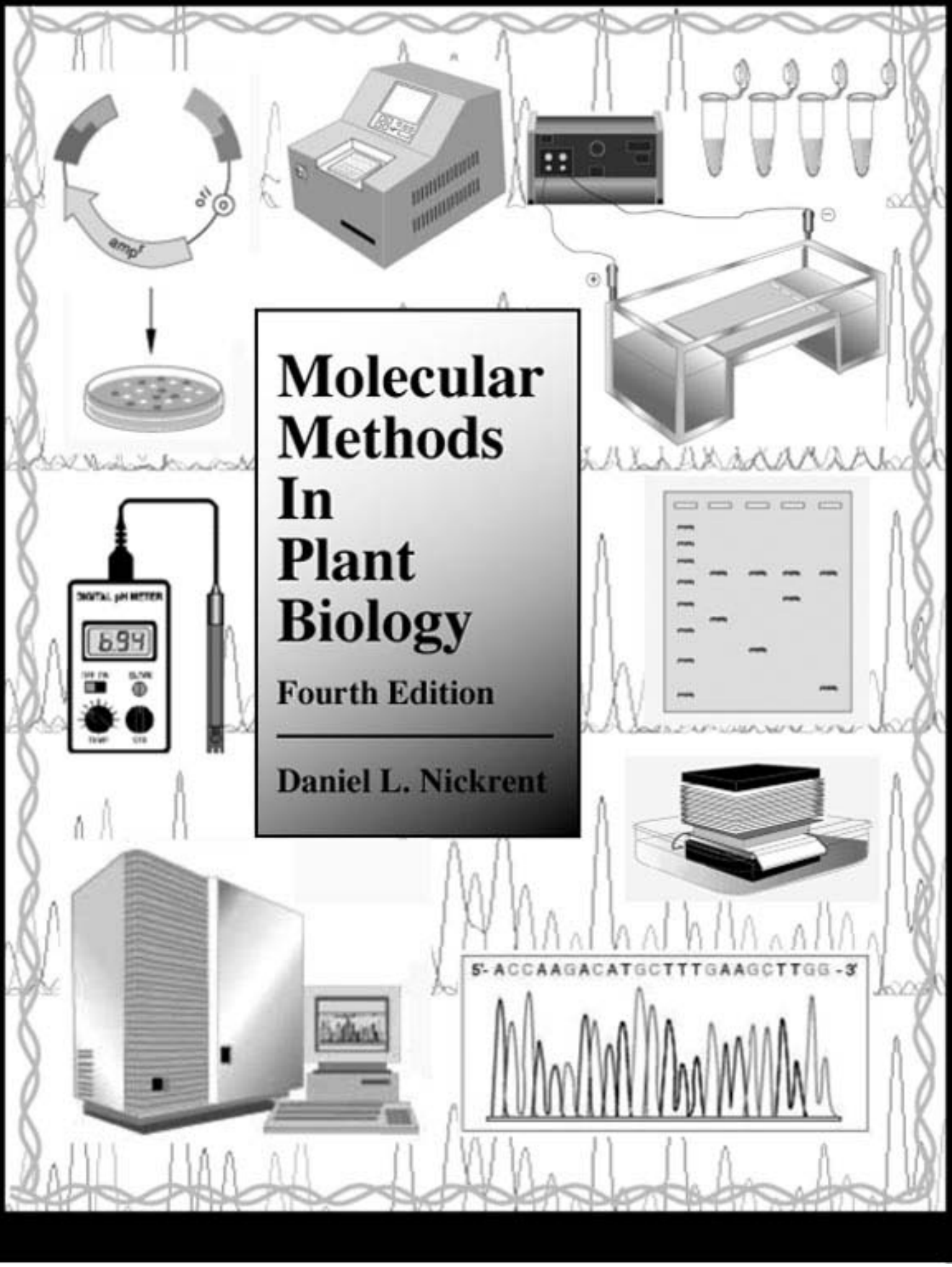

Molecular Methods In Plant Biology

Fourth Edition

Daniel L. Nickrent

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SECTION 1: LAB RULES AND SAFETY

Room Organization and Equipment

My laboratory is well-equipped to conduct projects involving isozymes and nucleic acids (RNA and DNA). This lab contains a number of sensitive and expensive pieces of equipment, each of which requires some knowledge for its proper operation. For the high-speed and ultra centrifuges you must be "checked out" by DLN prior to use. As a general rule, if you do not understand how to operate a piece of equipment, ***ASK*** before proceeding. If it appears that the instrument is malfunctioning (unusual noises, movements, etc.), please discontinue use and report the incident.

The rooms that are available to researchers working in my lab are listed below with their major equipment items.

• Rm. 1046 (main lab):
Balance, analytical (Ohaus GA-110)
Balance, top loading (Ohaus GR-4100)
Freezer, ultralow – 80˚ C (Revco)
Freezer, upright –20˚ C 22 cubic ft. (Lab Research)
Gel rigs, agarose electrophoresis, various sizes (Owl)
Heat block (Equatherm Temp-Blok)
Homogenizer (Brinkman Polytron)
Homogenizer (Omni)
Hotplate, stirring (Corning PC-320)
Incubator 37˚ C (Gravity Convect Fisher 655D)
Microcentrifuge (Sorval – in dairy case)
Microcentrifuge (Fisher accuSpin – room temp.)
Micropipets (Rainin and Oxford)
Microwave oven
pH meter (Fisher Acumet AB15)
Picofuge
Power supplies constant voltage (EC250-90)
Power supplies, not constant voltage (Fisher FB-105)
Refrigerator, three-door dairy case style (United)
Speed Vac (Savant)
Thermocycler (Applied Biosystems GeneAmp PCR System 9700)
Thermocycler (Perkin Elmer)
Thermocycler (RoboCycler Gradient 40 Stratagene)
Ultracentrifuge, bench-top (Beckman)
Vacuum pump (Savant - PLB)
Vacuum manifold (Promega)
Water bath, shaking (Belco Hot Shaker)
Water purification system (Barnstead Nanopure)

• Rm. 1046A (genomic DNA extraction and PCR)
Centrifuge, high-speed refrigerated floor model (DuPont RC-5B)
Freezer, upright 16 cu ft. –20 C (Frigidaire)
Heat block (Equatherm)
Hotplate, stirring (Corning PC-320)
Micropipets (Rainin and Oxford)
Picofuge (Qualitron)
Rotors for RC-5B (8, 12-place and swinging bucket)
Water bath (10 liter, Fisher)

• Rm. 1048. This room is shared with Dr. David Clark in Microbiology. We previously used this room when doing manual DNA sequencing that required the use of radioisotopes. Since we have converted to automated sequencing, we have not used the room for this purpose. On the south table I have a photo copystand. I also have a high temperature oven (Precision Gravity 18EG). The freezer is used by Dr. Clark for storing isotopes (such as ^{35}S).

• **Rm. 1047 (darkroom, shared with Microbiology**). In the past we used a typical UV transilluminator (Spectroline) to illuminate gels and captured the image on film using a gel doc system (Polaroid, using 667 film). We now have a computer connected to a digital camera (Nikon Coolpix 950) that is mounted on an enclosed UV transilluminator. Once the gel image is captured and transferred to the computer (Del Dimension XPS Pro200n), we open the file using NIH Image software. The image is then printed on a Mitsubishi thermal printer.

• **Rm. 1050 (common use).** Ice machine, dishwashers, dish drier, autoclave.

• **Rm. 1052 (collections, microscopy).** This room contains an herbarium case with my collection of dried plant parasites, a metal storage case with my fixed parasitic plant specimens, and a metal storage case with my 35mm slide collection. Equipment includes: Dissecting microscope Stereozoom (Olympus SZH-ZB10), light box (Picker) – for viewing/sorting slides, and a slide viewer (Caramate Singer).

• **Rm. 1054 (walk-in cold room).** Shared with Microbiology. Storage for chemicals, etc. Water bath (can be set at 17˚ C, useful for ligation reactions).

• **Rm. 1055 (store room).** Storage racks and a cabinet for lab supplies (tips, tubes, etc.). Ultralow – 80˚ C freezer (Revco). This contains a back-up genomic DNA collection and frozen samples (fresh frozen, silica dried) of various plants arranged in boxes by family.

• **Rm. 1057 (backup server).** This room formerly had various BioRad equipment items run by two Gateway computers. The room is now used only for the Plant Biology backup server (Macintosh iMac).

• **Rm. 1056 (phytotron).** This facility is being used by Dr. Stephen Ebbs in Plant Biology. The headhouse portion contains growth chambers, an autoclave and a dishwasher.

• **Available for use in room 1045**. Dr. Clark kindly allows us to use his shaking incubator (New Brunswick) when we are cloning. The key on the lab keychain will open this door.

In the past, the east side of Rm. 1046 was dedicated to isozyme work, however, our research focuses mainly on DNA these days. When isozymes are being run, shelf space in the dairy case is used to run starch gels. The dairy case in Rm. 1046 is currently the only refrigerator (4˚ C) space in that room. Should extra storage space be required, use the walk-in cold room (1054).

Room 1046A is a **non-PCR product** environment. This means that none of the pipetters, tube racks, ice buckets, etc. from Rm. 1046 should be brought into this room. Similarly, none of these items in Rm. 1046A should be left into Rm. 1046. Tube racks, buckets, pipets, etc. can be brought into the room on a short-term basis to run gels, but then promptly returned to Rm 1046A. To help all of us remember this, we have designated the red ice buckets and tube racks for 1046A and all other colors for 1046. It is very important that all users adhere to this segregational policy to avoid contamination. For example, one of the worst possible type of contamination would be to introduce a PCR product into a tube containing genomic DNA. Because our genomic DNA samples must be kept in an ultralow freezer, by necessity they are present in Rm. 1046 (instead of 1046A where, ideally, they would be housed). All genomic

DNA samples must therefore be brought immediately into Rm. 1046A when they are being used, not 1046.

A number of solutions made in our lab are intended for multiple use. All such solutions must be labeled with the name of the solution, it's molarity and pH, the date, and initials of who made it. These include the reagents needed for agarose gel electrophoresis, loading dyes, MW marker ladder solutions, TAE buffer for agarose gels, etc. It is more practical for every individual to have his or her own PCR reaction box that includes Taq, buffer, water, $MgCl_2$, nucleotides, etc.

Although we do not use the darkroom for developing film anymore, it is still important to keep it clean and organized. At times we conduct agarose gel electrophoresis in this room and sometimes this results in spilled buffer on the bench. This should be cleaned up immediately, especially if the buffer contains ethidium bromide. When taking pictures of agarose gels with the Polaroid camera, do not take the last photograph and leave the camera empty. Learn to load the film and have it ready for the next person. When the back of the camera is open, examine the rollers and make sure they are clean (dried gel from the film accumulates).

Specifics on Equipment Operation and Maintenance

• **Ultralow freezers.** We maintain two -80° C freezers. ***I cannot over-emphasize the importance of these freezers and the samples they contain to our research program***. The one in Rm. 1046 contains genomic DNAs of parasitic and nonparasitic plants, primer stocks, Taq polymerase, dNTPs, and some of the parasitic plant tissue samples. The majority of plant tissue samples (fresh, silica dried) are kept in the freezer in Rm. 1055. Any malfunctioning of these freezers should be reported to me (DLN) at any time, day or night. In the event of a power outage, the freezer in 1046 is connected to an emergency outlet that will continue to have power via the building's generator. Such is not the case for the freezer in 1055. If a power outage last for more than 10 hours, the samples must be moved to another location. Microbiology maintains some overflow freezers, albeit with limited space. In terms of daily use, it is generally a good idea to leave the freezer doors open for as short a period of time as possible. Wear gloves before handling any of the metal racks. Obtain the samples you need quickly and work with further sorting, retrieving, etc. with the lid closed. Monitor the digital readout to avoid excessive temperature change. If the temperature increases to –65° C, the alarm will sound and will not stop until the preset low temperature has been achieved. When working with boxes containing genomic DNA samples, it is also important to work quickly to avoid thawing the tubes. Attempts are made to keep the DNAs sorted alphabetically by genus, but some boxes are full and may require some searching. Use securing rings on all the Styrofoam Sarstaed boxes to prevent spilling the contents. Especially during the humid summer months, frost will accumulate around the Styrofoam sublids. Use the plastic ice scraper to keep this area clear. The rubber gasket on the lid will not fit properly if ice accumulation becomes excessive. Frost on the sublids can be quickly removed with hot tap water. Everyone should actively work to keep frost accumulation to a minimum on freezer and sample surfaces.

• **High-speed (Sorval) Centrifuge**. This centrifuge is mainly used during DNA and isozyme extractions, hence the temperature is set at 4° C. It is a good idea to turn on the machine at least a half hour prior to use to allow the chamber to reach the proper temperature. The rotor can then be installed. It is best to pre-cool the rotor (in the dairy case or walk-in coldroom) first. All centrifuge tubes must be balanced against their opposites. Use the twin pan balance on the top of

the freezer to do this. The rotor lid has two threaded rings - the outermost one secures the lid to the rotor (tighten this one first) and the innermost one secures the rotor/lid assemblage to the shaft (tighten second). The time and speed are set manually and the run commences after pushing the "start" button. The door cannot be opened until the rotor has essentially stopped spinning. The door light will then illuminate and the door will open after the button is pushed.

