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DNA, RNA, and Protein

RNA, DNA, AND PROTEIN are the bread and butter of the molecular biology laboratory. Until a few years ago, investigators worked in a "DNA lab," or an "RNA lab" or a "protein lab," and had great expertise only in that area. Increasingly now, investigators need to be familiar with techniques involving all three macromolecules, and laboratories are set up to accommodate this range. Even a small project can easily involve a bit of cloning, mRNA isolation, and Western blotting.

The problems associated with either DNA or RNA or protein are not the same, as the molecules have completely different properties. But thinking and working aseptically will help you avoid some of the major pitfalls of macromolecule work.

MOLECULAR BIOLOGY TIPS

- **Know the theory behind everything you do.** With kits and protocols readily available, it is particularly easy to do experiments in mol-

ecular biology without a thought in your head. Know the components of a kit, and know what they do. Know why you use high salt versus low salt, ionic versus nonionic detergents. If you don't, you will end up changing protocols instead of ever troubleshooting.

- **Don't endlessly protocol-shop.** In no field of science will you be able to get more help than in molecular biology. The manuals are plentiful and excellent, there are numerous on-line sources for advice, and many of the techniques are taught in college and grad-school labs. It is easy to become infatuated with a nifty new technique. But if you have a protocol that works, stick with it.
- **Purchase kits judiciously.** They are completely invaluable. But if they are not purchased wisely, they can be an enormous waste of time. Check out the lab's resources before you order one. If, for example, the lab already has 90% of the reagents needed, it doesn't make sense to purchase the kit. Or if a "kit" only consists of an easily made buffer, control DNA, and enzyme, buy the enzyme, make or buy a control, and make the buffer.
- **Consult with other scientists about results.** Because of the enormous amount of written material around, you may feel that you "shouldn't" ask questions. But here as in all fields, a bit of advice could save you weeks of effort and money.
- **Be vigilant about the effectiveness of your reagents.** The reagents you purchase are still made by human beings, and could be ineffective, labeled incorrectly, or have any number of things wrong. There is a tendency to assume that a prettily packaged, labeled, and boxed reagent *must* work, and that the failed experiment must be your own fault. (In most cases, of course, it is your fault.) Include controls, where feasible, for all reactions. Don't hesitate to call the manufacturer or supplier if you have a doubt about a reagent: The technical services will suggest ways to determine whether the reagent is functional and will arrange for an immediate replacement if it is not.
- **Label everything.** Each sample may pass through several tubes on its way to its final treatment, and the contents of even the intermediary tubes must always be clear.
- **Discard old tubes as you go.** Racks of microfuge tubes will pile up quickly in the refrigerator and freezer, and they can become confusing and depressing. Keep a tube only until you have the next step completed satisfactorily.

DNA

DNA is pretty tough, as the material that holds and transfers most genetic information would have to be. But don't get too casual. The main worry is that of contamination of samples or reagents with other DNAs.

DNA Isolation

- The DNA isolated will either be genomic or extrachromosomal.
- Investigate DNA isolation kits. These have been fine-tuned to isolate genomic and extrachromosomal DNA, as well as DNA from agarose gels and PCR products from multiple amplification reactions. They utilize a DNA-binding resin or membrane, and are often little microfuge columns. They are worth the expense. Their use usually eliminates the need for phenol extractions and CsCl centrifugation.

Read and follow carefully the instructions included with DNA isolation kits. Details that may seem minor—such as the O.D. of a bacterial culture—can be very important.

- Extrachromosomal DNA is isolated as phage or plasmid DNA.
- Large pieces of DNA, for example, genomic DNA, must be handled carefully to avoid breakage. It is isolated and stored differently from small DNAs.
- Preps may be referred to as mini, midi, and maxi (small, medium, and large). Mini preps are sufficient for a surprising number of uses.
- Find out what quality of DNA prep is required. Certain procedures such as DNA sequencing or transfection of mammalian cells may require a high quality of DNA to work well, whereas others, such as screening clones by restriction mapping, may be more forgiving.

Units of Measurement

1 kb of DNA = 6.5 x 10^5 Daltons of double-stranded DNA (sodium salt)
1 kb of DNA = 3.3 x 10^5 Daltons of single-stranded DNA (sodium salt)
1 kb of DNA = 3.4 x 10^5 Daltons of single-stranded RNA (sodium salt)
1 kb of DNA = 37,000 Daltons = 333 amino acids of coding capacity
Average MW of a deoxynucleotide base = 324.5 Daltons
Average MW of a deoxynucleotide base pair = 649 Daltons
1 µg/ml of 1 kb of DNA = 3.08 nM 5′ ends

TABLE 1. Genomic DNA Sizes

Organism	Base pairs/ Haploid genome	Copy number of single-copy genes
Escherichia coli	4.7 x 10^6 bp	1.8 x 10^8
Drosophila melanogaster	1.4 x 10^8 bp	6.6 x 10^5
Mus musculus (mouse)	2.7 x 10^9 bp	3.4 x 10^5
Homo sapiens	3.3 x 10^9 bp	2.8 x 10^5

PROTOCOL

Alkaline-SDS Plasmid Minipreps

Minipreps allow you to isolate and analyze plasmid DNA from a bacterial culture. 12 or 16—as many as can fit in your microfuge rotor—can easily be run at once.

Materials

- 5 ml overnight culture. Use LB plus the appropriate antibiotic, and inoculate with a single colony. Incubate at 37°C, shaking or rolling.
- Sterile microfuge tubes.
- Ice.
- Solution I (Lysis buffer: 25 mM Tris-HCl, pH 8.0, 50 mM glucose, 10 mM EDTA), hold on ice to chill.
- Solution II (Denaturing solution: 0.2 N NaOH, 1.0% SDS), made fresh for each preparation. Hold at room temperature.
- Solution III (Renaturation solution: 5 M potassium acetate. To make: Prepare 120 ml of potassium acetate. Add 23 ml of glacial acetic acid and 57 ml of H_2O for a total volume of 200 ml.) Hold on ice.
- TE.
- 70% and 100% ethanol.
- RNase A (DNase-free). Make up a 2 mg/ml solution, aliquot it, and store at –20°C. You can use and refreeze this. There are protocols to make Rnase A free of DNase, but you can (and should) just buy DNase-free RNase A.

Procedure

1. Add 1.5 ml of the culture to a microfuge tube.
2. Centrifuge 2 minutes at 10,000*g*, preferably in the cold. Aspirate the supernatant.
3. Resuspend the pellet in 100 µl of cold Solution I. Vortex for 2 minutes.

