 M stock of NaCl:
5.0 M x V1 = 1.0 M x 100 ml
V1 = 20 ml
Add 20 ml of 5.0 M to 80 ml of distilled water for a volume of 100 ml.
Either use sterile 5 M and sterile distilled water, or sterilize the final 100 ml by autoclaving or filtration through a 0.2 micron filter.

Dilutions are denoted differently from lab to lab. Generally, adding 1 ml of concentrate to 9 ml of diluent is written 1:10. This notation is confusing, as adding 1 ml of concentrate to 1 ml of diluent is written either as 1:1 or 1:2. It would be better to use 1/10 for 1 in 10 and 1:10 as 1 and 10.

Serial dilutions

Serial dilutions are the easiest way to **reduce the concentration** of a reagent, bacterial or cell sample, standard, or anything you want to use to test increasing or decreasing concentrations. Progressive dilutions are made, yielding concentrations differing from successive dilutions by the same factor.

Stock solution. The stock solution is the concentrated solution.

Dilution factor. Before setting up the dilutions, you must consult a protocol or the literature to decide on the dilutions you will need. 1:10 or 1:2 dilutions are the most commonly done. The serial dilution you choose will be based on how close in concentration you need your working dilutions to be. To survey for an effect, the dilutions will generally be spread, and you might use a factor of 1:10 or 1:100. To pinpoint the effectiveness of a particular concentration, a 1:2 dilution series would be more useful.

When considering the concentrations you will need, don't forget to take into account the dilution that occurs when you add the substance from the dilutions into the experimental containers.

Diluting medium. Do your dilutions in the medium or buffer you will actually be using in the final experiment. Don't just use water or the same solvent the stock solution is made of, unless they are what you require.

Oftentimes, the stock solution is made up in a solvent in which the substance could be dissolved but which is harmful for cells or for an enzymatic reaction. Dilution of the substance also serves to dilute the solvent to a nonharmful concentration.

Volume. The volume you use for dilutions and the size of the tubes are dependent on how concentrated you need your final volumes. Don't do dilutions in microfuge tubes if you will need to add 3 ml of the

dilutions to arrive at your final concentrations. Likewise, don't use 15-ml tubes if you are diluting 1-ml volumes.

Tubes. Set the tubes up, and add the diluting medium. Write the dilution on each tube, and/or record all dilutions in your lab notebook. Orient the test tube rack in the same way, every time, so you know which way you are working. Always pipet from left to right, to achieve decreasing concentrations. Set the tubes up to facilitate this.

The carryover from high to low concentration tubes can erratically make the actual concentration higher than you have calculated it to be. This is not the time to conserve pipets.

Pipets. Use a pipet or pipettor appropriate for the volume being transferred. Discard pipets and tips after you pipet into each tube!

Adding the dilutions to the working container. Add the required volume to your experiment or assay tubes in increasing concentrations. You may use the same pipet only if you are adding samples from increasing concentration dilution tubes, where the carryover effect will be negligible.

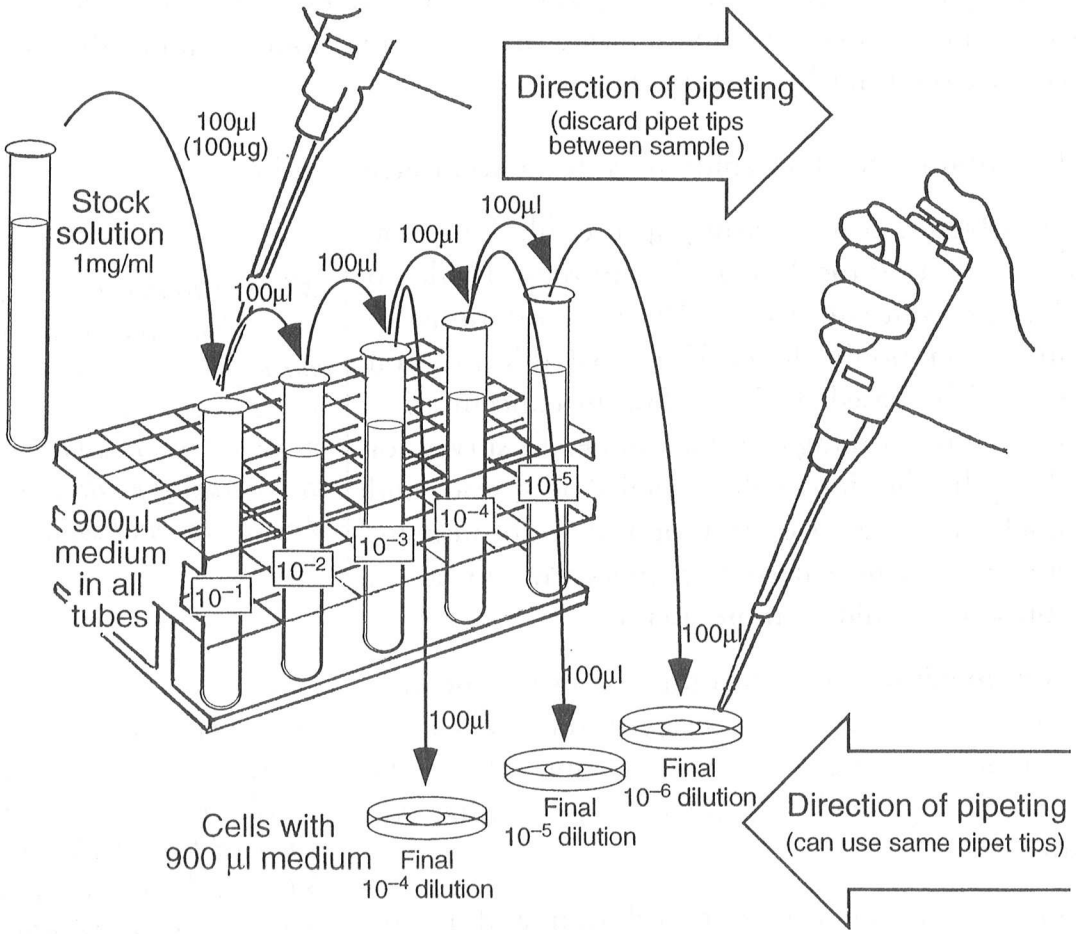


FIGURE 3.