• **Ultracentrifuge (Beckman).** This is probably the most sensitive (and expensive) piece of equipment in the lab! This is not meant to discourage its use, but all users should read the manual and be checked out by DLN prior to operating this piece of equipment. The most important factor to consider is that all tubes placed in the rotor are balanced ***exactly*** against the one opposite.

• **Thermal Cyclers**. At present, usage is not so great as to require a sign-up sheet. If this changes, we will implement such a policy. In the meanwhile, simply communicate with other lab users. Although we have three thermocyclers, it appears the Applied Biosystems machine is more popular than the others (mainly owing to the hot lid and small tube volume). The Perkin Elmer thermocycler block should be preheated to 94˚ C prior to inserting the tubes. This simulates a "hot start" and reduces the amount of nonspecific amplification. Check the listing of files for commonly used cycling parameters. For all thermal cyclers, if you see that the temperature is at 4˚C (on a soak cycle), feel free to turn it off and put the tubes in a labeled rack in the dairy case (even if they are not yours).

• **Water Baths**. The water bath in room 1046A is generally left off until it is needed. The two most common uses are for genomic DNA extractions and restriction digests. The bath is kept at 37˚ C, so if you readjust it, please return it to this temperature when finished. Keep an eye on the water levels and replenish when needed. A few drops of copper sulfate (small white bottle) can be added to keep algal growth down.

• **UV Transilluminator (darkroom).** The transilluminator should always be used with the protective plastic cover to keep the glass from becoming scratched. Be considerate of the next person and wipe off the surface with water and a paper towel. Do not leave ethidium bromide to dry on the cover. For viewing, always lower the glass cover or wear protective goggles. Photographs with the Polaroid camera can be taken on top of the glass cover.

• **Polaroid Camera**. The Polaroid camera is to remain with the transilluminator for use in fluorescent photography. The camera uses Polaroid 667 film which should be stored in the refrigerator, not the freezer. As mentioned above, the rollers will occasionally require cleaning to ensure smooth removal of the prints.

• **pH Meter**. Please follow the instructions in the booklet for proper calibration and use of the Fisher Acumet AB15 pH meter. The standard calibration solutions should be replaced monthly (and dated). Check the electrode often - it should be full of saturated KCl level solution. Replace if needed from the KCl bottles in the cabinet above. Remember, it is never necessary to place the electrode tip more than a centimeter into a solution. Immersing it deeper may result in the sample solution flowing into the electrode (bad news). Make sure the purple sleeve at the top of the electrode is turned so that the hole is not open.

• **Analytical and top-loading balances**. Be especially aware of spilled chemicals on and around the analytical balance, the top-loading balance, and the stirring hotplate. The next person who encounters some white powder may not know if it is dangerous or not, so CLEAN UP after yourself!

• **Nanopure water system.** When filling the carboys with deionized water, be sure to monitor the process carefully. I strongly recommend setting a lab timer and checking at various intervals to ensure the water does not overflow.

• **Oven.** This oven is used to bake glassware to destroy RNases. The temperatures required to accomplish this (200˚ C for several hours) are potentially dangerous. Never leave this oven at this temperature unattended for long periods of time (e.g. overnight). The oven should not be used to dry glassware or plasticware (use the drier in room 1050 for this).

Safety

Working in a molecular biology laboratory presents some hazards, hence everyone should be aware of the following safety considerations. The "red and white" safety sheet posted on the wall near the Rm. 1046 door explains emergency evacuation procedures, locations of fire extinguishers and emergency showers, etc. All users should read this notice.

• **Eating and Drinking**. No eating or drinking in the lab is allowed.

• **Proper Clothing**. Lab coats are suggested for all lab workers. This is especially important for anyone wearing shorts. No open-toe shoes or sandals are allowed in the lab. Take steps to avoid injury by using gloves, masks, and eyewear when appropriate.

• **Accidental Injury.** Cuts and burns are the most common injuries that occur in labs. For minor cuts, use the First aid kit, located along the east wall of Rm. 1046. For more serious injuries, go (with an attendant) to the Student Health Service. For accidental introduction of foreign substances in the eye, an eyewash station is located in the hallway just outside Rm. 1046. At this same location is an emergency shower (see below).

• **Chemical Spills.** Spilled acids and volatiles present real hazards, hence extreme care should be exercised when cleaning up. Most importantly, proper clothing (lab coat, etc.) and gloves should be worn to prevent incidental contact with the skin. To treat small areas of chemical contact, rinse with copious amounts of water in the sink. For large contact areas, use the emergency shower outside Rm. 1046. Remember, this shower is ***NOT*** to be used in case of contact with radioactive materials. Chemicals spilled on the floor and lab surfaces must be cleaned up immediately. For acids and bases, use the lab absorbers that are located on the top shelf in Rm. 1048.

• **Dry Chemical Disposal.** Under no circumstances should dry chemicals be poured down the sink or thrown away in the regular trash cans. We are required to have waste chemicals removed by the SIUC Center for Environmental Health and Safety (telephone 3-24343). The most

important thing is to make sure the container is properly labeled as to its contents. CEHS provides labels free of charge. For waste pickup, fill out the online form on the CEHS webpage (http://www.cehs.siu.edu/) . Also present on the web page is a list of chemicals for that are available for redistribution.

• **Liquid Chemical Disposal.** The same rules apply as with dry chemicals. Some liquids can be safely poured down the sink (e.g. Tris buffer, sucrose solutions, etc.). If in doubt, please ask. All waste liquid chemical (e.g. phenol, chloroform, etc.) should be stored in the hood in an appropriate container that is labeled with the red and white stickers provided by CEHS.

• **Ethidium Bromide**. Ethidium bromide is one of the most dangerous chemicals used in the lab. It is a powerful mutagen (cancer causing agent), therefore exercise extreme caution when using this reagent. If you spill any gel buffer containing EthBr, put on gloves and clean it up immediately with hot soapy water and a paper towel. Do ***NOT*** use the sponge that is used for general cleanup in the lab. Be especially aware of the bench and transilluminator surfaces in the darkroom. All gels, pipette tips, etc. that have come in contact with EthBr should be disposed of properly. Waste stained gels are collected in plastic containers (in the darkroom) for disposal by CEHS. Since the darkroom is now locked (same key as lab), arrangements must be made to allow CEHS personnel to gain access to dispose of waste gels.

• **Glass Disposal.** All broken glass, including used disposable pipettes, broken glassware, etc. should not be disposed of in the regular trash cans but should be placed in the waste glass disposal box located under the hood in Rm. 1046. When full, seal the box with tape, label, and place in the hallway for removal by the custodian. Remember to report any broken glassware so that it can be replaced.

• **Sharps Container.** For disposing of hypodermic needles, used razor blades, scalpel blades, etc., please use the red sharps container (Rm. 1046) provided by CEHS.

Chemicals

When new containers of chemicals arrive in the lab, it is standard procedure to label them with the date. Check the bottle label for proper storage conditions and then store properly. Save the Material Data Safety Sheet and the packing slip (often in a pocket on the outside of the box). The following is a list of the different chemical storage conditions maintained in my lab:

• **Dry Room Temperature Chemicals**. Most dry chemicals are stored in Rm. 1046A in the cabinets or on the black metal shelves (the latter for bulk items).

• **Dry Room Temperature Desiccated Chemicals.** Some chemicals must be stored in a desiccator but do not require freezing. These are housed in a large glass desiccator in the cabinet below the sink in room 1046A. Beware - some of these are hazardous.

• **Frozen/Desiccated Dry Chemicals.** A large number of chemicals used for isozymes are stored in small plastic desiccator containers in the –20˚ C freezer in Rm. 1046. A few chemicals used for DNA (e.g. DTT) also reside here. Check the Excel printout of lab chemicals if you do not know where a particular chemical resides.

• **Acids and Bases.** Acids are stored in the metal cabinets under the hood. Bases should not be stored in the same enclosed space as acids.

• **Organics.** Our small collection of organic liquids is housed under the center island sink in Rm. 1046.

• **Volatiles.** Volatile liquids, such as ethanol and methanol, are stored in the labeled metal cabinet under the counter in Rm. 1046. Rm. 1064 (near the Vivarium office) is also available for storing larger quantities of volatiles (we currently have methanol stored there). A key to this room can be obtained from personnel working in the Medical School stock room (upstairs, LSIII) or the Microbiology stock room (Edna Hand, Rm. 129 LS II).

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• Enzymes. All enzymes are expensive and most are very sensitive to temperatures above 0˚ C. For this reason, all enzymes should be kept on ice and returned to the freezer as quickly as possible after use.

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SECTION 2: INTRODUCTION

Molecular Methods in Plant Biology

Molecular biology is less of a discipline of its own than a tool used by biologists in diverse fields such as genetics, systematics, evolutionary biology, ecology, physiology, cell & developmental biology, agriculture, and many others. Many techniques first introduced in the realm of microbial genetics such as restriction endonuclease digestion, transformation, cloning, and sequencing are all now used in studies of higher plants.

This manual has a distinct bias toward those techniques I use in the field of plant molecular systematics and evolution. The publications in BioTechniques (Nickrent 1994, 1997) provided succinct summaries of the methods used in my lab over a decade ago. Some of these, such as the basic PCR reaction, have remained relatively unchanged, yet others, such as the method of obtaining DNA sequences from these products, has changed and will continue to change. Manual sequencing, where the radioactively-labeled DNA fragments were visualized via autoradiography (film exposed to the gel) was replaced by automated DNA sequencing methods. Here no radioactively labeled nucleotides are used. Instead, fluorescent dyes attached to the nucleotides are incorporated during the cycle sequencing reaction. These dyes fluoresce (one color for each of the four nucleotides) when excited with a laser and this fluorescence is detected by a sensor that produces a graphical representation of the sequence called an electropherogram. The electropherogram is read by computer software which generates a linear DNA sequence. Although the sequence still requires some manual editing, the speed of generating of DNA sequences is greatly increased over manually reading sequence from an autoradiograph.

But already, the ABI 377 automated sequencer we use is out-dated and will soon be replaced by a capillary sequencer (AB3730). Here, the chemistry is much the same as before (with fluorescent dyes), but the matrix through which the DNA fragments migrate is not polyacrylamide but polymer (POP-7™) contained within thin capillary tubes. Furthermore, this technology is certain to be replaced by other, higher throughput methods that promise to provide longer and more accurate reads at a lower price. Indeed, whole genomes are now being sequenced using radically different methodologies such as nanopores and pyrosequencing (see www.454.com).

Review of Metric Units

Molecular biological procedures require a thorough understanding of the metric system as it applies to mass, volume, and length. Since most protocols use very small volumes of liquids, you must be able to do calculations and conversions using the proper units. The following table gives the relationships between the commonly used prefixes:

Prefix	Abbreviation	Unit multiplication Factor	Exponential	Commonly Used Units
Kilo–	k–	1000 X	10^{3}	kg
Deci–	d–	0.1 X	10^{-1}	dm
Centi–	c–	0.01 X	10^{-2}	cm
Milli–	m–	0.001 X	10^{-3}	mm, ml, mg
Micro–	μ–	0.000 001 X	10^{-6}	μm, μl, μg
Nano–	n–	0.000 000 001 X	10^{-9}	nm, ng
Pico–	p–	0.000 000 000 001 X	10^{-12}	pg

Calculations to prepare dilutions from a stock solution

There is a simple formula to help you calculate amounts when preparing dilutions. It is:

$$C_1V_1 = C_2V_2$$

where C_1 is the initial concentration (or stock solution), C_2 is the final concentration, V_1 is the initial volume, and V_2 is the final volume. Initial volume is the amount from the stock that must be added to achieve that final concentration. The above equation can be rearranged to give you initial volume:

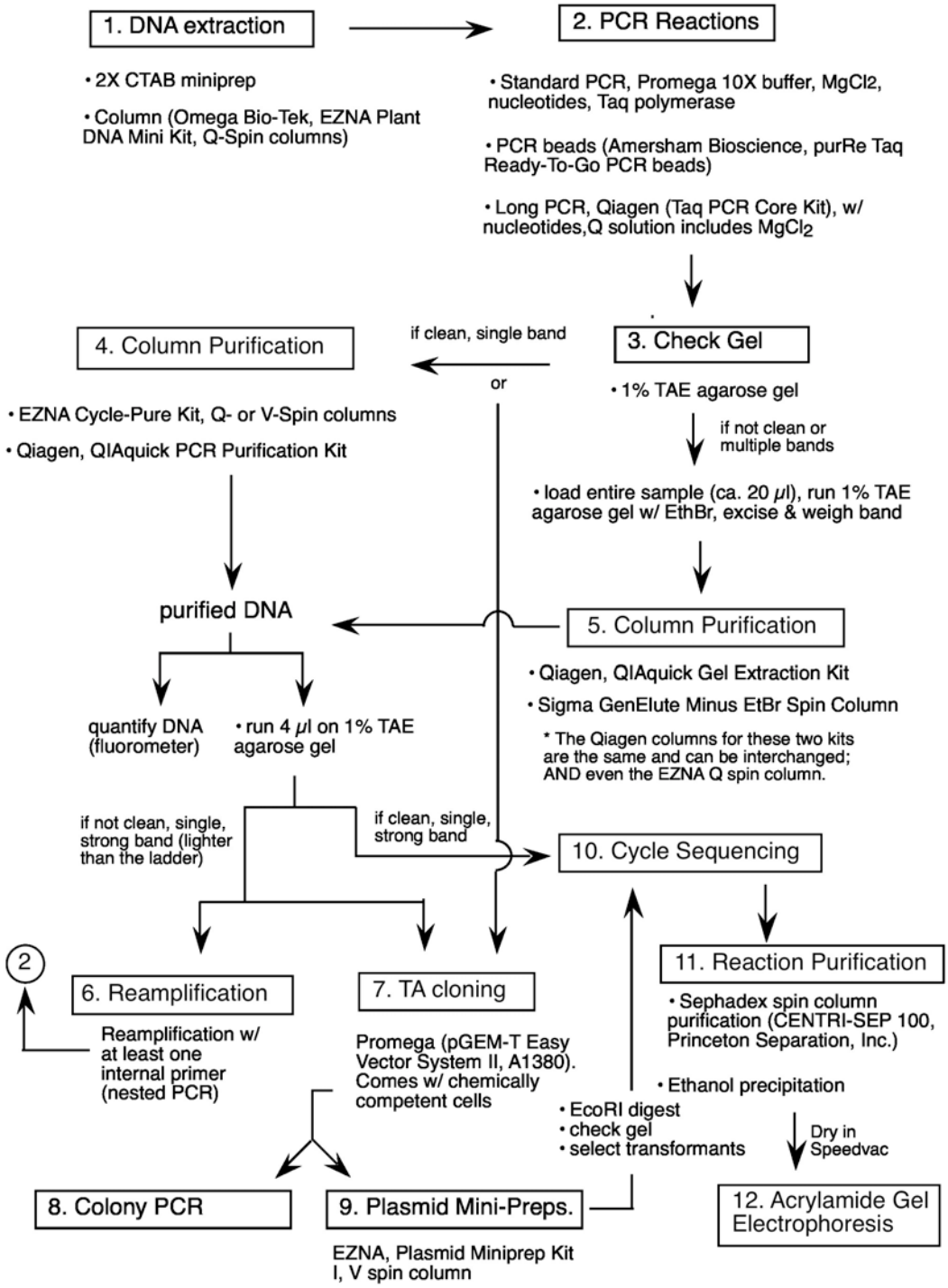
$$V_1 = \frac{C_2V_2}{C_1}$$

Flow Chart of Protocols – Plant Tissue to DNA Sequence

This manual provides protocols for many of the methods commonly used in my lab. These are presented sequentially, but in reality, there are often alternate directions one can proceed based upon the outcome of earlier steps. For example, we frequently produce sufficient PCR product to undergo direct cycle-sequencing using that PCR product as a template. But when the PCR product concentration is low, we may clone it into a plasmid, grow these in *E. coli* cells, extract and purify the plasmids, and then use these as templates for cycle sequencing. Part of becoming a competent molecular biologist is knowing what protocols are available to use given varying conditions. The overall rationale is to use the most efficient means (with respect to both time and money) to obtain the DNA sequence.

The following chart was prepared to show the various pathways one can follow to achieve a DNA sequence. Bear in mind that these pathways generally apply to phylogenetic studies of wild (not cultivated) plants that are far from "model organisms" such as *Arabidopsis* or maize. Typically the amount of DNA obtained from such plants, and its quality, is more variable, thus forcing the researcher to optimize conditions for each sample.

Protocol Flow Chart



SECTION 3: PLANT GENOMIC DNA EXTRACTION

General Considerations and Rationale

Three components necessary for successful nucleic acid extraction are 1) inhibition of nucleases, 2) removal of proteins, and 3) physical separation of the nucleic acid from other cellular components. Nuclease inhibition and removal of proteins are not mutually exclusive and often a particular step (such as phenol extraction) accomplishes both.

Inhibition of Nucleases

Detergents inhibit nucleases and help separate the proteins from the nucleic acids. A common detergent is sodium dodecylsulfate (SDS). Avoid the use of potassium salts or temperatures below 10 ˚C with SDS since this may cause precipitation of the detergent. One of the most widely used detergents for plant DNA extraction buffers is CTAB (cetyltrimethyl-ammonium bromide). CTAB is most often used in a 2% (w/v) solution (Rogers & Bendich 1985, Doyle & Doyle 1987, Nickrent 1994).

Removal of Proteins

Phenol is a strong denaturing agent for proteins. In phenol extractions, proteins partition into the organic phase (and interface) whereas nucleic acids partition in the aqueous phase. Chloroform is also an effective deproteinizing agent and also helps to remove lipids. When phenol is used first, it is often followed by a 1: 1 mixture of phenol: chloroform and then a final extraction with just chloroform. This last chloroform extraction helps to remove the last traces of phenol from the sample. Chloroform is usually mixed with isoamyl alcohol (24:1 ratio) because it helps with the phase separation, decreases the amount of material found at the aqueous and organic interface, and helps reduce foaming.

Antioxidants such as 8-hydroxyquinoline or ß-mercaptoethanol are often added to phenol. During phenol extractions, the pH of the buffer is important in determining whether DNA and/or RNA are recovered. At pH 5-6, DNA is selectively retained in the organic phase leaving RNA in the aqueous phase (hence water saturated phenol is useful for RNA extractions). At pH 8.0 or higher, both DNA and RNA are retained in the aqueous phase. Phenol can be stored under buffer for up to one month.

Although phenol is very effective in removing protein (many protocols designed for animal tissue use it), we have found that most plant tissues can be extracted using only chloroform (see 2X CTAB methods below). In addition, phenol contact with the skin can cause severe burns, thus it shouldbe considered hazardous.

Protease

The most common proteolytic enzyme used in nucleic acid extractions is protease K (e.g. Sigma P4755) which is generally made up in 50 units/ml aliquots and stored frozen for immediate use during extractions. Solutions are generally incubated with this enzyme at 37 ˚C for at least one hour.

Other Extraction Buffer Components

Two common reducing agents often found in extraction buffers are ß-mercaptoethanol or dithiothreitol (DTT). We prefer the use of DTT because it is not as volatile as ß-mercaptoethanol and is therefore easier on the nose. EDTA is also present to chelate Mg^{+2} ions thus mediating aggregation of nucleic acids to each other and to proteins.

Isopropanol and Ethanol Precipitations

The most common method of concentrating nucleic acids is with an alcohol precipitation. This occurs in the presence of a salt (see below) at low temperatures (-20 ˚C or less). Either 2.0 volumes of ethanol can be used to precipitate DNA or 0.6 volumes of isopropanol. lsopropanol is often used for the first precipitation, but not for final ones since it tends to bring down salts more readily than ethanol. Isopropanol can also be used when the volume of the tube will not allow the addition of 2 volumes of ethanol. The nucleic acid is collected by centrifugation at 10,000 rpm for ca. 15 - 20 minutes.

The most common monovalent cations used in nucleic acid precipitations are shown in the following table:

	Stock Solution	Final Concentration
Sodium acetate	2.5 M (pH 5.2 - 5.5)	0.25 M to 0.3 M
Sodium chloride	5.0 M	0.10 M (optimal < 0. 15 M = 1/50 vol.)
Ammonium acetate*	4.0 M	1.3 M

* filter sterilize, do not autoclave

The choice of salt is determined by the nature of the sample and the intended use of the nucleic acid. Samples with phosphate or greater than 10 mM EDTA should not be ethanol precipitated since the salts will come down with the nucleic acid.

Butanol Extractions

DNA can be recovered from dilute solutions by extracting with 2-butanol. The water from the sample moves into the butanol which is discarded, thus leaving a higher DNA concentration in

the aqueous phase. Water saturated butanol is also used to remove residual ethidium bromide from samples obtained via CsCl centrifugation or agarose gel purification.

Plant Genomic DNA Extraction using CTAB: Mini Prep

Record all information about the samples being processed, specifically tissue type (leaf, flower, etc.), state (silica gel dried, herbarium sample, fresh), weight, taxon name and collection number, on lab notebook paper (filed in DNA Extraction book). Remove aliquots of DTT and Protease K from freezer, place in tube rack, and put on ice once thawed. Turn on water bath (set to 37º C) and heating block (set to 94º C). Fill the heating block wells with distilled water. Label on caps enough 2.0 ml tubes as needed (number 1-12) and add 1.3 ml of 2X CTAB to each. Place these in heat block, two at a time, until the buffer is hot.

1. For fresh tissue, weight out 0.1 g. For dry samples (herbarium material, silica gel dried tissue), use 30-40 mg (0.03 g).

2. Grind tissue to a powder. For fresh tissue, liquid nitrogen is quite useful. For dry material, direct grinding in the mortar is often effective. If additional abrasion is needed for tough samples, add a small spatula tip of silica gel.

3. Add 1.3 ml of hot (94 ˚C) 2X CTAB. Continue grinding with pestle.

4. Using a 1.0 ml tip with the end cut off, pipet sample back into its 2.0 ml microfuge tube. Do not exceed 1.1 ml.

5. Add 60 μl Protease K (50 units/ml; i.e. 3 units) and 60 μl DTT. Cap and invert to mix.

6. Incubate sample for 60 minutes in the 37 ˚C waterbath. We have also had success speeding up the process by doing 30 minutes at 45 ˚C. It helps to mix the samples every 15 minutes or so.

7. Remove tubes from bath, place in rack and open all caps. Add 2/3 volume (0.79 ml) of chloroform:isoamyl alcohol (24:1). This will essentially fill the tube. Cap tubes tighly and place another rack on top. Invert to mix for 5 minutes.

8. Place tubes in refrigerated (4˚ C) centrifuge (dairy case, marked for "Genomic DNA Only"). Centrifuge for 15 minutes. While this is happening, label 1.7 ml tubes (1-12).

9. Gently retrieve the tubes from the centrifuge and place in rack being careful not to disturb the separate phases. Set a P-1000 pipet to 1.0 ml and attach a 1.0 ml barrier tip. Remove exactly 1.0 ml of the aqueous (top) layer, again being careful not to disturb the interface or underlying chloroform (if you do, centrifuge the tube again). Place the aqueous layer in the labeled 1.7 ml microfuge tube.

10. Add 2/3 volume (667 μl) of ice cold isopropanol (–20 ˚C) to each tube. Invert several times to mix, and place in a –20 ˚C freezer. These tubes can remain in the freezer for days to weeks. We generally precipitate the DNA overnight. One can speed up the process by precipitating at –70 ˚C for 20 minutes, but this sometimes results in precipitating salts as well.

11. Centrifuge the tubes for 15 minutes in the 4˚ C centrifuge (dairy case).

12. Remove the tubes to a rack. Pour off the propanol (into sink, with running water) and invert the tubes on a paper towel to drain. After a few minutes, the pellets can be dried further in the SpeedVac (set at medium heat).

13. Rehydrate the dried pellets in 300 μl of TE. Mix using a pipet tip until the pellet goes into solution. One can allow the pellet to hydrate at 4˚ C in the dairy case for ca. 1 hour if desired. If after this time there is still undissolved pellet in the tube, it is unlikely that this is DNA. To remove it, one can briefly centrifuge the tube and then transfer the supernatant to a new tube.

> *Optional: If one's sample has lots of RNA, and you want to remove it, RNase A (at 1 mg/ml) can be added to the sample tube. The tube is then incubated for ca. 1 hour at 37˚ C. Alternately, one can add RNase directly to the TE and proceed directly to the ammonium acetate step (next).*

14. Add 200 μl of 4M NH_4OAc (ammonium acetate). Mix thoroughly. Your total volume is now 500 μl.

15. Add 2 volumes (1.0 ml) of cold 100% ethanol (freezer), mix well, and store tubes at –20 ˚C for at least 1 hour. Longer periods (days) are OK. If one wants to proceed quickly, the precipitation can be done at –70 ˚C for 20 minutes.

16. Centrifuge tubes for 15 minutes, pour off the ethanol (into sink), drain on towels, and dry the pellet in the SpeedVac (all as above with the propanol step).

17. Resuspend the DNA pellet in 50-100 μl of TE (optionally w/ RNase A). Label using the ToughTags and a permanent pen (taxon name, collection number, Gen. DNA, and date).

Plant Genomic DNA Extraction using CTAB: Large-Scale

Before getting started, turn on the centrifuge to precool the chamber. The rotor is generally stored precooled. All glass and plasticware is assumed to be clean and sterile (autoclaved). You will need the following:

Equipment:
- centrifuge (e.g. Sorval RC-5B with SS34 rotor).
- centrifuge tubes, glass (e.g. Corex 30 ml)
- centrifuge tubes, plastic (e.g. Nalgene 50 ml polyallomer)
- cheesecloth (cut into 6" X 6" squares)
- funnels
- hotplate with beaker and water
- liquid nitrogen
- mortars and pestles
- thermometer
- waterbath set to 37 - 45 ˚C

Reagents:
- 2X CTAB buffer
- Protease K (stored in 50 units/1 ml aliquots in the freezer)
- Dithiothreitol (DTT) 0.5 M. Stored as 0.5 ml aliquots in freezer - added to buffer just before use.
- 1X TE buffer
- Ammonium acetate, 4.0 M.
- organics: chloroform:isoamyl alcohol (24: 1), Tris buffered phenol, cold isopropanol, cold 100% ethanol, and 70% ethanol.

The best DNA extracts come from young plant material. Attempt to conduct the extraction soon after collection if refrigeration is not possible. You will need ca. 3.0 g of leaves for this procedure.