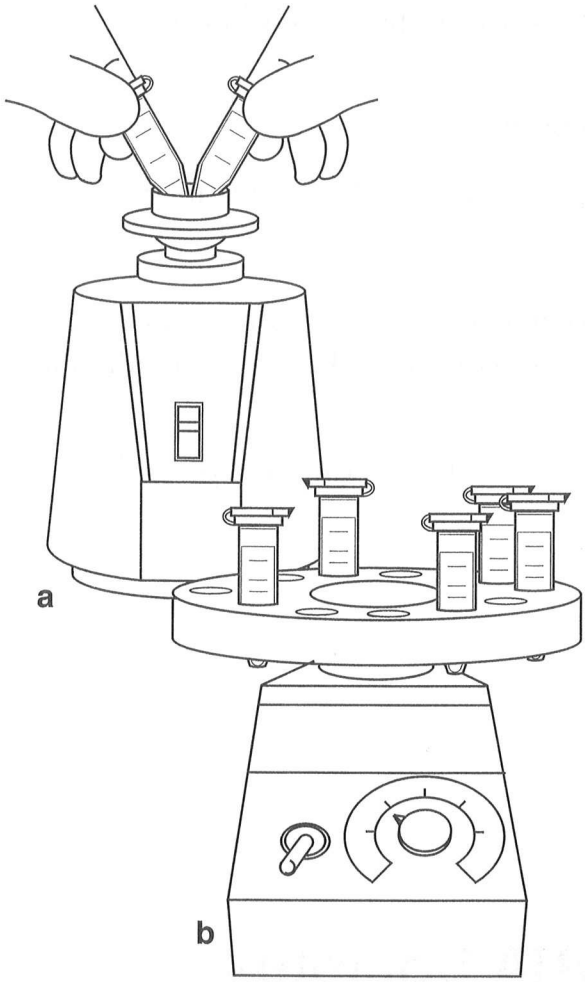

FIGURE 1.

Two tubes can be vigorously vortexed together by holding them bottom to bottom (*a*). If you have more than two tubes, use either a multitube vortexer or a standard vortexer with an adapter that holds multiple tubes (*b*).

4. Incubate the tube at room temperature for 5 minutes. The bacteria will be lysed and the DNA released.
5. Add 200 µl of Solution II and mix the tube by inversion for 5 seconds. Vortexing would damage the DNA.
6. Incubate the tube on ice for 5 minutes.
7. Add 150 µl of Solution III and mix the tube by inversion for 20 seconds.
8. Incubate the tube on ice for 5 minutes. Plasmid DNA is selectively renatured.
9. Centrifuge at 12,000*g* for 5 minutes.
10. Remove the supernatant (which contains the DNA) with a pipettor into a new tube.
11. Add 5 µl of 2 mg/ml RNase A (DNase-free) to the supernatant and incubate at 37°C for 5 minutes.

Some protocols call for the inclusion of lysozyme (Final 4 mg/ml) in Solution I to digest the bacterial cell wall, but this usually isn't necessary.

12. Add 450 µl of phenol-chloroform and extract (see protocol below) to remove the RNase A and proteins.
13. Extract with chloroform (see protocol below).
14. Add 1 ml of cold 100% ethanol and precipitate for 20 minutes at –80°C.
15. Aspirate the supernatant.
16. Wash pellet with 70% ethanol and dry under vacuum for 5 minutes. If you aren't in a hurry, invert the tubes onto a paper towel and let drain and dry for 30 minutes.
17. Resuspend the pellet in 25 µl of TE. Use 2–4 µl to run on a gel and store the rest at –20°C.

PROTOCOL

Phenol Extraction of DNA Samples

Phenol extraction is often done on samples of DNA (and RNA) to remove contaminating proteins. Phenol and water are not miscible, and will form separate phases when mixed. When the aqueous sample containing the DNA is mixed with phenol, the proteins partition into the phenol phase, the aqueous DNA is removed, and the sample is reextracted and concentrated by ethanol precipitation.

Materials

- Solvent-resistant plastic tubes. Try to work in as small volumes as possible. Most extractions can be done in microfuge tubes.
- TE-saturated phenol:chloroform:isoamyl alcohol 25:24:1 (see Chapter 7)
- Chloroform:isoamyl alcohol 24:1.
- Pipettor and tips.

Procedure

1. Add an equal volume of TE-saturated phenol-chloroform to the DNA sample. The total volume should not exceed 500 µl for a 1.5-ml microfuge tube.
2. Vortex the sample vigorously for 20 seconds.
3. Centrifuge the sample for 5 minutes at room temperature to separate the phases. This does not have to be a high spin, but it is often convenient to use the highest speed on the microfuge. Remove the samples carefully so you don't disturb the separated phases.
4. With the pipettor, remove as much aqueous layer as you can without disturbing the protein layer. Add it to a new tube.

If you have added hydroxyquinoline to the phenol, the phenol will have a yellowish color.

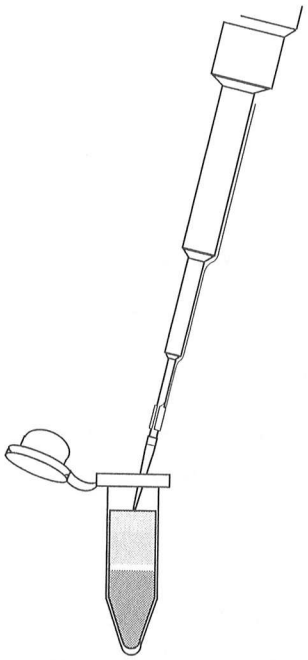

FIGURE 2.

After centrifugation, you should see two phases in the tube. The top phase is the aqueous phase, which contains the DNA. The bottom phase is the organic phase, containing the protein. There is usually an interphase of extracted protein, a thick or barely visible whitish band between the aqueous and organic phases. Don't touch the white junk when removing the aqueous layer!

To improve yield: If you think you have not gotten enough of the aqueous layer, you can reextract the phenol layer by adding a volume of TE, pH 7.5, approximately equal to the phenol layer. Vortex and centrifuge.

If the combined volume of the aqueous phases is under 500 µl, you may combine them in one tube. Usually, however, you will need another tube, and will end up with almost no DNA in that tube. It is not worth the time to routinely reextract the phenol layer.

To improve purity: If you think you might have picked up some of the protein layer, you can phenol extract the aqueous phase by adding a volume of phenol:chloroform approximately equal to the aqueous layer. Vortex, centrifuge, and carry on as in step 4. If you find that your enzymatic reactions with DNA aren't working, you probably need to do two extractions.

5. Add an equal volume of chloroform to the aqueous layer. Repeat steps 2, 3, and 4.
6. Label the tube. It is now ready for use or for concentration by ethanol precipitation.

Phenol-chloroform must be disposed of as hazardous waste. Do not discard it down the sink.

PROTOCOL

Ethanol Precipitation of DNA

Precipitating the DNA sample enables you to resuspend it in a smaller volume, and so, to concentrate it. Precipitation also removes residual chloroform, which will inhibit many enzymatic reactions.

Procedure

1. To a maximum volume of 450 µl of DNA in water, add 1/10 volume of 3 M Na-acetate, pH 4.8. Invert briefly to mix.
2. Add two volumes of 95% or 100% ethanol. Invert well to mix.
3. Precipitate the DNA by placing the sample in the cold. Precipitations can be done at –20°C overnight, –70°C for 30 minutes, or on dry ice for 5 minutes.

Using dry ice for ethanol precipitations

Use a mallet to pound the dry ice into a powder, and insert your tubes into the powder, or break the dry ice into pieces, and add to a freezing-resistant dish. Add 95% ethanol to make a slurry, and insert your tubes. The ethanol will wipe out all markings except those done with a permanent sharpie.