An example of a serial dilution used to dilute a 1 mg/ml stock solution to achieve final concentrations of 100, 10, and 1 ng.

Recipes

TABLE 1. Concentrations of Acids and Bases: Common Commercial Strengths

Substance	Formula	Molecular weight	Moles/ liter	Grams/ liter	Percentage by wieght	Milliliters/liter to prepare 1 M solution
Acetic acid, glacial	CH_3COOH	60.05	17.4	1045	99.5	57.5
Acetic acid		60.05	6.27	376	36	159.5
Formic acid	HCOOH	46.02	23.4	1080	90	42.7
Hydrochloric acid	HCl	36.5	11.6	424	36	86.2
			2.9	105	10	344.8
Nitric acid	HNO_3	63.02	15.99	1008	71	62.5
			14.9	938	67	67.1
			13.3	837	61	75.2
Perchloric acid	$HClO_4$	100.5	11.65	1172	70	85.8
			9.2	923	60	108.7
Phosphoric acid	H_3PO_4	80.0	18.1	1445	85	55.2
Sulfuric acid	H_2SO_4	98.1	18.0	1766	96	55.6
Ammonium hydroxide	NH_4OH	35.0	14.8	251	28	67.6
Potassium hydroxide	KOH	56.1	13.5	757	50	74.1
			1.94	109	10	515.5
Sodium hydroxide	NaOH	40.0	19.1	763	50	52.4
			2.75	111	10	363.6

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

TABLE 2. Approximate pH Values for Various Concentrations of Stock Solutions

Substance	1 N	0.1 N	0.01 N	0.001 N
Acetic acid	2.4	2.9	3.4	3.9
Hydrochloric acid	0.10	1.07	2.02	3.01
Sulfuric acid	0.3	1.2	2.1	
Citric acid		2.1	2.6	
Ammonium hydroxide	11.8	11.3	10.8	10.3
Sodium hydroxide	14.05	13.07	12.12	11.13
Sodium bicarbonate		8.4		
Sodium carbonate		11.5	11.0	

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

TABLE 3. pK_a Values of Commonly Used Buffers

Buffer	Molecular weight	pK_a	Buffering range
Tris[a]	121.1	8.08	7.1–8.9
HEPES[b]	238.3	7.47	7.2–8.2
MOPS[c]	209.3	7.15	6.6–7.8
PIPES[d]	304.3	6.76	6.2–7.3
MES[e]	195.2	6.09	5.4–6.8

(Reprinted, with permission, from Sambrook et al. 1989.)
[a]Tris(hydroxymethyl)aminomethane
[b]*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid
[c]3-(*N*-morpholino)propanesulfonic acid
[d]Piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)
[e](*N*-morpholino)ethanesulfonic acid

TABLE 4. Preparation of Stock Solutions

Solution	Method of preparation
10 M Ammonium acetate	Dissolve 770 g of ammonium acetate in 800 ml of H_2O. Adjust the volume to 1 liter with H_2O. Sterilize by filtration.
10% Ammonium persulfate	To 1 g of ammonium persulfate, add H_2O to 10 ml. The solution may be stored for several weeks at 4°C.
1 M $CaCl_2$	Dissolve 44 g of $CaCl_2 \cdot 6H_2O$ in 200 ml of pure H_2O (Milli-Q or equivalent). Sterile the solution by passage through a 0.22-micron filter.
1 M Dithiothreitol (DTT)	Dissolve 3.09 g of DTT in 20 ml of 0.01 M sodium acetate (pH 5.2). Sterile by filtration. Dispense into 1-ml aliquots and store at –20°C.
0.5 M EDTA (pH 8.0)	Add 186.1 g of disodium ethylenediamintetra-acetate$\cdot 2H_2O$ to 800 ml of H_2O. Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH (~20 g of NaOH pellets). Dispense into aliquots and sterilize by autoclaving.
Ethidium bromide (10 mg/ml)	Add 1 g of ethidium bromide to 100 ml of H_2O. Stir on a magnetic stirrer for several hours to ensure that the dye has dissolved. Wrap the container in aluminum foil or transfer the solution to a dark bottle and store at room temperature. **Caution:** Ethidium bromide is a powerful mutagen and is moderately toxic. Gloves should be worn when working with solutions that contain this dye, and a mask should be worn when weighing it out.
1 M $MgCl_2$	Dissolve 203.3 g of $MgCl_2 \cdot 6H_2O$ in 800 ml of H_2O. Adjust the volume to 1 liter with H_2O. Dispense into aliquots and sterilize by autoclaving. $MgCl_2$ is extremely hygroscopic. Buy small bottles (e.g., 100 g) and do not store opened bottles for long periods of time.
β-Mercaptoethanol (BME)	Usually obtained as a 14.4 M solution. Store in a dark bottle at 4°C.
Phenol chloroform	Mix equal amounts of phenol and chloroform. Equilibrate the mixture by extracting several times with 0.1 M Tris•HCl (pH 7.6). Store the equilibrated mixture under an equal volume of 0.01 M Tris•HCl (pH 7.6) at 4°C in dark glass bottles. **Caution:** Phenol is highly corrosive and can cause severe burns. Wear gloves, protective clothing, and safety glasses when handling phenol. All manipulations should be carried out in a chemical hood. Any areas of skin that come into contact with phenol should be rinsed with a large volume of water and washed with soap and water. Do *not* use ethanol.
10 mM Phenylmethyl-sulfonyl fluoride (PMSF)	Dissolve PMSF in isopropanol at a concentration of 1.74 mg/ml (10 mM). Divide the solution into aliquots and store at –20°C. If necessary, stock solutions can be prepared in concentrations as high as 17.4 mg/ml (100 mM). **Caution:** PMSF is extremely destructive to the mucous membranes of the respiratory tract, the eyes, and skin. It may be fatal if inhaled, swallowed, or absorbed through the skin. In case of contact, immediately flush eyes or skin with copious amounts of water. Discard contaminated clothing.
Phosphate-buffered saline (PBS)	Dissolve 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 in 800 ml of distilled H_2O. Adjust the pH to 7.4 with HCl. Add H_2O to 1 liter. Dispense the solution into aliquots and sterilize them by autoclaving for 20 minutes at 15 lb/sq. in. on liquid cycle.
1 M Potassium acetate (pH 7.5)	Dissolve 9.82 g of potassium acetate in 90 ml of pure H_2O (Milli-Q or equivalent). Adjust the pH to 7.5 with 2 M acetic acid. Add pure H_2O to 100 ml. Divide the solution into aliquots and store them at –20°C.