1. Heat a water bath on the hotplate to just below boiling (95 ˚C). Add 25 ml of CTAB buffer to the tubes.

2. Place 2 to 3 g of plant tissue in the mortar, cover with liquid nitrogen and grind to a fine powder.

3. Add 25 ml of the hot CTAB buffer and continue grinding for ca. 1 minute. Using a funnel with cheesecloth, strain the extract into a 50 ml polyallomer centrifuge tube.

4. Add 50 units of protease K and 0.5 ml of DTT, mix, cap and incubate sample in the 37-45˚ C water bath for 1 hour with occasional swirling.

5. Add 2/3 volume of chloroform:isoamyl alcohol (24: 1). Cap the tube and shake gently for ca. 2 or 3 minutes, intermittently releasing pressure.

6. Balance the tubes (w/ each other or a blank) using a twin pan balance. All subsequent centrifugation steps assume that you have balanced your tubes. Centrifuge at 8,500 rpm for 15 minutes.
7. Remove the top (aqueous) phase with a wide-bore pipet (P-5000 micropipets work well) and place it in a clean 50 ml polyallomer tube. Be very careful not to remove any chloroform or interface.

8. Add 2/3 volume ice-cold isopropanol and place in a –20 ˚C freezer for at least one hour (overnight OK).

9. Centrifuge for 20 minutes at 10,000 rpm.

10. Gently pour off supernatant, leaving the pellet on the bottom of the tube. Invert the tube on a Kimwipe to drain off excess isopropanol. If the pellet begins to slide down the tube, place upright. Allow the pellet to air dry until only slightly moist (at least one hour).

11. Add 3.0 ml of TE and 2.0 ml of 4.0 M NH4OAc. Using a 1.0 ml pipet tip that has the tip cut off, suspend the pellet in buffer. It is wise not to pipet up and down too vigorously for this shears the DNA.

This following phenol/chloroform step is optional, but helps remove any remaining protein. If omitted, simply go to the ethanol precipitation in step 13.

12. Add 3.0 ml of phenol (Tris saturated, pH 8.0 - bottom phase! and 3.0 ml of chloroform. Shake for one minute and centrifuge at 8,500 rpm for 15 minutes. Remove the aqueous (top) phase to a glass Corex centrifuge tube. Re-extract with an equal volume of chloroform. Centrifuge at 8,500 rpm for 10 minutes. Remove the aqueous phase to a new glass Corex centrifuge tube.

13. Add 2.0 volumes of 100% ethanol. Store at –20 ˚C for at least 1 hour.

14. Centrifuge at 10,000 rpm for 20 minutes. Pour off the ethanol, rinse briefly with 70% ethanol,
and dry the DNA pellet by inverting on a Kimwipe. Allow the DNA to dry completely at room temperature (overnight). Alternately, the pellet can be dried in a vacuum oven with gentle heat.

15. Resuspend the DNA pellet in TE, ca. 1.0 ml if the pellet is large, 0.5 ml if smaller, etc. Use a cut-off tip to pipet the DNA into a labeled 1.5 ml microfuge tube.

16. If desired, the RNA can be removed from the DNA sample by means of an RNase digestion. We use a combination of two enzymes, RNase A (at 10-50 μg/ml of sample) and RNase T1 (ca. 50 units per ml of sample). Incubate the samples for 30 minutes at 37 ˚C. Conduct a phenol/chloroform and then chloroform extraction as in step 12 above. Ethanol precipitate the DNA as in steps 13-15.

17. The quality (length) of the DNA should next be examined by running it on a 1% agarose gel. The quantity can be estimated from the intensity of the stained band in comparison to known standards.

Genomic DNA Extraction (Mini Prep) for High Carbohydrate Plants

1. Weigh out 0.1 g of fresh leaves (or 20-30 mg of dry tissue). Place in a small (2.5 inch wide) mortar.

2. Grind tissue to a powder using either liquid nitrogen or silica gel.

3. Add 1.3 ml of hot (94 ˚C) 2X CTAB containing 1.5% w/vol Polyvinylpyrrolidone (PVP-40). Continue grinding with pestle until well mixed.

4. Pipet extract into a 2.0 ml microfuge tube. Add (60 μl Protease K (50 units/ml; i.e. 3 units) and 60 μl dithiothreitol (DTT).

5. Incubate sample in a 37 ˚C waterbath for 30 minutes to one hour. Mix every 15 minutes.

6. Briefly centrifuge the sample to remove debris. Decant supernatant to a new labeled 2.0 ml tube.

7. Add 2/3 volume (ca. 0.8 ml) of phenol/chloroform:isoamyl alcohol (24:1). Shake well for 2 to 3 minutes.

8. Centrifuge for 15 minutes at 4 ˚C at maximum speed (ca. 10,000 rpm).

9. Carefully retrieve the tubes from centrifuge, remove the aqueous (top) layer using a wide-bore, barrier tipped 1.0 ml pipette (approximately 0.75 ml) and place in a 1.7 ml microfuge tube.

10. Add 2/3 volume of chloroform:isoamyl alcohol (24:1). Shake well for 2 to 3 minutes.

11. Centrifuge for 15 minutes at 4 ˚C at maximum speed (ca. 10,000 rpm).

12. Retrieve the tubes from centrifuge, remove the aqueous (top) layer using a wide-bore, barrier tipped 1.0 ml pipette (approximately 0.75 ml) and place in a 1.7 ml microfuge tube.

13. Add 0.66 volumes (ca. 0.5 ml) of ice cold (–20 ˚C) isopropanol. Invert several times to mix, and place in the –20 ˚C freezer for at least 30 minutes (overnight is OK).

14. Centrifuge the tubes for 15 minutes.

15. Pour off the isopropanol, being careful not to lose the pellet. Drain the pellet by inverting on a towel. Dry the pellet at room temp. or in the SpeedVac (medium heat).

16. Add 300 μl of TE (optionally w/ an RNase added) and 200 μl of NH_4OAc. Mix gently to get the pellet into solution.

17. Add 1.0 ml of 100% ethanol and store at –20 ˚C for at least 1 hour (overnight to many weeks OK).

18. Centrifuge for 15 minutes, pour off the liquid and dry the pellet in the SpeedVac.

19. Resuspend the DNA pellet in 50-100 μl of TE (RNase added).

Cesium Chloride Purification of Genomic DNA

Equilibrium density gradient centrifugation using cesium chloride (CsCl) is used to obtain very pure nucleic acids from crude homogenates (or to further clean up previous extractions). The procedure takes advantage of the fact that the buoyant densities of DNA, RNA, and protein are different in CsCl. Following ultracentrifugation (ca. 100,000 rpm), an RNA pellet forms at the bottom of the tube and DNA remains floating in the solution. Ethidium bromide is added to the CsCl so that the DNA can later be viewed and recovered under U.V. light.

It is a good idea to dedicate a mortar and pestle for use in grinding the CsCl. This should be done ahead of time and the powder stored in a sterile container (e.g. 50 ml disposable tubes). The following protocol is written for a Beckman TL-100 desktop ultracentrifuge using the 3.5 ml sealable tubes (Beckman, Quick-Seal polyallomer, bell-top, 3.5 ml capacity, 13 X 32 mm).

1. Before preparing your samples, it is good to have some CsCl/ethidium bromide solution available to top off the centrifuge tube (if required). For 100 ml of this solution:

83.33 g CsCl
81.1 ml of Tris (10 mM, pH 7.5)
2.23 ml of ethidium bromide (10 mg/ml)

The density of this solution should be 1.55 g/ml with a refractive index at 20 ˚C of 1.386.

2. Place a sterile 5 ml disposable plastic culture tube on the pan of an analytical balance. Tare the tube and add 2.5 g of powdered CsCl. Add 1.0 ml of Tris (10 mM, pH 7.5), 500 μl of genomic DNA, and 66 μl of ethidium bromide (10 mg/ml in dH20). Add an additional 0.934 ml of Tris. The final weight should be 5.0 g.

3. With a sterile Pasteur pipet, add the sample to the centrifuge tube. It is important that the tube be completely filled, otherwise it will collapse during centrifugation. If necessary, use the CsCl/ethidium bromide solution to top off the tube. Place the metal cap on the tube neck, apply the heat sealer, press until the cap melts down to the shoulder of the tube. Remove the heat sealer and immediately apply the heat sink tool to cool the cap. Take off heat sink. If all the tubes are filled equally, they should be the same weight.

4. Place the tubes in the centrifuge rotor, making sure to place a floating spacer on top of each tube. Screw on the rotor cap. Place the rotor in the centrifuge. Follow the instruction on use of the ultracentrifuge. Make sure you know how to use the centrifuge before starting it!

5. Centrifuge at 80,000 rpm for 6-10 hours. When the run is complete, the tubes must be removed with forceps.

6. Using long wave U.V. light, observed the position of the DNA band. The DNA can be recovered by using a hypodermic needle through the wall of the tube, but this is to be avoided since this will shear genomic DNA. Carefully cut off the neck of the centrifuge tube to allow

access with a micropipet tip. Very carefully, remove the top layer of CsCl until you reach the DNA band. Use a P
1000 tip to start and switch to a P-200 tip when near the band. Remove the DNA band and save in a microfuge tube (on ice). Discard all CsCl/ethidium bromide solutions in the ethidium bromide waste container.

7. The DNA can be separated from the CsCl salts either using dialysis or by means of a spin column. We use the Centricon®-100 microconcentrator (Amicon, Danvers, MA 01923). Place the filtrate cap on the sample reservoir and then wet the Centricon membrane with ca. 900 μl of Tris (10 mM, pH 7.5). Centrifuge at 4,000 rpm for 10 min. and discard the Tris.

8. Pipet the DNA sample into the reservoir, add the retentate cap. Balance with 10 mM Tris. Centrifuge at 4,000 rpm for 15 minutes. Discard the CsCI/ethidium bromide into the EthBr waste container.

9. To elute the DNA from the membrane, use 250 μl of High Salt NET buffer. Add the retentate cap and briefly vortex. Allow the liquid to stay in contact with the membrane for about 10 minutes. Gently vortex again, invert the tube so that the sample runs into the retentate cup. Centrifuge (opposite direction as previous one) for ca. 5 minutes at 4,000. Remove the liquid from the retentate cup into a microfuge tube.

10. To remove additional ethidium bromide, add 3X the volume (ca. 750 μl) of 1-butanol (H_20 saturated). Mix gently by inverting several times. Centrifuge for 5 minutes at 4 ˚C. Remove the top phase and discard in the butanol waste container.

11. To precipitate the genomic DNA, add 2.5 volumes of ice cold 100% ethanol. The tube can be stored at 4 ˚C for several hours at this stage if desired or centrifuged immediately. Centrifuge for 20 minutes, pour off the ethanol. Wash with 70% ethanol, centrifuge, pour off, and dry pellet. Rehydrate the pellet in TE (amount depends on starting amount of DNA and size of final pellet).

PEG Purification of Genomic DNA

Many molecular biological procedures (such as PCR, restriction endonuclease digestions, and sequencing) require relatively pure DNA. In PCR, for example, plant DNA contaminated with those ubiquitous secondary compounds and carbohydrates is especially difficult to amplify. For those of us interested in "quick and dirty" (or, should I say, "quick and clean") methods of purifying genomic DNA, this PEG method works amazingly well. Although the yield of pure DNA is not as high as with CsCl, this does not present a problem when PCR is the goal and only minute amounts of DNA are required. It is much faster than purification using long ultracentrifugations and uses 1000X less ethidium bromide!

The method is based upon the principle that most secondary compounds, RNA, etc. are smaller than genomic DNA and can be separated using gel electrophoresis. We have successfully used low melting point agarose (e.g. NuSieveTm) to purify genomic DNA whereby the band of interest is cut from the gel, diluted with water, and then used directly in a PCR reaction (see Nickrent 1994). A disadvantage of this method is that agarose is still present with the DNA sample and, from personal experience, the samples cannot be repeatedly frozen and thawed. NuSieve genomic DNA does remain useful for up to several weeks at 4 ˚C, but this seems to be variable based upon the species. We have also successfully isolated genomic DNA from agarose gels using DEAE membranes, but these are only effective in binding DNA to only 7 kb in length, thus much of the high molecular weight DNA is lost. For gel purification using PEG:

1. Pour a 1X GGB+ (i.e. with EDTA) gel. The size of the gel (really the comb teeth) depends upon the amount of DNA you want to purify. Add ca. 4 μl of ethidium bromide for every 250 ml of gel.

2. For every 100 ml of genomic DNA, add 10 μl of Ficoll dye. Mix thoroughly by stirring.

3. Load the DNA samples into the wells either submarine or, if you need to maximize the amount loaded per lane, load the well dry. For the latter, pour the electrode buffer only up to the top edges of the gel, run the samples into the gel, then cover with buffer for the remainder of the run.

4. Visualize the bands with long-wave UV light. If sufficient migration has taken place, turn off the power supply. Lower the buffer (by removing with a plastic disposable 50 ml syringe) till it just touches the top edges of the gel. Excess buffer can be blotted off the top of the gel with a Kimwipe.

5. Under U.V. light, use a sterile scalpel blade and cut a well just ahead of the genomic DNA band. It is OK to cut the well completely to the bottom of the gel mold - the buffer will not leak through. Work quickly - do not expose the DNA to U.V. light any longer than necessary. Remove any buffer that leaks into the well with a 1 ml micropipet and fill the wells with 25% PEG solution (20-25% polyethylene glycol (w/v) in 1X GGB+ buffer).