4. Centrifuge the sample at high speed (at least 12,000 rpm) for 15–30 minutes, at 4°C. If you don't have a refrigerated microfuge, place the microfuge at 4°C.
5. Decant or aspirate the supernatant. The supernatants can be discarded down the sink.
6. Drain the tubes by inverting the tubes and leaving them upside down on a paper towel on the bench.
7. Wash the pellet with cold 70% ethanol.

8. Dry as described in step 6, or use a vacuum concentrator.
9. Resuspend the DNA in TE, pH 8.0 (10 mM Tris-HCl, 0.1 mM EDTA). If the DNA does not seem to go into solution, add more TE. Store at 4°C.

Caution is needed when handling large-molecular-weight DNA (over 30 kb). The DNA should never be vortexed, but should be mixed by inversion or on a wheel. Instead of precipitating the DNA with ethanol, traces of chloroform should be removed by dialyzing the DNA solution against large volumes of cold TNE or by extraction with water-saturated ether.

PROTOCOL

Using a Vacuum Concentrator (Speed Vac)

If traces of ethanol remain in nucleic acids after ethanol precipitation, it is difficult to resuspend the pellets in water or buffer. If you are in a hurry, or have a large volume of volatile liquid to remove, a vacuum concentrator can be used.

A vacuum concentrator consists of a centrifuge, a pump used to create a vacuum, a heater, and a cooling trap. Models vary, and laboratory rules on usage vary even more.

Procedure

1. **Turn the cooling trap on at least 30 minutes before you are ready to use the concentrator.** In some models, you may have to add dry ice.
2. Turn on the vacuum pump.
3. Open the vent to release the vacuum inside the centrifuge.
4. Place your tubes in the centrifuge. They must be balanced, with the tops open. Cover the tubes with a piece of parafilm, and use a pin to poke a couple of holes in the parafilm. Theoretically, the tops can be left off, and the contents should be fine, as centrifugal force should keep the contents in the tubes. But if someone lifts the lid before the vacuum has been fully released and causes a bit of turbulence inside, the parafilm may help save your material.

If someone's samples are already spinning, slowly release the vacuum until the centrifuge is at atmosphere. Wait at least 10 seconds, a few seconds after you no longer hear the hissing of the air into the centrifuge. Then, and only then, can you turn off the centrifuge and load your own tubes.

5. Close the lid, and turn the centrifuge on.
6. Once the centrifuge has achieved full speed, open the vacuum vent to the centrifuge.
7. Turn the heater on only if you have a lot of volume to dry, or can't wait the 10 minutes it takes to dry DNA precipitation pellets. Oligos will require several hours of spinning with heat.
8. To stop the run, release the vacuum in the centrifuge. Only then should you turn the centrifuge off!
9. Open the centrifuge when the rotor has come to a complete stop.
10. Remove the tubes, checking by eye to be sure they are dry. Cap immediately, to prevent losing the dry pellets.
11. Turn off the vacuum pump.
12. Follow lab procedure on cleaning the trap. Some require that you clean after every use, others that it be done once a day. If you evaporated a lot of liquid, you should clean it right way.

PROTOCOL

Determining Nucleic Acid Concentration and Purity by UV Spectroscopy

1. Turn the spectrophotometer on.
2. Turn on the UV lamp 20 minutes before you will take your readings. The visible light lamps can be used immediately, but the UV lamp takes a while to become steady. The amount of warm-up time needed depends on the lamp and the spectrophotometer.
3. Your sample will be DNA or RNA in water or buffer, with a blank of water or the same buffer. The amount of nucleic acid you add will depend on the source, so ask someone in the lab for a recommendation on the amount of material and the dilution you need.

4. Put the sample and the blank in a matched set of quartz cuvettes.
5. Set the wavelength to 260 nm.
6. Blank the machine against the water (or blank manually, if only one cuvette at a time can be measured).
7. Read the O.D. at 260.
8. Set the wavelength to 280. Reblank and read the O.D. at 280.
9. Calculate the concentration of the nucleic acid, using the following information:

   1 A_{260} unit of double-stranded DNA = 50 µg (50 µg/ml has an O.D. of 1 at 260 nm)

   1 A_{260} unit of single-stranded DNA = 37 µg

   1 A_{260} unit of single-stranded RNA = 40 µg

Only quartz cuvettes, not glass or plastic, will allow you to take accurate readings in the UV range.

DNA concentration (µg/ml) = (OD_{260}) x (dilution factor) x $\frac{(50\ \mu g\ DNA/ml)}{1\ OD_{260}\ unit}$

Example: 10 µl of DNA is added to 390 µl of water, and the O.D. is 0.205.
0.205 x 40 x 50 = 410 µg/ml
The DNA concentration is 410 µg/ml.

10. Calculate the total yield of your preparation.

Yield = (DNA concentration in µg/ml) x (total volume in ml)
Example: If the 10-µl sample were taken from a 100-µl sample,
410 µg/ml x 0.1 ml = 41 µg or 0.41 mg
Note: If you removed 10 µl from the sample for assay, you would only have 36.9 µg left (41 – 4.1 = 36.9).

11. Estimate the purity of the prep by figuring the 260/280 ratio. The ratio between the readings at 260 nm and 280 nm gives an estimate of the purity of the nucleic acid. Pure preparations of DNA should have a 260/280 ratio of 1.8, RNA a ratio of 2.0. A higher ratio would suggest extraction with phenol-chloroform to remove protein impurities.

Example: If OD_{260} of the DNA prep was .205, and OD_{280} was .114, the 260/280 ratio would be 1.8. Right on the nose!

Restriction Enzymes

Restriction enzymes are usually pooled for a laboratory or one or more departments. Some enzymes are expensive and infrequently used, and single investigators can't afford to keep a large selection. There will be one person in charge, and most of the dialogue will take place via sign-up and comment sheets.

- Always bring an ice bucket, labeled tubes, a pipettor, a box of sterile tips, and a sharps discard box (if there isn't one near the freezer) with you when you go to the –20°C freezer where the enzymes are kept. Don't bring tubes with DNA to the enzyme freezer.
- Remove one enzyme only, and place it in your ice bucket. Keep the freezer door shut: Don't stand with your body in the door, pipeting away.
- Take what you need. Use aseptic technique.
- Replace the stock enzyme tube in the freezer.
- Sign the sign-up sheet, or make a note of what you used and the amount you took.
- Take another enzyme, if you need one.
- Report all empty or almost empty tubes to the person in charge. Don't merely leave a note.

Use tips luxuriously. Use a tip once, and discard it immediately, so there is no chance of inadvertently using it again. Contamination of one enzyme with another is the major worry when working with restriction enzymes.

If you need an amount of an enzyme that will deplete the stock, organize yourself to let the charge person know at least a few days before.

Buffers for restriction enzymes

The buffer needs of most of the restriction enzymes can be met with five buffers, differing in the kind and concentration of salt used. Companies often supply the appropriate buffer with the enzyme, but you may not be the one who gets one of those tubes. If there are common buffer sources for restriction enzymes in your lab, don't use them: You must have your own buffers, because this is another source of possible cross-contamination. Either get hold of the manufacturer's tubes (you can make a set from different manufacturers) or make your own. It is easy enough.