(*continued on following page*)

TABLE 4. (*continued*)

Solution	Method of preparation
Potassium acetate (for alkaline lysis)	To 60 ml of 5 M potassium acetate, add 11.5 ml of glacial acetic acid and 28.5 ml of H_2O. The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate.
3 M Sodium acetate (pH 5.2 and pH 7.0)	Dissolve 408.1 g of sodium acetate•H_2O in 800 ml of H_2O. Adjust the pH to 5.2 with glacial acetic acid or adjust the pH to 7.0 with dilute acetic acid. Adjust the volume to 1 liter with H_2O. Dispense into aliquots and sterilize by autoclaving.
5 M NaCl	Dissolve 292.2 g of NaCl in 800 ml of H_2O. Adjust the volume to 1 liter with H_2O. Dispense into aliquots and sterilize by autoclaving.
10% Sodium dodecyl sulfate (SDS) (also called sodium lauryl sulfate)	Dissolve 100 g of electrophoresis-grade SDS in 900 ml of H_2O. Heat to 68°C to assist dissolution. Adjust the pH to 7.2 by adding a few drops of concentrated HCl. Adjust the volume to 1 liter with H_2O. Dispense into aliquots. Wear a mask when weighing SDS and wipe down the weighing area and balance after use because the fine crystals of SDS disperse easily. There is to need to sterilize 10% SDS.
20X SSC (3.0 M Nacl and 0.3 M sodium citrate)	Dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 ml of H_2O. Adjust the pH to 7.0 with a few drops of a 10 N solution of NaOH. Adjust the volume to 1 liter with H_2O. Dispense into aliquots. Sterilize by autoclaving.
20X SSPE	Dissolve 175.3 g of NaCl, 27.6 g of NaH_2PO_4•H_2O and 7.4 g of EDTA in 800 ml of H_2O. Adjust the pH to 7.4 with NaOH (~6.5 ml of a 10 N solution). Adjust the volume to 1 liter with H_2O. Dispense into aliquots. Sterilize by autoclaving.
Trichloroacetic acid (TCA) 100% solution	To a bottle containing 500 g of TCA, add 227 ml of H_2O. The resulting solution will contain 100% (w/v) TCA.
1 M Tris	Dissolve 121.1 g of Tris base in 800 ml of H_2O. Adjust the pH to the desired value by adding concentrated HCl **pH** — **HCl** 7.4 — 70 ml 7.6 — 60 ml 8.0 — 42 ml If the 1 M solution has a yellow color, discard it and obtain better quality Tris. Allow the solution to cool to room temperature before making final adjustments to the pH. Adjust the volume of the solution to 1 liter with H_2O. Dispense into aliquots and sterilize by autoclaving. The pH of Tris solutions is temperature-dependent and decreases approximately 0.03 pH units for each 1°C increase in temperature. For example, a 0.05 M solution has pH values of 9.5, 8.9, and 8.6 at 5°C, 25°C, and 37°C, respectively.
50X Tris-Acetate-EDTA buffer	Dissolve 242 g of Tris base in 500 ml of H_2O. Add 100 ml of 0.5 M EDTA (pH 8.0). Add 57.1 ml of glacial acetic acid. Adjust volume to 1 liter with H_2O and sterilize by autoclaving.
Tris-buffered saline (TBS) (25 mM Tris)	Dissolve 8 g of NaCl, 0.2 g of KCl, and 3 g of Tris base in 800 ml of distilled H_2O. Add 0.015 g of phenol red and adjust the pH to 7.4 with HCl. Add distilled H_2O to 1 liter. Dispense the solution into aliquots and sterilize them by autoclaving for 20 minutes at 15 lb/sq. in. on liquid cycle. Store at room temperature.

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

PROTOCOL

Preparation of Phenol and Phenol Chloroform (Reprinted, with permission, from Sambrook et al. 1989)

Phenol

Most batches of commercial liquified phenol are clear and colorless and can be used in molecular cloning without redistillation. Occasionally, batches of liquified phenol are pink or yellow, and these should be rejected and returned to the manufacturer. Crystalline phenol is not recommended because it must be redistilled at 160°C to remove oxidation products, such as quinones, that cause the breakdown of phosphodiester bonds or cause cross-linking of RNA and DNA.

Caution: Phenol is highly corrosive and can cause severe burns. Wear gloves, protective clothing, and safety glasses when handling phenol. All manipulations should be carried out in a chemical hood. Any areas of skin that come into contact with phenol should be rinsed with a large volume of water and washed with soap and water. Do *not* use ethanol.

Procedure

Equilibration of phenol

Before use, phenol must be equilibrated to a pH >7.8 because DNA will partition into the organic phase at acid pH.

1. Liquified phenol should be stored at −20°C. As needed, remove the phenol from the freezer, allow it to warm to room temperature, and then melt it at 68°C. Add hydroxyquinoline to a final concentration of 0.1%. This compound is an antioxidant, a partial inhibitor of RNase, and a weak chelator of metal ions (Kirby 1956). In addition, its yellow color provides a convenient way to identify the organic phase.
2. To the melted phenol, add an equal volume of buffer (usually 0.5 M Tris•HCl [pH 8.0] at room temperature). Stir the mixture on a magnetic stirrer for 15 minutes, and then turn off the stirrer. When the two phases have separated, aspirate as much as possible of the upper (aqueous) phase using a glass pipet attached to a vacuum line equipped with traps.

3. Add an equal volume of 0.1 M Tris•HCl (pH 8.0) to the phenol. Stir the mixture on a magnetic stirrer for 15 minutes, and then turn off the stirrer. Remove the upper aqueous phase as described in step 2. Repeat the extractions until the pH of the phenolic phase is >7.8 (as measured with pH paper).
4. After the phenol is equilibrated and the final aqueous phase has been removed, add 0.1 volume of 0.1 M Tris•HCl (pH 8.0) containing 0.2% β-mercaptoethanol. The phenol solution may be stored in this form under 100 mM Tris•HCl (pH 8.0) in a light-tight bottle at 4°C for periods of up to 1 month.