6. Continue electrophoresis until the genomic DNA band migrates into the well. If. your well is cut close to the band, this should take place in about 10 minutes. If the band is very light, you

probably will not see it once it migrates into the well. Use the flanking bands to estimate when the light bands have entered the well.

7. Remove the PEG with DNA to a 1.5 ml microfuge tube. Add 0.5 volume of phenol (Tris buffered, pH 8.0) and 0.5 volume of chloroform. Mix briefly, centrifuge for 10 minutes, and remove aqueous (top) phase to new tube. Extract with an equal volume of chloroform. Save aqueous phase in new tube.

8. Precipitate the DNA with ethanol. Add 0.66 volume of ammonium acetate and then 2 volumes of 100% ethanol. Place in - 70 ˚C freezer for ca. 10 minutes.

9. Centrifuge (at 4 ˚C) for 20 minutes. Pour off ethanol. Rinse with 70% ethanol. Drain briefly on paper Kimwipe and dry pellets either at room temperature, in a desiccator, or in a Speed Vac.

10. Rehydrate samples in ca. 20 μl of TE.

Gel Spin Method of Cleaning Genomic DNA

Commercial Spin Columns

The GenElute™ Agarose Spin Column sold by Supelco (Supelco Park, Bellefonte PA 16823-0048; cat. no. 5-6500) is useful for fast recovery of DNA from agarose gels. These columns allow isolation of genomic DNA and PCR products for use in PCR and ligation reactions.

1. Place the spin column in a microfuge tube, put ca. 100 μl of TE in the spin column and centrifuge briefly (5 secs.) on high. Discard the TE. This washes and hydrates the membrane. Place the columns (in microfuge tube bottoms) on ice.

2. Run a TAE agarose gel (0.8 - 1%) that contains ethidium bromide with samples of interest (genomic DNA, PCR products). Visualize the bands on a transilluminator (lowest setting possible). Working quickly with a sterile scalpel blade, cut out the band of interest. Don't expose the DNA to UV too long. Cut the slice as close to the DNA as possible, minimizing the amount of agarose in the chunk. Place the gel chunk in the washed spin-column.

3. Centrifuge the spin column at maximum speed (ca. 12,000 x g) at room temperature for approximately 10 minutes.

4. The DNA is now in the buffer collected at the bottom of the tube. It can be used for PCR, cloning, etc. or can be precipitated using ammonium acetate and ethanol.

Home-Made Spin-Columns

For those on a budget, gel spin tubes can also be manufactured in the lab! This method is a modification of one described by He et al. (1992). It is especially useful as an alternative to CsCl gradient centrifugation for cleaning plant genomic DNA, especially if very small amounts of DNA are present in a sample (e.g. following extraction from herbarium specimens).

1. Cut off a P-1000 tip so that it fits within a 1.5 ml microfuge tube.

2. Stuff a little bit of glass wool (Pyrex filtering fiber, Cat. No. 3950), into the tip. Use only enough to keep a chunk of agarose from going through - do not overpack it or all the buffer you want to collect will get stuck in the wool.

3. Autoclave the tubes. You do they have to be completely dry before you use them, so dry in an oven afterwards. We found that microwaving them for 6-10 minutes also works OK (if you're in a hurry!)

4. Run an acetate agarose gel (as above) and isolate the band(s) of interest. Place the gel chunk in the spin columns.

5. Centrifuge at ca. 2800g for 45 seconds to one minute. We use setting 6 on an Eppendorf Micro Centrifuge 5415C which is 6000 rpm or 2940 relative centrifugal force (RCF).

6. Remove the tip/glass wool/agarose from the tube. There should be ca. 10-20 μl of buffer at the bottom that contains your DNA. We find that for genomic DNA, lots is still left in the wool/agarose present in the tip. We have experimented with adding a bit of TE (20 μl) and spinning again. We still get amplification from this! To take it further, we are now trying to reuse the spin tubes by freezing them in the ultralow (–70° C) and then thawing, re-centrifuging, etc.

Acacia DNA Extraction: Miniprep Method

(Modified from Byrne et al. 1993 Journal of Heredity 84, 218-220)

1. Grind in a mortar 25 – 50 mg leaves in liquid nitrogen until a fine powder.
2. Add 175-250 μl cold extraction buffer.
3. Continue grinding to fully suspend powder in buffer.
4. Centrifuge for 5 minutes in a 4° C microfuge at 2000g
5. Resuspend pellet in 20 μl cold wash buffer.
6. Add 5 μl 5M NaCl.*
7. Add 8 μl 8.6% CTAB / 0.7M NaCl. Mix gently.*
8. Add 10 μl 5% sarcosyl. Mix gently.*
9. Leave at room temperature for 15 minutes then incubate at 60° C for 15 minutes.
10. Add 40 μl chloroform/isoamyl alcohol (24:1), mix well and shake gently for 10 minutes.
11. Centrifuge at max (12,000 g) for 10-15 minutes
12. Transfer aqueous phase to a clean tube.
13. Add 2/3 volume isopropanol, mix gently by inverting tube.
14. Centrifuge at max for 15 minutes, pour off isopropanol.
15. Add 1 ml 50% isopropanol/0.3 ammonium acetate. Do not mix but allow pellet to soak in this solution for 30 minutes.
16. Gently pour off the solution and dry the DNA pellet in a SpeedVac.
17. Hydrate the DNA in 50-100 μl of TE.

*For samples that give problems with polysaccharides add the following instead of what is given in steps 6, 7 and 8:

6. Add 27 μl 5M Na Cl
7. 17.5 μl 8.6% CTAB/0.7M NaCl
8. 7.5 μl 10% sacosyl.

Extraction Buffer (100 ml)
3.2 g sorbitol
5.0 g PEG 6000
1.26 g sodium sulphite
2.5 ml 1M Tris pH 8.0
0.5 ml 0.5M EDTA pH 8.0
250 mg BSA (bovine serum albumin)
125 mg spermine
125 mg spermidine
250 μl ß-mercaptoethanol
Make up to 100 ml with H_2O

Wash Buffer (100ml)
6.4 g sorbitol
5 ml 1M Tris pH 8.0
1.26 g sodium sulphite
5 ml 0.5M EDTA pH 8.0
0.1 ml ß-mercaptoethanol
Make up to 100 ml with H_2O

EZNA* Plant DNA Miniprep: Short Method for Dry Samples

Have ready to use:

- waterbath set to 65 ˚C
- sterile water, at 65 ˚C
- ß-mercaptoethanol
- isopropanol
- ethanol (100%)
- RNase A stock solution at 20 mg/ml
- 10 mM Tris, pH 9.0 at 65 ˚C

1. Weigh up to 10 mg of dry tissue samples (silica gel, herbarium specimen). Place in mortar and grind to a powder using liquid nitrogen.

2. Transfer powder to a 1.7 ml microfuge tube. Add 600 μl of **Buffer P1**. Add 20 μl RNase (20 mg/ml). Vortex and incubate at room temperature for one minute. Add 10 μl of ß-mercaptoethanol and vortex to mix (disperse all clumps).

3. Incubate sample in 65 ˚C waterbath for at least 5 minutes, mixing twice (by inverting tubes) during this time. Miquel's modification for herbarium material: incubate overnight.

4. Add 140 μl of **Buffer P2** and vortex to mix. Centrifuge at $\geq$ 10,000 x g for 10 min.

5. Carefully remove 600 μl of supernatant to a new tube - do not disturb the pellet or transfer any debris. Add 300 μl of **Buffer P3** and one volume of 100% ethanol. Vortex thoroughly to obtain a homogeneous mixture. A precipitate may form but will not affect the procedure.

6. Add 800 μl of the mixture to the HiBind DNA column placed in a 2 ml tube (supplied with kit). Centrifuge for 1 minute. Discard the liquid. Add the remainder of the sample (including any precipitate that has formed) and centrifuge as above. Discard the liquid and the tube.

7. Place the column in a second 2 ml tube. Add 750 μl of **Wash Buffer** diluted with absolute (100%) ethanol. Centrifuge for 1 minute and discard the flow-through. Reusing the collection tube, repeat this step.

8. Centrifuge the empty column 2 minutes at maximum speed to dry (this removes residual ethanol).

9. Transfer the column to a clean 1.7 ml tube. Apply 100 μl of 10 mM Tris buffer pH 9.0 prewarmed to 65 ˚C. To increase DNA yield, incubate the column and buffer for 5 minutes at 65 ˚C. Centrifuge for 1 minute to elute DNA. Add another 100 μl of Tris buffer to the column, incubate, and centrifuge. This eluate can be kept separate from the first or combined with the first (lowers the total concentration of DNA).

* From Omega Bio-Tek (www.omegabiotek.com)
P.O. Box 47310
Doraville, GA 30362
800-832-8896; 888-624-1688 - fax

SECTION 4: PLANT RNA EXTRACTION

General Consideration and Rationale

RNA is quite prone to degradation due to the extremely resilient ribonucleases. RNase can survive even boiling temperatures, hence autoclaving is not entirely successful in removing it. To completely eliminate RNase, all glassware associated with RNA extractions should be baked at 180-200° C for at least 4 hrs. For removing RNase from solutions, the enzyme RNAasin can be used at 1000 units/ml. Blumberg (1987, p. 24) mentions the need to include at least 1 mm dithiothreitol for proper enzyme activity. Solutions used in RNA extractions should be made up in DEPC-treated water (0.01 %, let stand 24 hrs., autoclave). The one exception is ammonium acetate which should be filter sterilized (see Maniatis et al. 1982, p. 447).

Vanadyl-ribonucleoside complexes (also known as ribonucleoside-vanadyl complexes), are potent inhibitors of ribonuclease activity (Berger 1987). These complexes are useful in inhibiting ribonucleases until proteins can be quantitatively removed. The detergent sodium dodecyl sulfate (SDS) also inhibits nucleases and helps separate the proteins from the nucleic acids (esp. in ribosomes). When using SDS, avoid the use of potassium salts or temperatures below 10° C since this may cause precipitation of the detergent. Sarkosyl and sodium deoxycholate are sometimes used in the place of SDS. Polyvinyl sulfate and heparin are nonspecific inhibitors of ribonucleases (Blumberg 1987).

Phenol and chloroform are the most common reagents used for removing proteins. Other compounds, such as guanidinium isothiocyanate and guanidinium chloride are also used. These chaotropic agents disrupt protein secondary structure, including RNases (see MacDonald et al. 1987 and Cox 1968 cited in Maniatis et al. 1982).

High lithium chloride (LiCl) salt concentrations (e.g. 2 M or greater) will precipitate larger RNA (e.g. rRNA). The small RNA (tRNA and 5S rRNA) and DNA remain in the supernatant. Wallace (1981) recommends adding one volume of 8 M LiCl (= 4M LiCl in the sample). The hot borate method of Hamby et al. (1988) uses 1/4 volume of 10 M LiCl (= 2.5 M).

More recently, the Tri Reagent TM (Molecular Research Center, 5645 Montgomery Rd., Cincinnati, OH 45212) has proven useful in rapidly isolating RNA from a variety of organisms including plants. The tissue is homogenized in Tri Reagent (guanidine thiocyanate and phenol in a mono-phase solution), centrifuged to separate the phases, and the RNA is then precipitated using isopropanol.

RNA Extraction Using Hot Borate Buffer

This method was borrowed and slightly modified from Dr. Elizabeth Zimmer who modified the protocol described by Hall et al. (1978). Zimmer freezes the plant tissue on liquid nitrogen and then stores at -80 ˚C in airtight plastic bags until ready for extraction. I found that for some plants, the freezing adversely affected RNA yield. If at all possible, I suggest doing the extraction starting with fresh material. The best results are obtained with embryonic or meristematic tissues. You will need to get the following:

Equipment and supplies:
- Centrifuge and rotor precooled to 4 ˚C (e.g. Sorval with SS-34 rotor)
- Centrifuge tubes, glass, 25 ml Corex (one tube each per sample)
- Centrifuge tubes, polypropylene, 50 ml, sterile (Corning 25330) --or next --
- Centrifuge tubes, polyallomer, 50 ml, Nalgene (autoclaved)
- Funnels, glass [baked]
- Liquid nitrogen
- Miracloth (or cheesecloth), 9" X 9" squares (autoclaved)
- Mortars and pestles [baked]
- Pasteur pipets [baked]
- Pipets, plastic, disposable, 10 ml and bulb
- Polytron (Brinkman) homogenizer with the 2.0 cm diameter generator [autoclaved]
- Spoonulas or spatulas [baked]
- Squirt bottle filled with Sarcosyl rinse solution
- Test tubes, glass, 2.5 X 20 cm [baked]
- Water bath set at 37˚ C and another at boiling

Reagents
- Ammonium acetate (NH_4OAc, 4 M)
- Lithium chloride (LiCl, 2 M)
- Lithium chloride (LiCl, 10 M)
- Potassium acetate (KAc, 2 M, pH 5.5)
- Potassium chloride (KC1, 2 M)
- Protease K powder (stored in 50 units/1 ml aliquots in the freezer)
- Sodium borate buffer (0.2 M borate, 30 mM EDTA, 5 mM dithiothreitol)
- Sodium dodecyl sulfate (SDS, 20% w/vol)
- STE buffer (salt, Tris, EDTA)
- TE buffer
- Organics: chloroform:isoamyl alcohol (24:1), Tris buffered phenol, cold 70% and 100% ethanol

1. Make up the appropriate volume of borate extraction buffer for the number of samples you wish to process (25 ml per sample). The SDS is best added after the samples have been homogenized to minimize foaming. Place the buffer in the boiling water bath to achieve 90-95 ˚C.