PCR (Polymerase Chain Reaction)

PCR, an invention made by Kary Mulis at Cetus Corporation in 1985, is an in vitro method of DNA synthesis that allows a particular segment of DNA to be copied and amplified.

TABLE 2. Restriction Enzyme Buffers

Buffer	Label	10x Stock
Low salt buffer	x10 L	100 mM Tris-HCl, pH 7.5 100 mM $MgCl_2$ 10 mM dithiothreitol
Medium salt buffer	x10 M	10 mM Tris-HCl, pH 7.5 100 mM $MgCl_2$ 10 nM dithiothreitol 500 mM NaCl
High salt buffer	x10 H	500 mM Tris-HCl, pH. 7.5 100 mM $MgCl_2$ 10 mM dithiothreitol 1000 mM NaCl
Potassium buffer (KCl)	x10 K	200 mM Tris-HCl, pH 8.5 100 nM $MgCl_2$ 10 mM dithiothreitol 1000 mM KCl
Tris acetate buffer BSA-free	x10 T	330 mM Tris-acetate, pH. 7.9 100 mM Mg-acetate 5 mM dithiothreitol 660 mM K-acetate

(Reprinted, with permission, from Amersham Pharmacia Biotech, UK Limited.) Make 10x stocks of restriction buffers, and freeze in 1-ml aliquots. With 1-ml stocks of 0.1% BSA and 0.1% Triton X-100, the buffer needs of most restriction enzymes can be met.

The DNA template is first denatured by high temperature. The temperature is lowered, and two oligonucleotide primers that flank the DNA fragment to be amplified are annealed to their complementary sequences on opposite ends of the target sequence. With a DNA polymerase in the solution, the temperature is increased slightly: The primers are extended and the region between the primers is synthesized. The strands are again denatured, new primers are annealed, and DNA synthesis is allowed to take place again ... and again and again, 20 to 50 times.

A temperature cycler, a programmable water bath, is used to achieve the rapid changes of temperature the process requires. The DNA polymerase used is *Taq* DNA polymerase, which is derived from the thermophilic bacterium *Thermus aquaticus* and can thus function at high temperature. The process is brilliantly simple, but the problem is that any contaminating DNA can be amplified, sometimes in preference to the desired template.

Contamination of one DNA with another is the bane of the PCR user. With amplification possible with such minute pieces of DNA, it is horrendously easy to find spurious bands resulting from DNAs accidentally introduced during sample preparation.

The major source of contamination seen in PCR laboratories is the DNA obtained as a product from previous PCR procedures, which arises from the aerosols generated during the pipetting and manipulation of the PCR samples. Because of this, many labs have a separate sample preparation area, physically distant from the

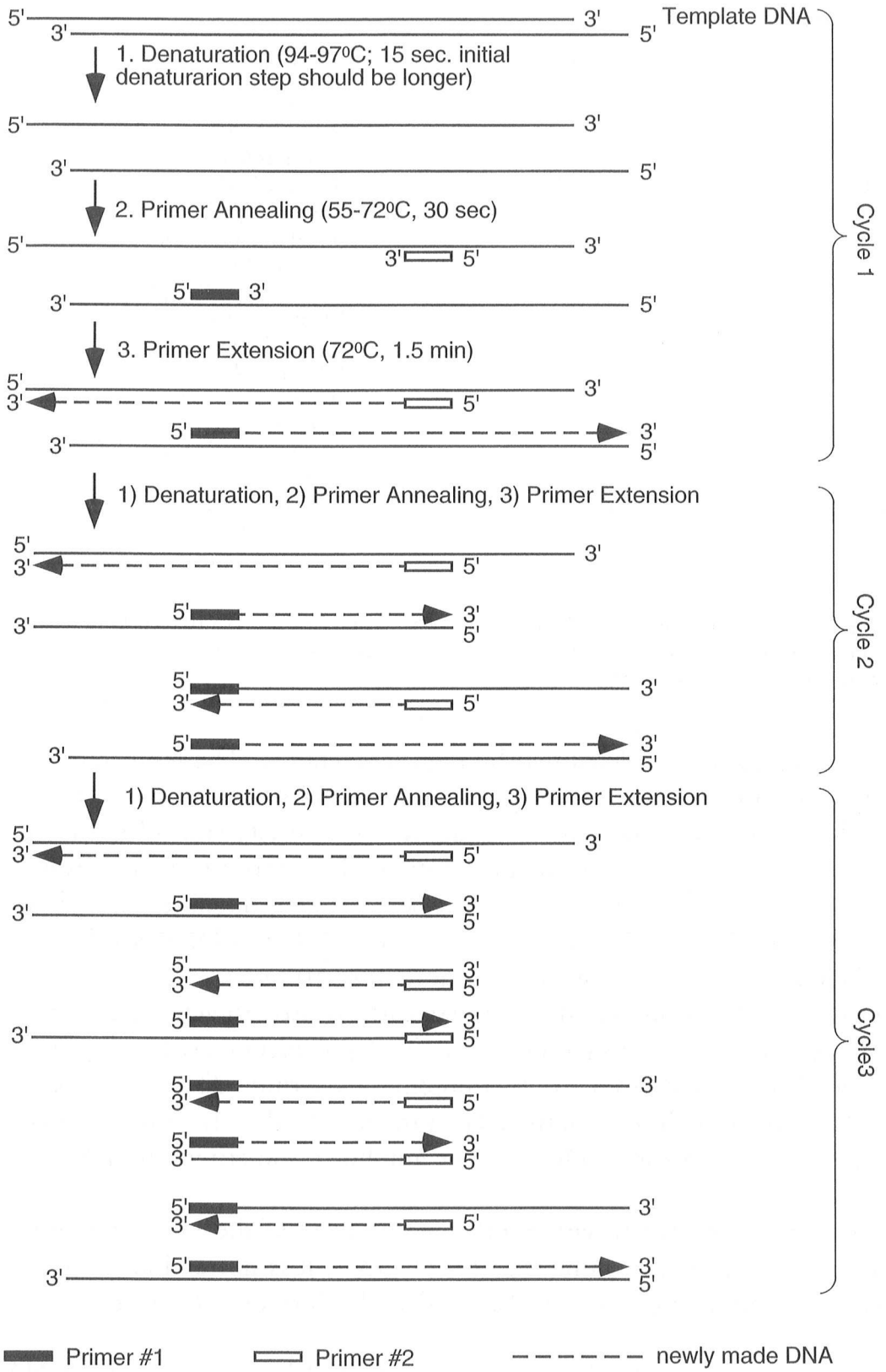

FIGURE 3.
Amplification of a specific sequence of DNA with PCR. After the first two or three cycles, the single-strand overhangs are ignored and the vast majority of the product is double-stranded target sequence. (Redrawn, with permission, from Zyskind and Bernstein 1992.)

PCR machine and from any area in which the post-PCR tubes will be opened. Another source of contamination of mammalian DNA is DNA from skin cells. Wear gloves.

Basic PCR Rules

- Prepare samples away from the PCR machine. Most labs have a separate sample preparation area, physically distant from the PCR machine and from the area where tubes will be opened after PCR. Whether or not there is an area dedicated to sample preparation, never prepare your samples near the PCR machine or where further manipulation of the sample will be done. Try to get a consensus to establish strict rules about sample preparation.