Phenol:Chloroform:Isoamyl Alcohol (25:24:1)

A mixture consisting of equal parts of equilibrated phenol and chloroform isoamyl alcohol (24:1) is frequently used to remove proteins from preparations of nucleic acids. The chloroform denatures proteins and facilitates the separation of the aqueous and organic phases, and the isoamyl alcohol reduces foaming during extraction.

Neither chloroform nor isoamyl alcohol requires treatment before use. The phenol:chloroform:isoamyl alcohol mixture may be stored under 100 mM Tris•HCl (pH 8.0) in a light-tight bottle at 4°C for periods of up to 1 month.

WEIGHING AND MIXING

Weighing out ingredients to make a buffer isn't one of the hardest things you will do, but it is one of the most important. Gather *everything* you need before you start, so you don't have to leave your powder uncovered on the balance while you frantically search for a clean beaker.

Never weigh a sample directly on the balance pan.

You will need

Weighing paper (for solids under a gram and smaller than a golf ball) or

Weighing boats. These come in several sizes, so take one larger than you need. A *small beaker* can be used for liquids or a large amount of powder.

Spatulas. Spatulas also come in several sizes, in reusable metal and disposable plastic, and you should use a large one for large amounts, small ones for small amounts. You can't make a mistake, you can only have a slightly awkward time.

A clean graduated cylinder of a volume as close to the target volume as you can get. Err on the side of a cylinder larger than the desired volume, rather than measuring multiple times from a too small cylinder.

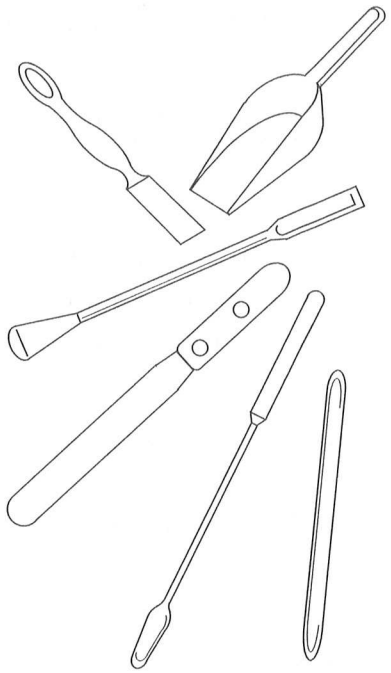


FIGURE 4.

Weighing utensils are known by various names in different labs and companies. Spatula, microspatula, scoop, and spoon are some of the contenders. Most labs have one name for all the utensils, but you will be understood with any one term.

Beaker or Erlenmeyer flask, whatever you will make the buffer in. Choose a size that will allow room for mixing, and for pH-ing: For a 500-ml volume of solution, use a 1-liter beaker.

Magnetic stir plate and stir bar. The magnetic stir plate does not need to have a heater. The stir bar should be the largest that will rotate freely in the beaker or Erlenmeyer flask.

Don't use a plastic beaker or flask on a hot plate!

Place to dispose of spatulas, magnetic stir bars. This is lab dependent—just look to see whether they are washed immediately, or placed somewhere for washing.

Kimwipes. This is lab tissue paper, used for delicate wiping and blotting.

Distilled water. Your solvent is assumed to be water. Use filter-purified or glass-distilled water. Do not use tap water.

Weighing

1. Put on surgical gloves. In general, this protects you and protects the contents of the jars you are dipping into. Gloves are expensive, and if you must limit glove use, put them on to protect yourself from potentially harmful substances.
2. Read the bottle, and see if you need to wear a mask or heavyweight gloves. Many substances are, at the least, powdery, and no matter how careful you are, may form

a little duststorm and fly into your nasal passages. Others are blatantly toxic.

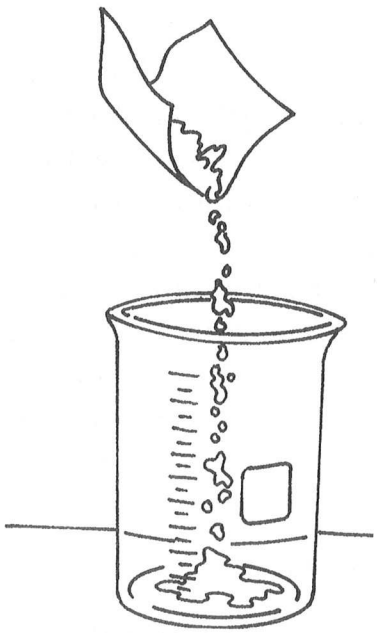
3. Turn on the balance. Depending on the balance, and the lab, some balances are left on all day, perhaps on standby. Most are not. If the balance isn't clean, clean it before you weigh anything.

4. Tare the weigh paper/boat. Place it on the balance, and push the tare or weigh button. The scale should then read zero.

5. Weigh your material. With the spatula, remove a small amount of material from the tilted open jar, and place it on the weigh boat/paper. Once you have a feel for what amount weighs how much, you can move a bit faster. As you get closer to the desired weight, add less material. Resist the temptation to pour from the jar, until you know the feel and properties of an inert substance and what will or will not make a mess.

   If you put too much on the paper/boat, you can put it back in the jar *if* (1) The material is not hydroscopic. Hydroscopic material absorbs water, and might have done so in the time it has been out of the jar. (2) The material has not touched anything but a clean spatula or weigh paper/boat.

Some balances may not have an automatic tare feature. If not, you must tare the weighing vessel manually.
1. Weigh the beaker or weigh boat.
2. Record the weight.
3. Add the boat/beaker weight to the target weight of the material.
4. Weigh the material and adjust to the weight calculated in step 3.



6. When you have the correct amount of material, pour it into the beaker or flask. To do this, grasp the boat or paper by opposite ends, gently bending the ends toward each other to make a funnel-like opening through which you can pour. Pour slowly. If any material sticks to the paper/boat, knock it off on the side of the beaker.

7. Close the lid of the stock jar. The longer it is open, the more dust can get in, the more likely that someone might knock it over or spill something in it. **Replace the jar** as soon as possible, even if you are still weighing other things. The more clutter you can remove, the less chance of a mistake or a mess.