2. Clean the Polytron generator with 95% ethanol, acetone, and then the Sarcosyl rinse solution.

3. Weigh out 5 gm of young leaves (wash and blot dry if especially dirty).

4. Place the leaves in the mortar, add enough liquid nitrogen to cover the leaves (ca. 1/2 full), and grind. Continue grinding vigorously after the nitrogen has all evaporated until the tissue is a fine powder.

5. Transfer the powder to a 2.5 X 20 cm glass test tube with the spoonula. Quickly add 25 ml of the hot borate buffer. Homogenize the sample with the Polytron using relatively short bursts to keep foaming to a minimum. Filter the sample into the Nalgene centrifuge tube using a funnel with Miracloth.

6. Add the 1.25 ml of 20% SDS solution and the Protease K solution to the centrifuge tube using a micropipeter. Be sure to add these solutions to the liquid below the foam. Cap and place in the 37 ˚C water bath to incubate for one hour. Mix occasionally during this time.

7. Place the tube on ice and add 1.0 ml of 2 M KCl. Allow to chill for 5-10 minutes.

8. Centrifuge the sample at 8,500 rpm at 4˚ C for 10 minutes. Place a Kimwipe in a glass funnel and place the funnel in a baked 30 ml Corex centrifuge tube. Remove the samples from the centrifuge and pour the supernatant through the Kimwipe into the Corex tube. Add 1/4 the volume (ca. 3.0 ml) of 10 M LiCl. Freeze the sample briefly (on dry ice or in -70 ˚C freezer) and then place in refrigerator (4 ˚C) for 4 hours.

9. Centrifuge at 9,000 rpm for 15 minutes at 4 ˚C. As soon as the rotor stops spinning, pour off the supernatant (keep the pellet).

10. Add 5.0 ml of ice cold 2 M LiCl to the pellet. Resuspend and wash the pellet by moving it up and down in the pipet.

11. Centrifuge at 9,000 rpm for 15 minutes at 4 ˚C. As above, pour off supernatant as soon as possible.

12. Resuspend the pellet in at least 2.0 ml of 2 M potassium acetate (pH 5.5). Use more for exceptionally large pellets. Resuspension at this stage often requires vortexing and possibly gentle heating (in 37 ˚C water bath).

13. Add 2.5 volumes (ca. 6.0 ml) of ice cold ethanol. Store sample at -20 ˚C for 2-4 hours (overnight is OK).

14. Centrifuge the Corex tube at 8,500 rpm for 15 minutes at 4 ˚C. Pour off the supernatant and let the pellet dry briefly. Dissolve the pellet in 5 ml of STE buffer.

15. Add ca. 2.5 ml of Tris buffered phenol (pH 8.0). Add an equal volume of 24:1 solution of chloroform/isoamyl alcohol (use a glass pipet). Cap, mix thoroughly, and let it stand on ice of 5 mins.

16. Centrifuge at 7,000 rpm for 10 minutes at 4 ˚C. With a sterile Pasteur pipet, carefully remove the top (aqueous) layer and place in a clean, sterile Corex tube. Be careful not to draw any of the interface or lower phenol layer into the pipet.

17. For every 3 ml of supernatant, add 1 ml of 4 M ammonium acetate and 2.5 volumes of ice cold ethanol (10 ml). Cap, invert to mix, and store 2-4 hours at -20 ˚C.

18. Centrifuge the sample at 8,500 rpm for 15 minutes at 4˚ C. Pour off the supernatant and let the pellet air dry for a few minutes (until just moist, no ethanol odor). Dissolve the RNA pellet in 1.0 ml of DEPC treated water (or TE buffer if you wish). Store the RNA at –20 ˚C or colder.

TRIzol RNA Isolation Method

Reagents

• TRIzol Reagent: (final concentration):
Phenol in saturated buffer (38 %): 380 ml/liter
Guanidine thiocyanate (0.8 M): 118.16 g
Ammonium thiocyanate (0.4 M): 76.12 g
Sodium acetate, pH5 (0.1 M): 33.4 ml of 3M stock
Glycerol: 50 ml
H_2O: to 1 liter

Other Reagents:
• 0.8 M sodium citrate / 1.2 M NaCl
• Isopropanol
• Chloroform
• 75% EtOH
• DEPC-treated ddH_2O

Homogenization

1. Grind 1 g tissue in liquid nitrogen in a motar and pestle. For some tissues, the preparation will need to be scaled up or replicated to obtain enough total RNA to recover 5μg poly A+ (see below) This must be determined empirically.

2. Put tissue into a plastic screw-cap centrifuge tube containing 15 ml TRIzol reagent. Incubate samples for 5 min at room temp or 60° C.

Phase Separation

1. Homogenize tissue with homogenizer for 15 sec., repeat once.
2. Centrifuge samples at 12,000 X g at 4° C for 10 min.
3. Put supernatant into new screw-cap tube. Discard pellet (or save if you want DNA).
4. Add 3 ml chloroform to each tube.
5. Shake tubes vigorously with vortex for 15 sec.
6. Let tubes sit at room temp 2-3 min.
7. Centrifuge tubes at 10,000 X g at 4° C for 15 min.

RNA Precipitation

1. Carefully pipette aqueous phase into a clean screw-cap centrifuge tube; discard interphase and lower phase.
2. Add isopropanol and 0.8 M sodium citrate/1.2 M NaCl, 1/2 volume of the aqueous phase each. Cover tube and mix by gentle inversion.
3. Let sit at room temperature for 10 min.
4. Centrifuge tubes at 10,000 X g at 4° C for 10 min.

RNA Wash

1. Discard supernatant. Wash pellet with 20 ml of 75% ethanol by votexing briefly.
2. Centrifuge at 10,000 X g at 4° C for 10 min.
3. Discard supernatant; briefly dry pellet (5-10 min; not longer).
4. Add 250 μl of DEPC-treated H_2O to pellet. Resuspend by pipetting up and down a few times.
5. Also add 200 μl RNase inhibitor (1 μl). Incubate at 55 –60˚ C for 10 min to resuspend pellet. Transfer sample to microfuge tube.

6. Centrifuge samples at 4° C for 5 min (to pellet the material that would not resuspend).

7. Pipette supernatant into new microfuge tube.

8. The concentration and quality of the RNA can now be determined, e.g. with a spectrophotometer. For optimal spectrophotometric measurements, RNA aliquotes should be diluted with water or buffer with a pH > 7.5. Distilled water with pH < 7.5 falsely decreases the 260/280 ratio.

Simple Plant RNA Extraction

Modified from Ems et al. Plant Molecular Biology 29:721-733, 1995

1. Weigh out 2 to 4 g of plant tissue, place in mortar.

2. Grind tissue to a powder on liquid nitrogen.

3. Add frozen powder to baked glass Corex centrifuge tube (30 ml) containing hot (60° C) phenol emulsion [5 ml phenol plus 6 ml extraction buffer - below]. Stir at 60° C for 5 minutes. [or, incubate in hot water bath and mix occassionally]

4. Add 5 ml of chloroform/isoamyl alcohol (24:1), stir for 5 minutes.

5. Centrifuge in RNase-free tube at 10,000 rpm for 10 min. at room temperature. Transfer the aqueous phase to a new tube. Add 5 ml of chloroform/isoamyl, mix, centrifuge as above.

6. Transfer aqueous phase to new tube. Adjust to 2 M NH_4OAc [i.e. add equal volume of 4 molar NH_4OAc].

7. Add one volume of isopropanol (room temperature), incubate at room temp. for 15 minutes.

8. Centrifuge at 10,000 rpm for 20 minutes. Wash pellet twice with 70% ethanol and dry.

9. Resuspend pellet in 1.0 ml or less of DEPC-treated dH_2O.

Extraction Buffer
100 mM Tris pH 8.0
20 mM EDTA
0.4% SDS
0.5 M NaCl
0.1% β-mercaptoethanol

NUCLEIC ACID CONCENTRATION: FLUOROMETRY

Nucleic acids can be quantified using spectrophotometry or fluorometry. For the former, the amount of nucleic acid in a sample is quantified by measuring the absorbance at 260 nm. The fluorometer measures the fluorescence of a dye (Hoechst H 33258) in the presence of DNA. This method is preferred over spectrophotometry because it does not require as much DNA (hence less is wasted). Ed Heist (Zoology) has a fluorometer that he let's us use to measure the quantity of DNA.

Calf thymus DNA will be used as a concentration standard. The concentration of the calf thymus DNA standard is 100 ng/μl. We will dilute each sample 1000 fold with low range assay buffer. The final concentration of the standard will be 100 ng/ml.

The fluorometer will provide us with measurements of DNA concentration in ng/ml. Because our samples are diluted 1000X, the concentration of the DNA in our tubes will be equal to 1000X the concentration provided by the machine. For example, if the fluorometer tells us the concentration of our diluted DNA is 25 ng/ml, we know that the concentration of DNA in our tube is 25 ng/μl.

When the fluorometer is calibrated with low range assay buffer and 100 ng/ml calf thymus DNA, accurate measurements can be made in the range of 10-500 ng/ml diluted (10-500 ng/μl concentrated).

Quantify each sample twice. In practice, results that agree within 5% should be achieved. You can record your results in the table like the one shown below.

Sample	Reading 1	Reading 2	Mean

SECTION 6: AGAROSE GEL ELECTROPHORESIS

Minigels are run to check for the presence of nucleic acids. This is done for example, after an extraction protocol to determine whether DNA (or RNA) was successfully isolated or following a PCR amplification to determine whether the reactions were successful. Both DNA and RNA will stain with ethidium bromide and will migrate to different positions based on their molecular weight. Lower agarose percentages (0.6-0.3%) can resolve DNA in sizes ranging from 1 to 60 kb (Maniatis et al., 1982). Higher percentage agarose (e.g. 2%) works in a range of 0.1-3 kb. Most of our applications work somewhere in the middle, thus we use a 1% Tris-acetate EDTA (TAE) gel.

Preparing Minigels

1. It is convenient to prepare agarose in buffer ahead of time and store the solution in small Erlenmeyer flasks for later use. The agarose is then simply melted in a microwave oven prior to pouring. The agarose can be stored at room temperature for several weeks.

2. Choose the appropriate size of minigel rig/comb combination based upon the number of samples to be run. The 30 ml Owl Scientific rigs (Model B1A) have combs to accommodate either10 or 12 samples. Note that one can also "double load" by placing two combs in a single gel (hence 20 sample lanes).

3. Turn the gel mold so that the gaskets are against the inner walls of the electrode chamber. Position the comb in the mold (depth preset). Place gel rig in dairy case on flat tray. Make sure the surface is level (use floating bubble level and adjust with towels under tray if necessary).

4. Microwave the flask with agarose, checking occasionally to see whether all the agarose has melted. If a microwave is not available, melt the agarose in a beaker of water on a hotplate.

5. Measure out the appropriate volume of hot agarose into a graduated cylinder (for the small Owl rigs, 30 ml). Cool the cylinder by running cold tap water along the outside of the flask. When only warm to the touch, pour the agarose into the mold inside the dairy case.

6. The gel is ready to run after the agarose is solidified, ca. 15 mins. in the dairy case. Gels can also be poured at room temperature, but they take longer to solidify.

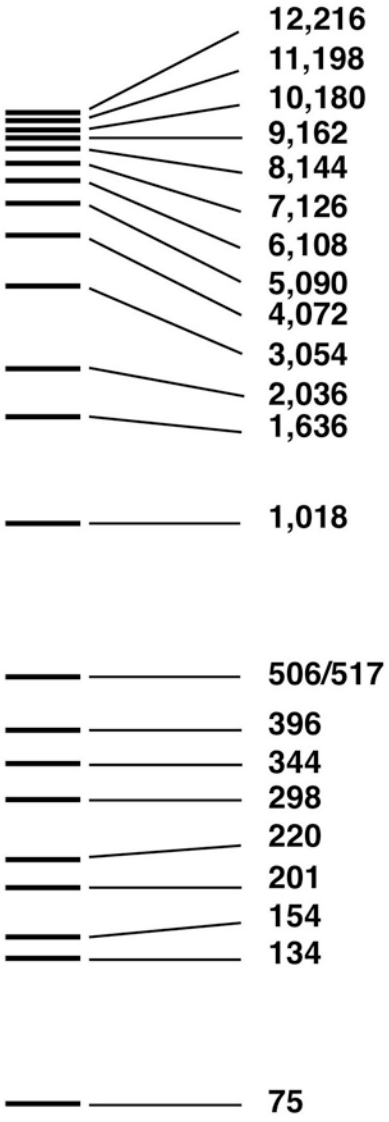
Preparing Samples and Running Minigels

1. Gently remove the comb by pulling evenly upward.

2. Position the gel mold in the electrode chamber and cover with buffer (1 X TAE).

3. Since the agarose gel is "submarine," the samples will be loaded into the lanes under buffer. This requires that the samples be heavier than buffer. This is accomplished by mixing the samples with glycerol or Ficoll. The most frequently used loading dye is a 1 X dye/buffer/glycerol solution (see Solution Recipes).

4. An easy way to mix the dye with the sample on a piece of Parafilm. Using a permanent marker, number the Parafilm with the number of samples to be run. Spot out 10 μl of dye near each of the numbers. Add an extra spot for the molecular weight standard (see Figure) that should be run next to your samples.