Briefly centrifuging the tube prior to opening will reduce aerosols.

- Keep separate pipets and other supplies for setting up PCR reactions. A positive displacement pipettor will prevent aerosols and reduce the chance of sample carryover. Pipettor tips with filters will also prevent carryover from sample to sample.
- Wear gloves and change them frequently. This will help prevent contamination of the sample with epithelial cells from the hands.
- Add the DNA last to all reaction tubes.

Contamination can be removed physically and enzymatically (Dieffenbach and Dveksler 1995), but it is best to prevent it.

Oligonucleotides

Oligonucleotide primers are synthesized in the lab, at a departmental facility, or by a company. It may be obtained as a crude, dried-down product, column- or HPLC-purified. You will be given instructions on how to further purify the product, if necessary, and how to store it. The more pure the product, the more expensive it will be.

There are software packages available to help in designing oligos. You can sit down with a pen and paper and do it, but a computer program can really help in designing the best primers and giving the optimum annealing temperature. Companies that supply oligos can help you with analysis. You could go on line, and use one of the programs available on the WWW (see Resources), or ask for a program recommendation from a newsgroup or another investigator.

PROTOCOL

Ethanol Precipitation of Oligonucleotides

1. Add 1/10 volume of 3 M NaOAc, pH 6.5, and three volumes of cold 95% ethanol.
2. Place at –70°C for at least 1 hour.

INTRODUCING DNA INTO CELLS AND MICROORGANISMS

After isolation, DNA is not only isolated and analyzed, but is often introduced into cells and microorganisms with the intention of altering the genotype of the recipient or harvesting quantities of the donated DNA or its translated protein.

Prokaryotic Cells

Transformation is the genetic change in a bacterium after exposure to and recombination with an isolated DNA. This is routinely done in order to amplify cloned DNA. Another common reason is to obtain quantities of a particular protein: The bacteria are transformed with DNA contained in an expression vector, and will pump out large amounts of the protein coded for by the DNA.

There are two main methods of bacterial transformation:

- *Incubation* with high concentrations of Ca^{++} ions, which causes the bacterial plasma membrane to admit foreign DNA. This is simple and takes a few minutes at the bench.
- *Electroporation.* Higher efficiencies of transformation are possible with electroporation. The conditions for maximum electroporation are different for different species and even different strains.

With either method, the bacteria must first be rendered competent; that is, able to take up DNA. The bacteria must be grown to a particular density, harvested, and washed with salts: Competent cells can then be aliquoted and frozen. There are slight variations between the methods for producing chemically competent versus electroporation-competent bacteria. Competent cells are available commercially.

Eukaryotic Cells

There are several methods of gene transfer for eukaryotic cells, and the success of a particular method is very species- and strain-specific. The process is termed transfection, a hybrid of transformation and infection: Virally mediated gene transfer is also called infection.

In a stable transfection the cells which have taken up the DNA are selected by expression of a reporter gene. (Traditionally, this is antibiotic resistance to hygromycin or neomycin: There are many alternatives, such as FACS selection of cells expressing Green Fluorescent Protein [GFP].) Cell lines can be made from individual clones of expressing cells. Transient transfections are more likely to be done to gather immediate information about the effect of the DNA on the cell. In general, transient transfections are of limited use since variable numbers of cells contain the gene of interest (the precise number is governed by the transfection efficiency), and the cells that do contain the appropriate DNA have variable copy numbers. In addition, results can be difficult to interpret since the recent transfection is extremely traumatic, and this damage is hard to control for. Analysis of transient transfectants can be of use in defining an early, preliminary sense of the phenotype that can be expected in the stable transfectants.

Transfection of eukaryotic cells is done in many ways, including:

- Calcium phosphate coprecipitation.
- DEAE-dextran-mediated transfection.
- Lipid-mediated transfection.
- Electroporation.
- Microinjection.
- Virally mediated transfer. Adenovirus, retrovirus, and SV-40 can be used to introduce DNA into cells.

Reporter genes are genes used to locate, identify, or analyze another gene. They may be coupled to the upstream sequence of another gene and transfected into cells to study the regulation potential of the potential upstream sequence: This is the more stringent definition of a reporter gene. The CAT gene, coding for chloramphenicol acetyltransferase, is the classic gene used to evaluate gene regulation in eukaryotic cells. More loosely, reporter genes may be transferred into cells or bacteria just to monitor whether or not the foreign DNA is being expressed: antibiotic resistance genes are an example.

Other reporter genes are β-galactosidase, β-glucuronidase, and alkaline phosphatase (if the enzyme is present and interacts with the substrate, a colored or fluorescent product results). GFP, an autofluorescent protein from the jellyfish Aequorea victoria, is becoming the reporter gene of choice, since it requires no activation or enzymatic activity to be visualized.

Since the efficiency of DNA transfer is so dependent on the cell type, you must investigate the transfection protocols used for your cells before you waste a lot of time. Check the literature, go on line, and make phone calls to ask about protocols.

Recommendation: Make sure you have access to an electroporator. You will be able to transform bacteria, yeast, mammalian cells, and many exotic cell types. Some older models lack the capacity to electroporate mammalian cells, but an attachment that allows this can be purchased.

The vector is of critical importance in determining the outcome of the transformation or transfection. In choice of vector, you will have a great deal of help. Companies such as Invitrogen or Promega offer vectors with inducible promoters, reporter genes, the ability to be propagated both in eukaryotes and in bacteria, and technical help.

RNA

RNA degradation was such a serious problem that, for years, people would avoid all RNA work like the plague. But a few rules, similar to those of aseptic technique, keep everything running smoothly.

Basic rules

- Autoclave all plasticware and glassware that will touch anything that will touch the RNA.
- DEPC (diethylepyrocarbonate)-treat all water that will be used to make DNA buffers. Add DEPC to final 0.1%, leave overnight at room temperature, and autoclave for 15 minutes. Don't use DEPC for Tris buffers, as the DEPC will decompose into ethanol and carbon dioxide.
- Avoid alkaline buffers, as the hydroxy group in RNA makes the molecule very sensitive to alkali.
- Keep RNA buffers separate from other buffers, so they won't be accidentally used and contaminated with RNases.

RNA Isolation

Reagents such as guanidine hydrochloride or guanidinium isothiocyanate act as chaotropic agents and maintain the integrity of RNA. For RNA isolation, it makes sense to use a kit or prepared reagent. Many companies now offer an RNA-isolation reagent that allows the simultaneous isolation of DNA, RNA, and protein from cells or tissue samples. Even if you only need the RNA, these reagents are a worry-free way to work with RNA.

PROTOCOL

Ethanol Precipitation of RNA

Usually, RNA can be precipitated as can DNA.

Procedure

1. Add 1/10 volume of 1 M NaOAc, pH 4.8, and 2.5 volumes of cold 95% ethanol.
2. Precipitate overnight at –20°C.
3. Wash the pellet with 70% ethanol.

Note: When you are working with small amounts of RNA (under 5 µg), add a carrier or coprecipitant to the RNA before precipitation. This material will precipitate with the RNA and give you a visible pellet that is much easier to work with. Two common carriers are molecular-biology-quality glycogen and yeast RNA.