8. Clean the balance when you are finished. There may be a brush there that you can use to sweep any stray bits off the pan. Otherwise, use a Kimwipe or a paper towel. Wipe any liquid drops

with a Kimwipe. If you spilled something, remove the pan, wash it with distilled water and dry it, and replace it on the balance. Make sure the entire weighing area is clean—no weighing papers, beakers, bottles, paper towels, or Kimwipes should be left, and the surrounding counter space should be wiped if necessary.

If you can't add water immediately, cover the top of the beaker with foil or plastic wrap, LABEL IT, and place it on your lab bench.

Label, label, label! Any container with any substance (even water) in it should be labeled. Even if you are putting it on the bench for "one minute." Post-Its are handy for temporary labels.

Mixing

Theoretically, all you have to do now is to add the water, but there are several schools of thought on this simple procedure. For example, there are those who will add the material to the water in the graduated cylinder, cover it with plastic wrap, and shake. Don't do it! Even if you don't break the cylinder or spray the lab with water, you probably will not get a very well mixed solution. Others add the powder and the water directly into a beaker, and use the beaker as a cylinder, to measure the amount of water added. Don't do that, either: It just isn't accurate enough.

Remember the old chem lab adage when mixing acids and water:

Do as you oughta.

Add acid (and base) to watah.

For most solutions

1. Add 80% of the total final volume of water to the beaker, and then add the solid. For a liter, add 800 ml of water, measured with a graduated cylinder. *Note:* For concentrated solutions—say, over 3 M or 5X—there will be too much powder to get the stir bar moving. In these cases, pour the powder slowly into a beaker, in which there already is 80% of the desired volume of water and a spinning stir bar. Pour slowly, around 2 tablespoons worth at a time, making sure all powder has dissolved before you add more. If you are having considerable trouble getting things to go into solution, find a stir plate with a heater and warm the solution mildly (a setting of 2–3, or a one-quarter turn of the dial) while stirring. *The solution must be cooled to room temperature before you pH it* or you may get an erroneous pH.
2. Gently drop in a magnetic stir bar, as large as will spin freely.
3. Put the beaker on the magnetic stir plate first, and turn on the stirrer very slowly. If you do it too fast, or turn on the stirrer before you put the beaker on the plate or the stir bar in, the stir bar will jump wildly enough to break the beaker.

If the stir bar loses its rhythm, turn off the stirrer, wait until the stir bar settles, and turn the stirrer on slowly.

4. Stir until the powder is completely in solution.
5. Pour the solution into the graduated cylinder or volumetric flask to check the volume and bring the volume up to 90% of the desired final volume.
6. Pour the solution back into the beaker or Erlenmeyer flask for pH-ing.
7. Label the contents of the beaker. Label with tape only. Never write directly on a bottle, cylinder, or flask.

If you don't need to pH the mixture, bring the volume to 100% and pour it immediately into a container suitable for autoclaving or prepare for filter sterilization.

After pH-ing the solution, pour it into a graduated cylinder or volumetric flask and bring the solution to the desired volume with water.

MEASURING PH

It is critical that all buffers and solutions used in biology be at the appropriate pH. Solutions are pH-ed before autoclaving, with the use of a pH meter.

pH tips

- The pH meter has a sensitive electrode that measures the H^+ concentration in a solution. The electrode is protected by a plastic sleeve, but be very careful not to bang the electrode on the glassware or with the stir bar. When not in use, the electrode is left in a small beaker or medicine cup containing a neutral buffer (the soak buffer). The pH meter, especially if it is fairly new, will have many buttons for functions you don't need to know about to determine a pH. The only buttons you will probably need to use are the standby, standardize, and pH buttons.

- There are several kinds of electrodes; some filled with a gel or liquid, some not; some requiring maintenance, some not. In many labs, no one even knows what kind of electrode they have until there is a problem and they must search through the drawers for the package literature to figure out what to do. Check with a knowledgeable lab member or with the manufacturer before you have problems.

- The pH meter should be calibrated daily, using two buffers of known pH. From these calibrations are derived the pH determinations, so don't scrimp

(and render your pH reading inaccurate) by standardizing only to one buffer of pH 7. There is a lot of inertia and worry about calibrating the pH meter, but it is necessary, it does make a difference, and it is ridiculous to go through the trouble of pH-ing at all if it isn't done correctly.

Don't use the pH paper you may find in the drawer to routinely pH your solutions. It is used to check the pH of small volumes, or to do a quick pH check before use of the pH meter. Always remove a drop from the solution to add to the paper; never dip the pH paper into the solution.

- If you are pH-ing a series of solutions, you don't need to recalibrate between solutions. Do recalibrate if someone known to be sloppy calibrated the meter.

- pH will affect solubility. An example of this is 0.5 M EDTA, a common molecular biology reagent. It is usually made at a pH of 8.0, and won't even go into solution until that pH is approached.

- pH is dependent on temperature. Be sure the buffer you are pH-ing is the same temperature as the buffer standards you use to calibrate the pH meter. And be sure the buffer and the standards are at the temperature at which the buffer will be used.

Calibrating the pH Meter

You will need:

At least two pH standard solutions. Near the pH meter you should find standards of 4, 7, and 10. In most labs these are purchased ready-made and are stored in 500-ml bottles. Generally, using standards of 4 and 10 is sufficient.

3 or 4 50-ml beakers or medicine cups, glass or plastic.

Kimwipes.

Choose your standards according to what you will be pH-ing. If you will be pH-ing a solution of extreme pH (that is, above 10 or below 4) you should standardize with buffers closer to the target pH. For example, if you will be pH-ing a buffer to 3, it would be better to standardize to buffers of pH 2 and 4, or 2 and 7. After, recalibrate to pH values of 4 and 10.

1. Raise the electrode out of the soak (or storage) buffer, and rinse it with distilled water from the wash bottle. Rinse the electrode over a beaker used only for washes. Do not rinse the electrode into the soak beaker. The pH meter should still be set on standby. Touch electrode gently with a Kimwipe, to remove excess fluid.

Calibration with one buffer

If you are going to measure buffers of approximately the same pH, you could standardize the pH meter with just one buffer.

- Use a standard of 7.0 for neutral buffers, 10 for basic buffers, or 4 for acidic buffers.
- Calibrate the pH meter and measure the pH as described for 2-buffer standardization.

Should you need to pH a buffer of a different pH, you will have to restandardize the pH meter.