5. Pipet 4 μl of your sample and add to the dye spot of the correct number. Hold the tip and eject. Place the tip on another pipeter set to 14 μl and draw up the entire sample/dye mixture. Pipette the mixture into to the appropriate minigel lane so that when photographed lane number one is to the top left. Continue until all the samples and the M.W. standard DNA is loaded.

6. Replace the cover to the electrode box (which simultaneously attaches the electrodes) and plug in the wires to the power pack. The samples should be run towards the red or anode wire. Turn on the power pack and, using the low milliamperage setting, run the gel at ca. 75 ma. Some power packs automatically adjust the voltage needed to achieve a particular number of milliamps, but others do not. For the later, milliamperage will drop during the run, but be careful not to overheat the gel if you adjust the voltage upward. Run times will vary slightly, but the Owl rigs are normally finished in 40 minutes. Stop the run before the bromophenol blue dye exits the gel.

Staining and Imaging Minigels

1. Put on gloves. After turning off the power, gently remove the gel mold from the electrode chamber and slip the gel into the plastic staining box containing ethidium bromide (EthBr). Be aware that EthBr is a toxin (mutagen). *Always wear gloves when working with EthBr.*
2. The EthBr is kept in a dark bottle in the refrigerator in a 10 mg/ml solution. For the staining box, add 20 μl of EthBr to ca. 40 ml of 1X TBE buffer. The stain box stays inside the foil-lined box to exclude light. Stain for ca. 15 minutes in the dark.
3. To visualize EthBr stained DNA, one must illuminate with U.V. light. NOTE !! Whenever you turn the U.V. light on, be sure to wear protective goggles for your eyes!! U.V. light will cause bums on exposed skin, so work quickly and carefully when the light is on. Lower the hinged protective shield on the Fisherbrand U.V. transilluminator before turning on the U.V. light. Darken the room lights and view the gel.
4. The ethidium bromide stained gels are imaged using the Nikon950 digital camera. After transferring the image to the Del computer, the image is later printed on a thermal printer.

Capturing Gel Images as Digital Computer Files

1. Place gel on the transilluminator opening with the long axis of the gel oriented left to right (lanes on left).
2. Position the camera (mounted on the black hood) over the transilluminator opening.
3. Plug in the power cord at the junction on the top of the camera. Turn camera on by rotating the switch to the "M" (manual) position. Turn off the flash. The camera should be in close-up mode such that you see a flower (not mountain) on the LCD screen. The camera should also be set to take Black and White photos, which you can select in the menu (under Special Exposure Mode).
4. Turn on the transilluminator and dial the reostat to maximum.
5. Use the zoom button to frame the gel as you wish. [Technically, one is supposed to take the photo in close-up mode only when the zoom reaches the "sweet spot", indicated when the flower turns yellow. We have found that this doesn't matter, i.e. the photos are fine even when the flower is white.] Take the photo.
6. Plug in the serial cable (right side of camera). The LCD monitor will go black.
7. On the computer, double click the Nikon View icon. In the window, double click Digital Camera. It will take some time to connect to the camera. Eventually you will see 100 Nikon. Double click this and your photo(s) will appear in the window. Drag your photo(s) to the correct folder on the desktop. We have three as follows: **Genomic DNA**, **PCR**, and **Purifications**. This will take some time to transfer.
8. Open the folder that contains your photo file and rename it. It will come with a name such as DSCN###. Name it appropriately (e.g. PCR####).
9. When you are sure you have an acceptable photo, throw away the photo file from the 100 Nikon folder. "Control A" selects all files, hit the "Delete" key, and say "Yes". Close the window. Leave the computer on (it goes to sleep eventually).
10. Turn off the digital camera and unplug the power and serial cables.
11. Dispose of the gel in the waste container and clean the surface of the transilluminator.

Dispose of the agarose gel in the waste bucket. Clean off the surface of the transilluminator with the dH_20 squirt bottle.

The photograph files can be printed on a Thermal printer (Sipes lab).

Inactivating Ethidium Bromide

One of the more hazardous chemicals that exists in a molecular biology laboratory is ethidium bromide (EthBr). This reagent is indispensable since it can intercalate within the stacked bases of DNA and RNA and, when illuminated with U.V. light, produce fluorescence that indicates the presence of nucleic acid. Unfortunately, EthBr is also a potent mutagen. Gloves should always be worn when handling gels, etc. that contain this chemical. Clean up any spills - no matter how small. EthBr solutions are light sensitive so should be kept in dark bottles away from the light.

A number of different ways have been proposed to inactivate EthBr - some are effective and others are not. Many labs simply add sodium hypochlorite to the solution containing EthBr, however, this does not eliminate the mutagenicity (see Lunn and Sansone, 1987). A more effective way is as follows:

1. Dedicate a large-mouth jar (1.0 liter or larger) to EthBr waste - *place in hood area*.

2. For every liter of buffer containing EthBr, add 16 ml of 50% hypophosphorus acid and 3 g of sodium nitrite ($NaNO_2$). For proper neutralization of EthBr, the solution should be below pH 3.0.
Gently stir the solution and allow to sit for 24 hours.

3. Add ca. 3 g of sodium bicarbonate to neutralize the solution. Allow to stand for ca. 1 hour. If the liquid is clear, it is safe to pour down the sink. If not, repeat above procedure.

****

For Agarose Gels That Contain Ethidium Bromide

Agarose gels contianing ethidium bromide are placed in a large-mouth container that is labeled with the red and white sticker provided by the **Center for Environmental Health and Safety**. Their campus telephone number is 453-7180. Personnel from this office will pick up and dispose of this waste after a request is made (form filled out) on their website at: http://www.cehs.siu.edu./

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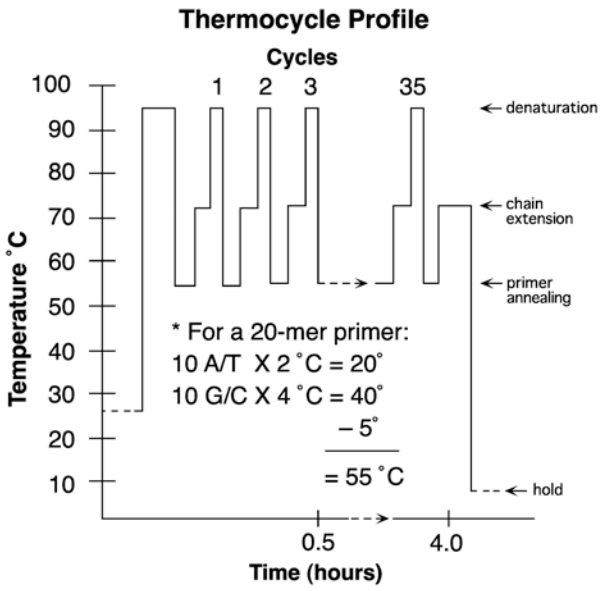
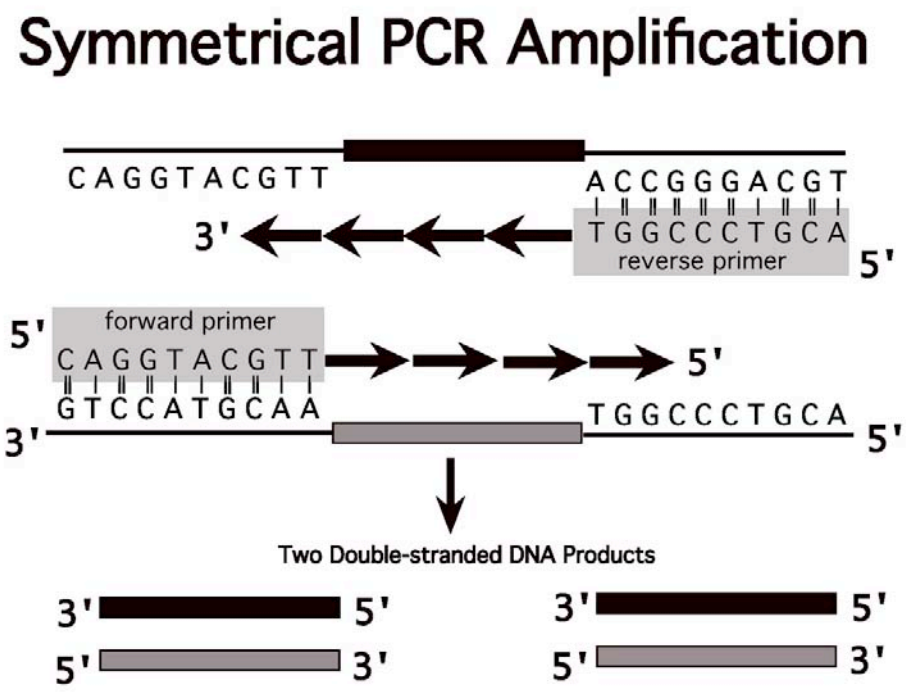
SECTION 7: POLYMERASE CHAIN REACTION

How PCR Works

The polymerase chain reaction (PCR) is an in vitro method of enzymatically amplifying (increasing) a small amount of target DNA as much as 108-fold. This amplified DNA can then be used for cloning, sequencing or other molecular applications. The process is composed of three steps: **DNA denaturation, primer annealing**, and **chain extension** (see figure below). These processes are facilitated through the use of a thermal cycler - a programmable machine that can achieve temperatures from 0° C to 100° C, can rapidly change from one temperature to the next, and can hold any temperature for a specified amount of time. The enzyme that can survive these extreme temperature changes without significant degradation is Taq polymerase - isolated from *Thermus aquaticus*, a thermophilic bacterium.

During denaturation, the target DNA is "melted", i.e. the strands of the double helix come apart. This typically occurs at 94° C. Oligonucleotide primer annealing is optimal at lower temperatures, typically 37-55° C, depending upon the primer length and A/T - G/C content. For a symmetrical PCR amplification, two primers (a forward and reverse) must be present that bracket the gene of interest (see figure below). Chain extension via Taq polymerase occurs from the 5' ends of each of the two primers and this step has an optimum temperature of 72° C. This new chain is complementary to the sequence of the template DNA. The time required for denaturation and primer annealing is generally from 30 seconds to one minute each, whereas chain extension requires 1 - 3 minutes. Once one cycle is complete, the amount of target DNA in the sample is doubled. With increasing numbers of cycles, the DNA that is produced can also serve as a template, hence the concentration of target DNA increases logarithmically and then tapers off (limited by the amount of primer and nucleotide in the original solution). For asymmetrical amplifications, as occur during cycle sequencing, only one primer is used. Therefore, the number of templates available for the primer to anneal to is less and is determined by the initial concentration of template. The increase in DNA amount through the PCR reaction is arithmetic (linear), not logarithmic. For more discussion of the PCR process, see Kessing et al. (1989), Saiki et al. (1988), Mullis (1990) and Dieffenbach and Dveksler (1995).





In the Nickrent lab there are three thermocyclers: Perkin-Elmer Cetus, RoboCycler Gradient 40 temperature cycler (Stratagene), and Applied Biosystems GeneAmp PCR System 9700. For the first two machines, mineral oil must be placed above the sample to prevent excessive evaporation of the reaction mix. The GeneAmp thermocycler does not require mineral oil in the tubes because the lid is heated, thus preventing condensation from accumulating on the inside of the sample tubes. Refer to the manuals for proper operation of these machines.

The RoboCycler is very useful for optimization of PCR reaction conditions. This machine has four independent heating/cooling blocks (3 heating, 1 cooling) and a robotic arm that moves the sample tubes between blocks. Most importantly, it also has a temperature gradient block that allows for a linear temperature gradient range of 14 ˚C. This feature can be used to determine, in one set of reactions, optimum primer annealing temperature.

Symmetrical Amplifications

The following protocol gives a typical recipe for a symmetrical amplification of a gene. This reaction mix can be used for a variety of genes by simply substituting the appropriate forward and reverse primers and by using different thermocycler programs.

1. Turn on the thermocycler (lid begins heating). Remove the 10X PCR buffer, water, primers, genomic DNA and dNTPs from the freezer to begin thawing. We keep sterile water frozen in aliquots in the freezer to prevent the growth of fungi, etc. that may take place more readily at room temperature. Place the tubes on ice when thawed. Keep the dNTPs on ice at all times once thawed! Leave the Taq polymerase in the freezer until you are ready to use it and return it to the freezer promptly when finished. The Taq may be used undiluted or diluted (e.g. 1:1 to 1:9) with Taq dilution buffer depending upon its activity. We use "home-made" Taq polymerase, hence we do not know the exact Unit/μl concentration. We simply determine empirically the activity on a batch-by-batch basis. Communicate with lab members to keep up on what dilution is to be used for the Taq.

2. Label (on the lip of the lid!) the proper number of thin-walled 0.2 ml microfuge tubes - one for each sample being amplified.

3. Dilute the genomic DNA samples (e.g. 1:9 w/ dH_20). Leave the pipet tip in the tube for later use.

4. If all of the tubes use the same primers, label a cocktail tube and determine the amount of dH_20, 10X PCR buffer, etc. that can be pooled by multiplying each volume by the number of tubes required. Obviously the DNA is omitted from the cocktail if the tubes are to contain different samples. When doing more than 10 tubes, add one more to account for pipetting error.