PROTOCOL

Selective RNA Precipitation

This protocol has been adapted, with permission, from Epicentre Forum 1996, p. 10, Epicentre Technologies, Madison,Wisconsin. If you have a DNA–RNA mix (for example, after in vitro transcription reactions), you can precipitate just the RNA with ammonium acetate as the salt.

Procedure

1. Add 5 M ammonium acetate to the RNA to a final concentration of 2.5 M.
2. Chill the mixture on ice for 15 minutes.
3. Centrifuge in a microfuge at high speed for 15 minutes at 4°C.
4. Remove the supernatant and wash the pellet with 70% ethanol.
5. Resuspend the RNA pellet in the desired volume of RNase-free water or buffer.

Note: Since ammonium acetate decomposes by loss of ammonia (and base is harmful to RNA), solutions should be prepared only from the pure salt which has been kept cool in a closed container. Sterilize the solution by filtration and store it at 4°C.

mRNA Isolation

mRNA is a small (generally 5–10%) proportion of the total RNA in a cell, the bulk of which is ribosomal RNA. Eukaryotic mRNA isolation methods take advantage of the polyadenylated tail present only on most of the mRNAs (there are a few mRNAs without poly(A) tails). Poly(A) RNA will bind to a resin made of oligo(dT)—cellulose will bind the mRNA under high salt conditions, and will be eluted with a low salt wash. This can be done in batch or in small columns. Some mRNA isolation kits allow the direct isolation of mRNA from cells.

Determining RNA Concentration

RNA concentration and purity can be determined by UV spectroscopy, as described for DNA. There are two differences:

- One A_{260} unit of RNA is 40 µg. The RNA concentration (µg/ml) =

$$(OD_{260}) \times (\text{dilution factor}) \times \frac{(40\ \mu g\ \text{of RNA/ml})}{1\ OD_{260}\ \text{unit}}$$

- Sample volume is usually kept as low as possible, to limit the use of the RNA to measure O.D. You can buy and use mini UV cuvettes, or determine the minimum volume your UV cuvettes can hold and give accurate readings.

PROTEIN

Degradation is the fear permutating protein work.

Basic rules

- *Ice, ice, ice.* Always have a bucket of ice handy when you are doing any protein work, and put tubes on ice immediately when removing them from freezers, centrifuges, etc.

- *Spin cold,* unless otherwise noted. Centrifuges can get quite warm.

- *Know your protein.* The properties of individual proteins vary greatly. Is it denatured by heat? Does it have disulfide bonds? Can it be repeatedly frozen and thawed? If you don't know, assume that it cannot be frozen and thawed, and that it can be heat-denatured.

- During and after cell lysis, include the appropriate *protease inhibitors* in all buffers.

Isolation

The ease of producing large amounts of protein with a bacterial or baculoviral expression system is not always accompanied by ease in isolating your protein. Despite the tricks for binding expressed fusion proteins, the characteristics of the particular purified protein can alter the way the system should work. Before you scale-up production, be sure you can isolate the protein effectively.

There are a lot of tricks to protein isolation. Although DNA, and even RNA, work can pretty much be done by following a manual, protein work is more complicated: Each protein has its own profile and personality. Seek help from someone in a protein lab.

Chromatography

Proteins, as well as DNA and RNA, are routinely separated and isolated by chromatography. This need not conjure up images of huge columns in the cold room or HPLC machines, as chromatography can as well be performed in a microfuge tube. The main kinds of chromatography done are gel filtration, ion exchange, and affinity chromatography.

- **Gel filtration:** The separation of compounds on the basis of molecular size.

  How it works: The stationary phase contains pores, which trap only smaller molecules.

  Example of materials: Sephadex (cross-linked dextran), Sephacryl (cross-linked copolymer of allyl dextran and *N,N'*-methylenebis(acrylamide), Sepharose (beaded agarose).

  Example of use: Removal of unincorporated nucleotides from a nick translation reaction on a G-50 (a kind of Sephadex) column.

- **Ion exchange:** The separation of compounds on the basis of charge.

  How it works: Association of the protein or other material with the charged groups of a solid support, followed by elution with an aqueous buffer of higher ionic strength. Column material can be anionic, cationic, or mixed bed (both).

  Example of materials: DEAE (Diethylaminoethyl) cellulose, amberlite, Dowex, CM-Sepharose.

  Example of use: Isolation of DNA from a gel by electophoresis onto DEAE paper.

- **Affinity chromatography:** The separation of compounds on the basis of natural binding site.

  How it works: The molecule to be purified is specifically and reversibly adsorbed by a ligand immobilized on an insoluble support.

  Example of materials: Oligo(dT) cellulose, biotin, heparin.

  Example of use: The binding of antibodies to Protein A.

Many chromatography materials need to be soaked and swollen before use, or they won't work effectively. Some materials require an overnight incubation in buffer.

Dialysis

Dialysis is done to remove salts or other impurities from a sample. The sample is placed in a porous tubing: The size of the pores in the tubes will permit only molecules of sizes smaller than the pores to exit. The material in the dialysis bag is placed in a large volume of water or buffer, allowing the outward passage of contaminants and the eventual replacement of the buffer contents of the tubing by the dialysis buffer.

PROTOCOL

Preparing the Tubing

Prepared dialysis tubing is a good item to share, as few single investigators can use it up before it should be discarded. Make an arrangement with other lab members to do this.

The tubing must be cleaned and prepared before use. Wear gloves for all manipulations.

1. Choose tubing of the appropriate MW cutoff.
2. Prepare the dialysis tubing. This can be done to a package, and the prepared tubing can be stored at 4°C.
3. Place the tubing in a large volume (a liter for a full package) of 5 mM EDTA, 200 mM sodium bicarbonate in a flask.

Sodium bicarbonate	FW 84.01	use 16.85 g/l
EDTA	FW 372.24	1.86 g/l

4. Boil for 5 minutes.
5. Pour off the bicarb/EDTA, rinse the tubing briefly with deionized water, add another large volume of bicarb/EDTA, and boil again for 5 minutes.
6. Discard the second wash. Rinse the tubing well with deionized water.
7. Add a large volume of deionized water, and cover the flask with aluminum foil.
8. Autoclave for 10 minutes on the liquid cycle (slow exhaust).
9. Store at 4°C in a sterile container with an opening large enough to permit easy removal and replacement of the tubing. If the tubing will be stored for longer than a few days, add sodium azide to a final 0.02% to the water and tubing.

Sodium azide will inhibit bacterial growth. Weigh it out very carefully, as it blocks the cytochrome electron transport system. Be aware that it can also inhibit some enzymatic reactions. Keep a small volume in the refrigerator of filter-sterilized 2% azide in water, and add 1 µl for every 100 µl of solution.

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Setting up Dialysis

1. Cut a piece of tubing large enough to contain the material you want to dialyze. Allow approximately 2 inches at each end (4 inches in total) for tying off the tubing. (Special chambers can be purchased for "micro" dialysis.)