2. Pour approximately 1.5 inches of standard pH 4 (it could be 10; there is no order necessary) into a small beaker or medicine cup. Recap the bottle of standard 4, and then immerse the tip of the electrode into the beaker.
3. Press "standardize" and wait until the display reading has stabilized at pH 4.
4. Press "standby," raise electrode, and rinse and dry it as in step 1.
5. Pour the standard pH 10 into a fresh beaker, immerse the electrode, and press "standardize." Wait until the display reading has stabilized at a pH of 10.
6. Press standby, rinse and dry electrode, and leave it immersed in a beaker of storage buffer.
7. Read the pH of the standardization buffers. If the pH is not as it should be, repeat the calibration until the pH reads as it should. The pH meter is now standardized for most buffers and solutions.

Determining pH

Have ready the same materials needed for calibration, as well as a magnetic stir plate, stir bars, and pasteur pipets. You will also need acids and bases to actually determine pH: concentrated HCl (12.1 M) and 1 M HCl, and NaOH (5 or 10 M) and/or 0.1 M NaOH should be found next to the pH meter.

1. Stir the solution to be pH-ed on a magnetic stir plate. The stir bar should be stirring as slowly as possible, to lessen the chance of electrode damage should the electrode mistakenly come in contact with the bottom of the flask or beaker.
2. Raise the electrode out of the soak beaker, rinse it with distilled water from the wash bottle, and blot dry gently with a Kimwipe. The pH meter should still be set on "standby." Do not rinse the electrode into the soak beaker.
3. Immerse the tip of the electrode into the solution you want to measure. Make sure the bar clears the electrode before you turn on the magnetic stirrer.

What should I use to pH buffers?

Most labs use **HCl and NaOH** to adjust the pH on the acid or basic form of routine buffers. (Other strong acids or bases could also be used.) It is true that using HCl or NaOH to pH a buffer adds anions (Cl^-) or cations (Na^+) to your buffer, and that these could interfere with certain experiments. But it usually doesn't, and it is often assumed that you will do it that way. A Tris-Cl buffer means that after dissolving the base form of Tris, the pH was adjusted with HCl. A Tris-acetate buffer means that the pH was adjusted with acetic acid.

One could also add the acid and base forms of a buffer separately to obtain the desired pH. Phosphate and acetate buffers are often made this way. If you know the pKa, you can calculate the ratio you need of base form to acid form of the acid-base pair for a particular pH. You will probably never have to do this. The protocol or recipe will specify the amounts of acid and base you must add to achieve the desired pH, or you can consult one of the many tables found in catalogs.

4. If the pH meter has a function switch, change it from standby to pH. (If you need to take the electrode out of the beaker to mix by swirling, turn the function switch to standby first.)

5. Wait for the readings to stabilize. Read the pH, and adjust it by adding NaOH if the pH is too low, and HCl if the pH is too high. Use a pasteur pipet and bulb, or a transfer pipet, to add the pH-ing solutions: Use a separate pipet for each solution. Start with one drop at a time: Add a drop to the stirring solution and wait until the pH has changed and stabilized before adding another drop.

   If the pH is off by more than a unit, use concentrated HCl (12.1 M) or NaOH (5 or 10 M). Otherwise, use 1 M HCl or 0.1 M NaOH.

   Continue to add acid or base, dropwise, until you have reached the target pH. You will need more drops as you approach the pH at which a solution is buffered. If the pH isn't budging, use a more concentrated solution to pH it, but be very, very careful to do so slowly and dropwise.

6. Turn function switch back to standby.

7. Raise the electrode out of the solution, rinse the electrode with distilled water, and wipe it gently with a Kimwipe.

8. Leave the electrode immersed in storage buffer.

9. Pour the solution into a graduated cylinder or volumetric flask and bring the volume up to 100%.

Be sure the stir bar is not autoclaved with the solution in the bottle. Few things infuriate lab personnel more than seeing a row of autoclaved reagents, complete with stir bars inside; most labs don't have unlimited numbers of stir bars. Should you accidentally pour the stir bar into the bottle, retrieve it before autoclaving with the long, magnetized rod that you should find near the sink.

10. Pour the solution into a glass or plastic bottle with a cap (see below for autoclavable plastic.) Do not store buffers in flasks, beakers, or cylinders, covered with parafilm.

11. Label the bottle. Use a piece of tape and a Sharpie or other marking pen. Write the date (including the year), the components, the concentration, the pH, and your initials.

12. Put a piece of autoclave tape on the bottle. It is heat sensitive and will darken visibly only after being autoclaved. If you are unable to autoclave immediately, store the buffer at 4°C to reduce the chance of contamination.

If readings are erratic, check

- **The electrode.** Some electrodes contain a buffer, which must be maintained. If so, fill it with the correct buffer (check the manual) and close. The electrode may also be cracked or broken, and need to be replaced.

- **The standards.** People often pour the standards back into the stock bottle after use, arguing that it was used so little that it is as good as new. Not true, and this practice will have a negative effect. Try a new bottle of standard buffer.

- **The temperature** of the buffer. Since pH is dependent on temperature, be sure all buffers are at room temperature before reading the pH. Common culprits are freshly distilled (too warm) or refrigerated water (too cold), just-made exo- or endothermic buffers, or the heat of the hot plate stirrer.

- **The storage solution.** If the storage solution in the beaker has dried up, salt may be encrusted around the tip of the electrode. Rinse the electrode well with water, and store the electrode in a beaker of fresh storage buffer.

Tris buffers can be difficult to pH, giving unstable readings. A few electrodes give spurious readings for Tris-containing solutions. In particular, silver/silver chloride reference electrodes used with Tris solutions containing protein can be inaccurate.

After you pH your Tris solution, wait 10 minutes and pH it again. If your readings are different, call the electrode or pH meter manufacturer to find out whether that electrode is compatible with Tris buffers. If not, Tris-compatible electrodes are available, of course.

Most of the Tris solutions you will make will not contain protein, and there should be no problem pH-ing those buffers.

- **Your technique.** Rinse electrodes well between buffers. Always stir moderately while pH-ing.

STERILIZING SOLUTIONS

Autoclaving or filtration? Most buffers are sterilized before use and storage, to prevent bacterial and fungal growth. And most buffers are autoclaved: Although filtration would be as effective, the large volumes in which buffers are usually made would make filtration extremely tedious.

Even if you require the buffer for a nonsterile application, it should still be sterilized, because microbial growth can cause changes in pH and in the nature and function of the buffer.