2. Either knot one end of the tubing, or use a dialysis clamp to close one end.
3. Open the other end with a gloved finger, and keep one finger in the tubing. Pipet or pour the material into the tubing carefully.
4. Tie off or clamp the other end. Remove air bubbles by pushing them before you clamp. Check for leaks, especially at the ends.
5. Place the filled tubing in as large a beaker or Erlenmeyer as you can find: 2–4 liters is a good size.
6. Add a stir bar and place on a magnetic stirrer, usually in a cold room. If the tubing sinks to the bottom of the container, and that worries you, tie the tubing on one end with string, hang it from the container, and attach it to a weight. You could also allow an excess of dialysis tubing at one end, which can be taped to the outside top edge of the beaker, with the filled tubing left suspended in the beaker. But unless the dialysis tubing is very turgid and immobilized on the bottom of the container, a gently turning stir bar will not break it.
7. Usually, dialysis is performed overnight. Change the buffer once if you have a 2–4 liter container, more often for smaller containers.

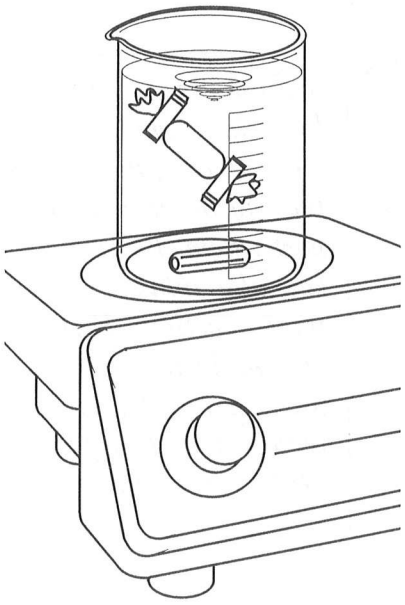

FIGURE 4.
Dialyze your material against as large a volume as is practical.

Cell Lysis

When isolating a protein, the cell or bacteria containing the protein must first be lysed.

Physical lysis. This can be done physically, with machines of various disruptive potentials. For example:

- *Nitrogen cavitation bomb.* Nitrogen decompression is one of the gentler ways to open up eukaryotic cells. Nitrogen under pressure is allowed to equilibrate within cells. When the pressure is released, the nitrogen comes out of solution and the popping bubbles break the individual cell's membrane. No further disruption of the cell occurs, and organelles can be preserved intact. In addition, the reducing atmosphere and cool temperature provided by the nitrogen protect the proteins from degradation.

- *Homogenizer.* Basically a blender, subjecting the cells to shearing forces. Not suitable for bacteria, unless glass beads are added.

- *Ultrasonic processer (sonicator).* Sonic pressure waves create microbubbles, which can not only break open cells, but can shear DNA. Glass beads can be added to disrupt bacteria.

- *Freeze press.* In the freeze press, frozen cells are forced through a narrow orifice, causing shear stress and explosive decompression powerful enough to break open tough cell walls.

- *Bead mill homogenizer.* Bacteria, spores, or yeast, usually, are vigorously vortexed in a tube containing glass or zirconium beads. The movement against, and bombardment of, the cells by the beads breaks even tough cells open within minutes.

Cell lysis is relatively easy for eukaryotic cells, compared with yeast, bacteria, spores, and plant cells, with tough cell walls, and is usually accomplished by detergent lysis, nitrogen decompression, or homogenization.

Detergent. Detergents are sufficient to lyse many eukaryotic cells. The detergent used is dependent on the application for the lysed cells. Check the source of the detergent carefully, because the quality and purity of the detergent have a big effect on the success of protein isolation.

- Examples of anionic detergents are the salts of cholic acid, caprylic acid, sodium dodecyl sulfate (SDS), and deoxycholic acid.
- Examples of cationic detergents are cetylpyridinium and benzalkonium chloride.
- Examples of zwitterionic detergents are CHAPS and phosphatidylcholine.

- Examples of nonionic detergents are digitonin, Tween-20 (polyoxyethylenesorbitan, monolaurate), and Triton X-100.

Nonionic detergents are much milder, and don't perturb the nuclear membrane: They are often used to lyse cells prior to immunoprecipitations. A 0.1% Triton X-100 solution in water works to lyse most mammalian cells, and up to 0.5% will not harm most enzymes being isolated. Many enzymes, such as Proteinase K, remain active in the presence of Triton X-100.

Protease inhibitors. When a cell is lysed and the contents released, proteases and other degradative enzymes are released as well. Unless proteases are included in the lysis buffer, the cell's own proteases will break down the cellular proteins.

Other inhibitors, specific to the protein you are studying, may also be used. For example, sodium vanadate, an inhibitor of protein phosphatases, is added to lysis buffers when a phosphorylated protein is being isolated.

TABLE 3. Protease Inhibitors

Inhibitor	Protease target	Effective concentrations	Stock solution	Comments
Aprotinin	Serine proteases	0.1–2 µg/ml	10 mg/ml in PBS	Avoid repeated freezing
EDTA	Metalloproteases	0.5–2 mM	500 mM in H_2O, pH 8.0	
Leupeptin	Serine and thiolproteases	0.5–2 µg/ml	10 mg/ml in H_2O	
α-Macroglobulin	Broad spectrum	1 unit/ml	100 units/ml in PBS	Avoid reducing agents
Pepstatin	Acid proteases	1 µg/ml	1 mg/ml in methanol	
PMSF	Serine proteases	20–100 µg/ml	10 mg/ml in isopropanol	Add fresh at each step
TLCK	Trypsin	50 µg/ml	1 mg/ml in 50 mM acetate, pH 5.0	Chymotrypsin unaffected
TPCK	Chymotrypsin	100 µg/ml	3 mg/ml in ethanol	Trypsin unaffected

Derived from Boehringer Mannheim Biochemicals (1987). (Reprinted, with permission, from Harlow and Lane 1988.)

Determining Protein Concentration

Which to choose

The most common methods of protein determination are the Bradford, BCA, and absorbance at 280 nm. Labs tend to be dedicated to a particular assay: **Try the lab assay first,** before you order new reagents. The Bradford is the best all-purpose assay to use.

- You cannot directly compare the results of one assay method with another. You must get used to working with the relative concentrations determined by one method. For example, BSA gives a value about twofold higher than its weight for the Bradford assay.
- The nature of your protein sample will also suggest which assay to use. If you know you have a purified protein without tryptophan, you shouldn't rely on absorption at 280 nm. And if you must have detergent in the protein sample, you must choose a method that is not particularly detergent sensitive, or you must remove the detergent (see below).
- For all methods you must run your unknown samples against a standard curve, every time you perform the assay. Any purified protein can be chosen as a reference standard, if only relative protein concentrations are desired. Bovine serum albumin (BSA) and IgG are commonly used: Use BSA unless you are measuring antibodies.

BCA

How it works. Copper sulfate, added to an alkaline solution of BCA (bicinchonic acid), gives an apple-green colored complex. When this solution is added to a protein solution, the Cu^{++} ions are converted to Cu^{+} by interaction with the peptide bonds of the protein, changing the color of the complex to purple with an absorbance maximum of 562 nm. Pierce makes a BCA assay reagent.

Advantages. Fast, sensitive, accurate.

Disadvantages. Subject to interference by agents such as detergents and organic solvents. Time dependent, color develops for 24 hours.

Bradford

How it works. Utilizes the dye Coomassie blue G-250, which is red-brown at a pH below 1 but turns blue when binding to protein causes a shift in the pKa of the bound dye. Blue color is measured at 595 nm. The Bradford reagent is available from Bio-Rad.

Advantages. Fast, sensitive, accurate. Not time dependent.

Disadvantages. Detergent concentrations over 0.2% interfere with the assay.

Absorption at 280 nm

How it works. The aromatic amino acids, especially tryptophan, absorb strongly around 280 nm. All proteins that contain aromatic residues (or UV

absorbing cofactors) have a unique extinction coefficient at 280 nm.

Advantages. Speed. Sample is not destroyed.

Disadvantages. Not as accurate as other methods.

Biuret

How it works. Measures the peptide bonds. O.D. read at 540 nm.

Advantages. Rapid. Good for monitoring protein separation, since salt interferes less than with the Bradford.

Disadvantages. Not very accurate for low protein concentrations.

Lowry (Folin-Ciocalteu)

How it works. Similar to BCA. O.D. read at 750 nm.

Advantages. Need very little material. Bio-Rad's detergent-compatible assay is based on the Lowry.

Disadvantages. Depends on the presence of tyrosine in the protein.

Detergent in the samples?

Detergents are a part of life with proteins, since they are used to lyse cells and to denature proteins. But they can interfere with determination of protein levels and with protein function.

To determine the protein level in a sample containing detergents, you have two options:

- Include the same percentage detergent in the standard curve. This is a necessity, and you may be lucky—you may get an accurate determination. More likely, however, the addition of detergent severely reduces the linear readings of your standards: You may be able to dilute samples (and hence, the detergent level) to be in the linear range, but this is not possible for low protein levels.

- Use a protein assay that is compatible with detergents. Several companies have reagents that can be used for samples with or without detergents. For example, the usual Bio-Rad protein assay is based on the Bradford, but does not accommodate detergent in the samples. However, the Bio-Rad DC Protein assay, based on the old Lowry, is compatible with both ionic and nonionic detergents.

If you are isolating a protein, you probably will have to remove the detergent. The method of detergent removal will depend on the detergent, the protein, and the buffer. Generally, detergents with a high critical micelle concentration (CMC) are easy to remove by dilution, and those with low CMC can be removed on the basis of molecular weights. You will need to seek advice for your particular situation: Especially if you have a hard-to-get protein, this is not the time to experiment. The wrong temperature or salt concentration can very quickly turn your precious solution into crystals or mud.

Possibilities for detergent removal (adapted from Harlow and Lane 1988, p. 688)

1. Ionic detergents
   - Use gel filtration on a G25 column. For some proteins, equilibrate column in another detergent below its CMC.
   - Add urea to 8 M, then bind detergent to an ion-exchange column. Protein flows in 8 M urea: Dialyze to remove urea.
   - For ionic detergents with a relatively low micelle size and high CMC: Dilute as much as possible and dialyze. Add mixed bed resin to dialysate to increase the exchange rate.
2. Nonionic detergents
   - Use gel filtration on a G200 column. For some proteins, equilibrate column in another detergent below the CMC.
   - Dilute if possible, dialyze extensively against DOC, then slowly remove DOC by dialysis.
   - Use velocity sedimentation into sucrose without detergent.
   - Bind protein to affinity matrix or ion-exchange column, wash extensively to remove detergent, then elute protein. For some proteins, equilibrate column in another detergent below the CMC.
3. Amphoteric (Zwitterionic) detergents.
   - Dilute if possible, dialyze.

Antibodies

Antibodies are proteins secreted by lymphocytes and directed against foreign molecules. They are an important component of the immune system, and a vital tool of the lab.

Polyclonal antibodies are raised against an antigen in vivo by injection of an animal with the antigen. Polyclonal antibodies are a heterogeneous mixture of immunoglobulins of various affinities directed against different epitopes of the same protein.

Monoclonal antibodies are made in vitro, the product of a cell fusion between an immortalized myeloma cell and an antibody-secreting plasma cell. The entire culture will produce just one antibody, of one class (usually IgG), directed against one epitope.

Obtaining antibodies

- Antibodies can be obtained commercially or from other investigators, or you can make them yourself. See Chapter 9 for suggestions on sources of antibodies which can be obtained from many of the same places as cells. The WWW site, The Antibody Resource Page, contains links to many antibody sources.

- Polyclonal antibodies are relatively straightforward to make. If you will require a regular supply of a polyclonal antibody, you should make (or have made) antibody. Don't attempt to make monoclonal antibodies unless you are in a lab or department in which this is done routinely.

- Be considerate when you ask other investigators for polyclonal antibodies. Unlike monoclonals, they are in limited supply. If your experiment is successful and you will need more antibody, you must make or buy your own.

Uses. Antibodies can be labeled with a radioactive or enzymatic tag, allowing the protein they are recognizing to be visualized and quantitated.

- *Cell staining.* Labeled antibody can be used to localize cell proteins.

- *Immunoassays.* The antibody is a reagent used to test the function or presence of an antigen.

- *Immunoblots.* Also known as Western blots. The presence and amount of a protein can be detected on a sample immobilized on a filter.

- *Immunoaffinity.* The protein against which the antibody is directed can be isolated and purified.

TABLE 4. Immunochemical Techniques, Polyclonal versus Monoclonal Antibodies

Technique	Polyclonal antibodies	Monoclonal antibodies	Pooled monoclonal antibodies
Cell staining	Usually good	Antibody dependent	Excellent
Immunoprecipitation	Usually good	Antibody dependent	Excellent
Immunoblots	Usually good	Antibody dependent	Excellent
Immunoaffinity purification	Poor	Antibody dependent	Poor
Immunoassays			
labeled antibody	Difficult	Good	Excellent
labeled antigen	Usually good	Antibody dependent	Excellent

(Reprinted, with permission, from Harlow and Lane 1988.)

Storage

- Antibodies are best stored at –20°C, in aliquots: freeze-thawing is not good for most antibodies.
- A working aliquot can be stored at 4°C for at least 6 months.
- Sodium azide can be added to a final volume of 0.02% to inhibit bacterial growth.

Tips

- Know the animal from which your antibody has been derived, as this will determine the secondary antibody used in some assays. Polyclonals are usually from rabbit or donkey: Monoclonals are usually from mouse, rat, or hamster.
- Quickly spin your antibody in a microfuge before use to remove any precipitated material. This can prevent high background in a variety of assays.
- Protein A, a 42-kD polypeptide isolated from the cell wall of *S. aureus,* and Protein G, a 30–35-kD polypeptide isolated from some β-hemolytic streptococci, bind strongly to antibodies, and are useful tools to immunoprecipitate or localize antibodies. Protein A and G can be bound to beads, or labeled, and are good for most (not all) subclasses of antibodies.

RESOURCES

Antibody Resource Page.
http://www.antibodyresource.com

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BioGuide-PCR
J. Weizmann Institute of Science, Genome and Bioinformatics
http://bioinformatics.weizmann.ac.il/mb/bioguide/pcr/contents.html
Lists of programs for designing PCR primers.

Cell and Molecular Biology Online.
http://www.tiac.net/users/pmgannon/faq.html#technique
Recommended for its many links to protocol and reagent sources.

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