Be sure the solution can be autoclaved: Heat-labile ingredients cannot be heated, and a solution containing such an ingredient must either be filter-sterilized or autoclaved without the ingredient (which can be filtered and added later to the autoclaved material).

Autoclave

- Most buffers.
- Undefined bacterial and yeast media.

Do not autoclave

- Buffers with detergent, such as 10% SDS, because they will boil over.
- Organic solvents, including phenol.
- Heat-labile ingredients such as serum and vitamins, antibiotics, and proteins (BSA).
- Mammalian, plant, and insect media.
- HEPES-containing solutions.
- Dithiothreitol (DTT)- or β-mercaptoethanol (BME)-containing solutions.

If a heat-labile or otherwise non-autoclavable ingredient must be added to an autoclavable buffer, autoclave the buffer first. When the buffer has cooled to room temperature, add the filter-sterilized ingredient.

Using the Autoclave

Autoclaves work by subjecting the material to high heat (121°F) and pressure. If you are unfamiliar with the autoclave, ask someone to demonstrate its use. This is not a machine to experiment with, although it is quite safe when used correctly.

Be sure the flask or bottle is borosilicate glass or autoclavable plastic. Glass will generally be used for buffers, because repeated autoclaving is rough on most plastics. A rough rule of thumb is that the more brittle-feeling plastics aren't suitable, but you will have to check the catalog or ask someone about a particular item.

1. Leave at least a quarter of the total flask or bottle volume as free space. This leaves plenty of room for boiling liquids.
2. Place containers in a shallow metal or autoclavable plastic pan to catch anything that might break. Traditionally, a couple of inches of water are placed in the pan to reduce the chance of glass breaking with a sudden pressure change: This is not necessary, but you may be considered to be crazy for not doing it.
3. Be sure caps are loose to prevent buildup of pressure. If you are using tin foil as a cap, tape one side of the foil to the flask to prevent the top from getting knocked off.
4. Stick a small piece of autoclave tape on each item to be autoclaved. This is pressure-sensitive tape, which will show a design or color change only after autoclaving. On the cap or just above the identification label are logical places to put it. Write the date on the tape.
5. Close and tighten the autoclave door. It should be tight, but not so tight that it will be a strain to open. If it is a two-door autoclave, be sure both doors are closed.
6. Adjust appropriate settings and turn on. Most autoclaves are automatic, and will have a minimum of a liquid (solutions in glass containers require a controlled rate of cooling and slow release of pressure) and a dry setting (which requires no cool-down period). The newer programmable autoclaves may have numbered programs, as well as manual settings. If you have large volumes, the cycle will take longer than for small volumes.

   For a 2-liter Erlenmeyer flask containing 1 liter of medium, set 30 minutes of sterilization time.
7. Be sure the autoclave has returned to ambient pressure before opening. Open very slowly, standing back from the door, to avoid contact with any escaping steam.
8. Put on heat-resistant gloves. The gloves should be large, heavyweight gloves. Don't use potholders or oven gloves.

To avoid precipitates, browning, and substrate breakdown

- Autoclave glucose separately from amino acids/peptones or phosphate components.
- Autoclave phosphates separately from amino acids/peptones or other mineral salt components.
- Autoclave mineral salt components separately from agar.
- Avoid autoclaving media at a pH greater than 7.5. Autoclave at neutral pH and adjust to the desired pH with a sterile base solution after cooling.
- Avoid autoclaving agar solutions at less than pH 6.0.

(Reprinted, with permission, from Cote and Gherna 1994.)

9. Tighten caps before taking the bottles out of the autoclave. The liquid may boil over as you remove them.

10. Let the bottles sit at room temperature until cool. If you put the bottles immediately into the cold, the glass may crack. It usually works out well to leave the bottles on the bench overnight.

Filter Sterilization

If a liquid is heat-labile or volatile, or less than 20 ml in volume, it should be sterilized by filtration. The solution is passed (aided by gravity, force, or vacuum) through a filter of a pore size small enough (0.2 or 0.1 µm) to exclude most microorganisms. Note that viruses are not removed by filter sterilization. Use 0.4-µm filters only as a prefilter for a viscous solution: A passage through 0.4 µm will not sterilize a solution, and so must be followed by passage through a 0.2-µm filter.

0.2 µm is typically used for buffer sterilization and most medium sterilization. 0.1 µm is used for some tissue culture media.

There are many reusable and disposable filtration systems commercially available. Some labs or departments make their own media and have large-scale, pump-driven filtration units. There are several kinds commonly used at the bench for individual filtration needs:

- Non-disposable filtration apparatus, vacuum driven: volumes 20 ml–1000 ml.
- Disposable filter cup units, vacuum driven: volumes 15 ml–1000 ml.
- Disposable filtration units with storage bottle, vacuum-driven: volumes 15 ml–1000 ml.

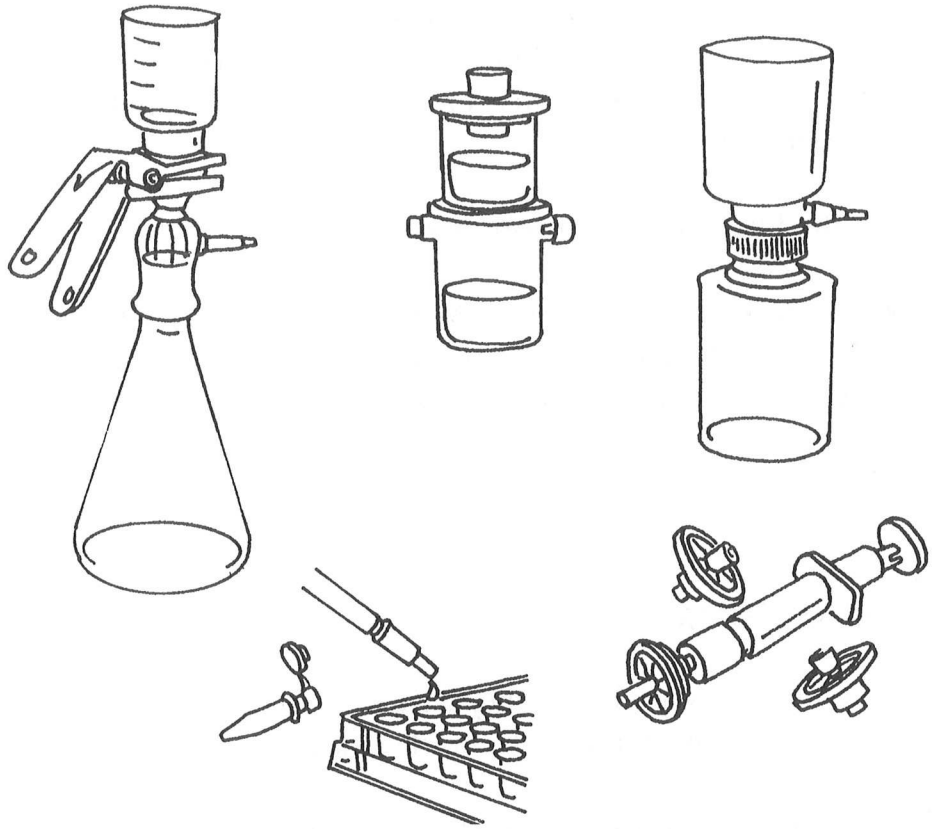


FIGURE 5.

Clockwise, from left: A nondisposable unit; a disposable cup unit; a disposable filtration unit with storage bottle; syringe and micro-syringe filter units; and a microfuge tube filter unit.

- Syringe filters: volumes 1 ml–20 ml.
- Micro-syringe filters: volumes less than 1 ml.
- Spin filters, microfuge tube filters: volumes less than 1 ml.

Work aseptically. Doing the filtration in a biosafety cabinet adds an extra measure of security. See Chapter 9, Working without Contamination, for filter-sterilizing techniques.

STORING BUFFERS AND SOLUTIONS

Store media at 4°C.

Buffers can be stored at 4°C or at room temperature. Some buffers, especially concentrated ones, will precipitate in the cold: This is usually not a problem, as heating at 37°C for a few minutes will redissolve the precipitate. Storage in the cold will dampen the growth of contaminants that might

be introduced during use. There is a psychological component as well, for people tend to be more careful with things stored in the refrigerator rather than on the bench. In general, store buffers for culture in the cold, buffers for biochemical assays at room temperature.

Store concentrated solutions at room temperature.

Light-sensitive reagents should be stored at the appropriate temperature in a brown bottle, or in a bottle kept in a box or covered with foil and tape. See Chapter 8 for more details on the storage of solutions.

When to Discard Buffers

Many buffers can be used for years, especially if they have been sterilized and kept in the cold. But there are several reasons to throw the bottle away.

- **Discoloration.** If a buffer looks discolored—that is, if it has acquired a tinge of *yellow*—discard it. It is probably okay, but using it will always leave a feeling of unease that isn't worth saving the effort of making another bottle. Make sure it isn't a buffer that is normally colored. For example, MOPS, one of the Friend zwitterionic buffers, is always yellowish above a 1 molar concentration.
- **Contamination.** A sure reason to immediately discard a buffer is contamination. This is usually *mold*, and will appear as a discrete, ball-shaped item, sometimes furry, often white or blue-greenish. If a clear, colorless bottle becomes cloudy, discard it. Desperate people have filtered the mold away and used the buffer, but it isn't worth it: Even if the solution is now sterile, it might have been changed by metabolic by-products.

  Bacterial contamination is less likely, and will either settle on the bottom of the container or will make the solution appear cloudy. Get into the habit of swirling your bottles and eyeballing them as you get ready to use them, to check for contamination.

  When in doubt, throw it out. There is too much resting on the quality of your reagents to try to save time and money here.
- **Precipitation.** It is sometimes difficult to tell bacterial contamination from precipitated salts. If you are in doubt, place the buffer in a 37°C water bath for 20 minutes and see if the cloudiness dissolves. If it does, storage in the cold is causing some salt to precipitate, and you can warm it up before use. (But you should

plan on replacing it soon.) If the cloudiness remains, you don't know whether it is a stubborn precipitate or contamination, but it doesn't matter—throw it away immediately.

If you really need to know whether it is precipitate or contamination, put a drop of it on a slide with a coverslip, and look at it on a microscope at 100X. A precipitate will appear as large *crystals,* contamination as tiny and uniform shapes.

RESOURCES

A.L.E.R.T. (Allergy to Latex Education and Resource Team). 1997. http://www.execpc.com/~alert/news.html/

Bateman R. and Evans J. 1996. *Biochemistry laboratory manual.* I. Solution preparation. http://www-chem.st.usm.edu/biochem/lab_manual/lab.html/

Bateman R. and Evans J. 1996. *Biochemistry laboratory manual.* II. Buffer preparation and testing. http://www-chem.st.usm.edu/biochem/lab _manual/lab_manual.html/

Cote R.J. and Gherna R.L. 1994. Nutrition and media. In *Methods for general and molecular bacteriology* (ed. Gerhardt P. et al.), pp. 155–178. American Society for Microbiology, Washington, D.C.

Gershey E.L., Party E., and Wilkerson A. 1991. *Laboratory safety in practice: A comprehensive compliance program and safety manual.* Van Nostrand Reinhold, New York.

Heidcamp W.H. 1995. *Cell biology laboratory manual.* Gustavus Adolphus College, St. Peter, Minnesota. http://www.gac.edu/cgi-bin/user/~cellab/phpl?index-1.html/

Lenga R.E. 1988. *The Sigma-Aldrich library of chemical safety data,* edition II, vol. I and II. Sigma-Aldrich Corporation, Milwaukee.

Lide D.R., ed. 1997–1998. *CRC Handbook of Chemistry and Physics,* 78th edition. CRC Press, Boca Raton, Florida.

Maniatis T., Fritsch E.F., and Sambrook J. 1982. *Molecular cloning: A laboratory manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Sambrook J., Fritsch E.F., and Maniatis T. 1989. *Molecular cloning. A laboratory manual.* 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sigma Chemical Company. 1996. *TRIZMA. Tris(hydroxymethyl)aminomethane; Tris. Technical Bulletin no. 106B.* St. Louis, Missouri.

UW GenChem Pages. 1996. Department of Chemistry, University of Wisconsin-Madison. http://genchem.chem.msg.edu/labdocs/

Windholz M., ed. 1976. *Merck index,* 9th edition. Merck and Co., Inc. Rahway, New Jersey.