17.0 μl	dH_20
2.5 μl	10X PCR buffer*
1.5 μl	$MgCl_2$ **
0.5 μl	dNTP (= dATP, dCTP, dGTP & dGTP; 2.5 mM each)
1.0 μl	forward primer ***
1.0 μl	reverse primer ***
0.5 μl	Taq polymerase
1.0 μl	Genomic DNA (straight or diluted, e.g 1:9)
25.0 μl	Total

* Promega (M1901): 500 mM KCl, 100 mM Tris-HCl (pH 9.0), 1% Triton® X-100.
** Promega (A3513): 25 mM
*** 1:19 dilution of stock solution (1250 μg/ml) = 62.5 μg/ml $\approx$ 10 μM

In the final PCR reaction mixture, the concentrations are: Promega buffer (50 mM KCl, 10 mM TrisHCl), 1.5 μM $MgCl_2$, 50 μM dNTPs, 0.4 μM forward and reverse primers, 1 Unit Taq polymerase, 30–50 ng genomic DNA.

5. Dispense the correct amount of the cocktail mix to each numbered tube. Add the genomic DNAs directly to the solutions. The program parameters used for various genes vary considerably (see attached Table). The following represents a standard amplification protocol that works with many genes:

Denaturation: 94˚C for 5 min.
35 Cycles: 94˚C for 40 sec., 50˚C for 40 sec., 72˚C for 2.5 minutes.
Final Extension: 72˚ C for 10 minutes
Hold (indefinite time): 4˚ C

A typical **step-up procedure** (e.g. for *matK*), is shown below. The general idea here is to first amplify relatively nonspecific templates and then increase the annealing temperature to realize higher stringency. This protocol should be tried if there are mismatches at primer binding sites.

Denaturation: 94˚C for 5 min.
5 cycles: 94˚C for 1 minute, 46˚C for 1 minute, 72˚C for 2 minutes.
35 cycles: 94˚C for 30 sec., 50˚C for 30 sec., 72˚C for 1.5 minutes.
Final Extension: 72˚ C for 10 minutes
Hold (indefinite time): 4˚ C

A typical **touch-down procedure** (e.g. for *trn*T-L-F), is shown below. The general idea here is to first amplify specific templates and then decrease the annealing temperature to allow the polymerase to function more efficiently on the specific templates.

Denaturation: 94˚C for 5 min.
5 cycles: 94˚C for 30 seconds, 52˚C for 30 seconds, 72˚C for 1 minute.
33 cycles: 94˚C for 30 sec., 48˚C for 30 sec., 72˚C for 1 minute.
Final Extension: 72˚ C for 10 minutes
Hold (indefinite time): 4˚ C

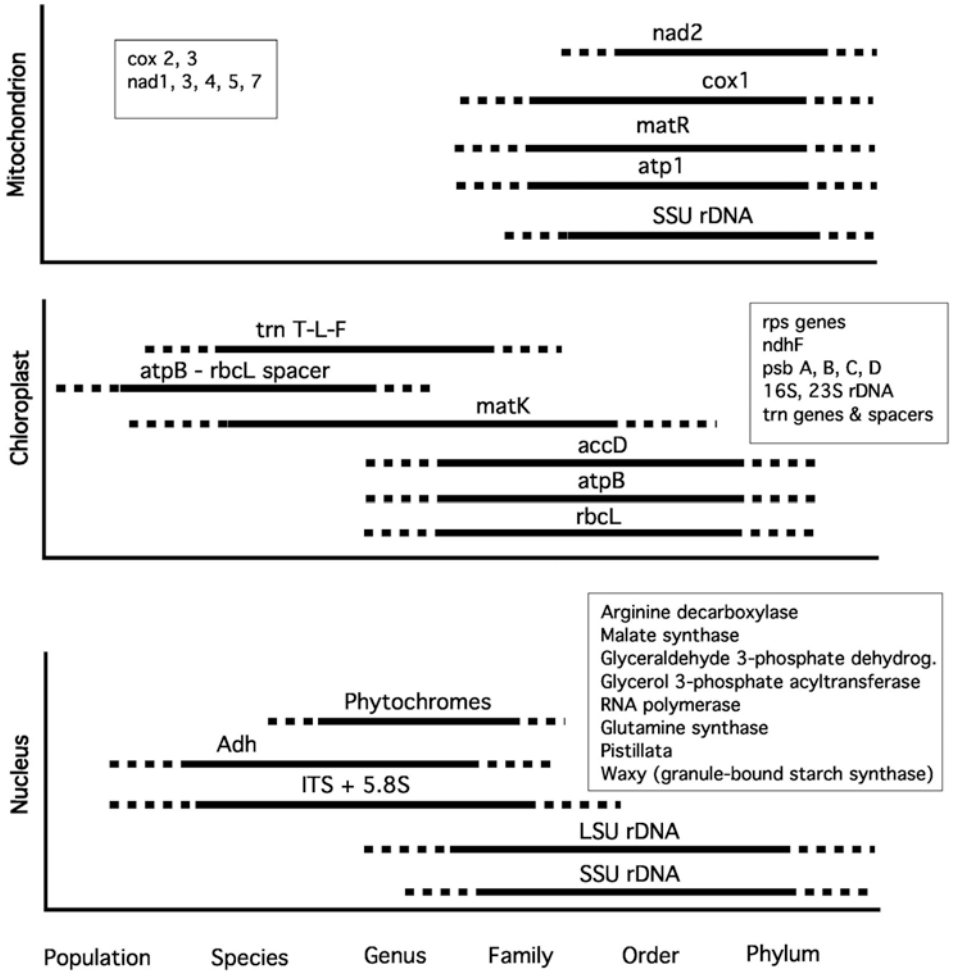
When the thermocycler (GeneAmp) has the lid heated (ca. 5 minutes), place the plastic tube rack on the heat block. Place the tubes in the wells and use the roller to ensure the lids are tightly closed. Close the heated lid and begin the thermocycle program.

To determine whether the amplification was successful, the PCR products are run on a 1% TAE agarose minigel. Remove 4 μl of the PCR product, mix with 10 μl of loading dye, and load into the well of the agarose gel with a molecular weight size standard.

When conducting PCR amplifications from an existing PCR product, there are **MANY** considerations to be aware of. The major problem is contamination of pipets in Room 1046A. The best way to do this is set up the PCR reaction in 1046A and move the tubes to 1046. Then, using the pipets from that room, with filter tips, add the PCR product to your tubes. You should always include a negative control as well as a tube that was the negative from the original PCR (negative of the negative).

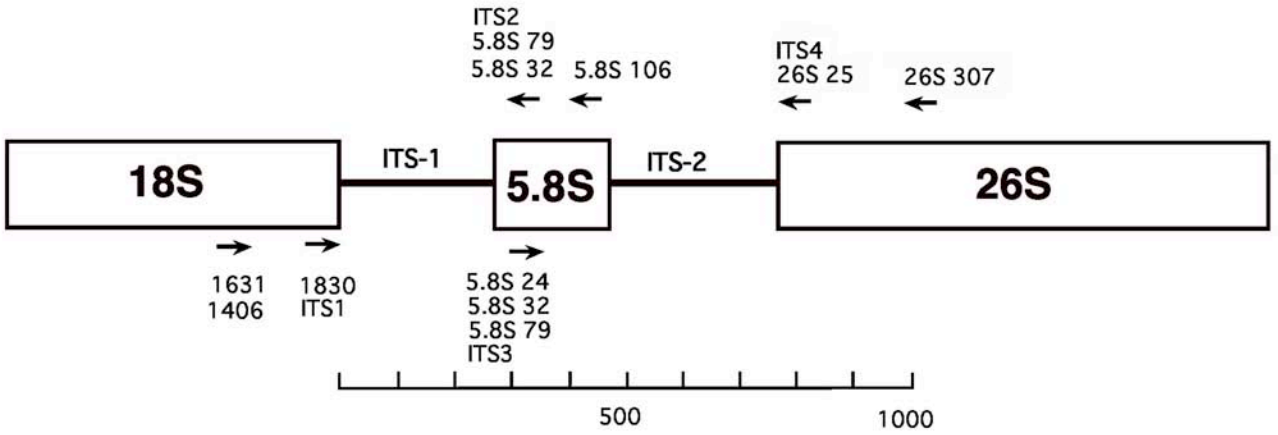
Genes Commonly Amplified

It is important to consider that each gene has a different range of applicability with regard to resolving divergences among taxa (figure below). For example, *rbcL* may work very well to resolve families in an order or even genera in a family, but it is unlikely to provide sufficient numbers of substitutions to examine species within a genus. In general, we typically attempt to maximize the number of substitutions (*K*) between taxa we are trying to resolve – but not to the point of site saturation. Frequently we combine information from more than one gene. But here it is important to make sure the phylogenetic relationships resolved by the individual genes (partitions) are not in conflict, that is, that they are congruent. If they are congruent, then there is good reason to analyze them together in one data matrix. For plants, it is also helpful to know whether the nuclear, chloroplast, and mitochondrial genes are congruent. If they are, then one can feel more confident that the organismal phylogeny is being tracked as opposed to the phylogeny of just one gene. These can be very different, for good biological reasons (hybridization, introgression, chloroplast capture, lineage sorting, and horizontal gene transfer).



The following pages give schematic diagrams of the genes commonly amplified in plant phylogenetic studies and their primers.

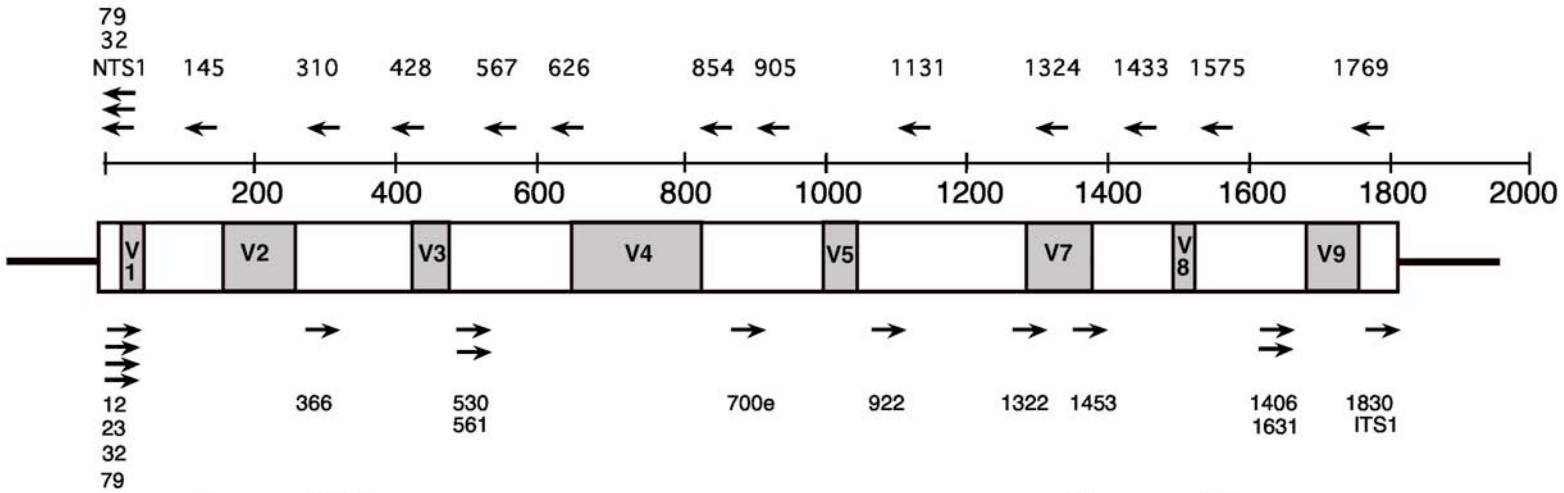
Nuclear Internal Transcribed Spacer (ITS) Ribosomal DNA



Primer Sequence 5'-3'	Size	Position	Name
TTT GTA CAC ACY GCC CGT CG	20	1631-1650	18S 1631 for
TGY ACA CAC CGC CCG T	16	1633-1648	18S 1406 for
AAC AAG GTT TCC GTA GGT GA	20	1769-1787	18S 1830 for
TCC GTA GGT GAA CCT GCG G	19		ITS1 for
GAA GAG GAA TGA CAA AGA AC	20		OWDM ITS1 for
TCT TGA CTC TCG TAT CGA TG	20	24-44	DM 5.8S 24for
CGA AAC ATA TCA AGA AAC CTG	21		OWDM ITS2 rev
GCA TCG ATG AAG AAC GTA GC	20	32-52	5.8S 32 for
GCA GAA TCC CGT GAA CCA TC	20	79-99	5.8S 79 for

Primer Sequence 5'-3'	Size	Position	Name
GCT ACG TTC TTC ATC GAT GC	20	32-52	5.8S 32 rev
GAT GGT TCA CGG GAT TCT GC	20	79-99	5.8S 79 rev
AGG CGC AAC TTG CGT TCA AA	20	106-126	5.8S 106 rev
TAT GCT TAA AYT CAG CGG GT	20	26-45	26S 25 rev
TCC TCC GCT TAT TGA TAT GC	20	41-60	40 rev = ITS4
GCA TTC CCA AAC AAC CCG AC	20	267-285	26S 268 rev
TTG GGC TGC ATT CCC A	16	278-293	307 rev
AGA TGA GGG GAC ATG TTC AC	20	-	Apo C ITS rev
AGC TTT AGC ACC ACA ACT GG	20	-	Apo D ITS rev

Nuclear Small-Subunit (18S) Ribosomal DNA



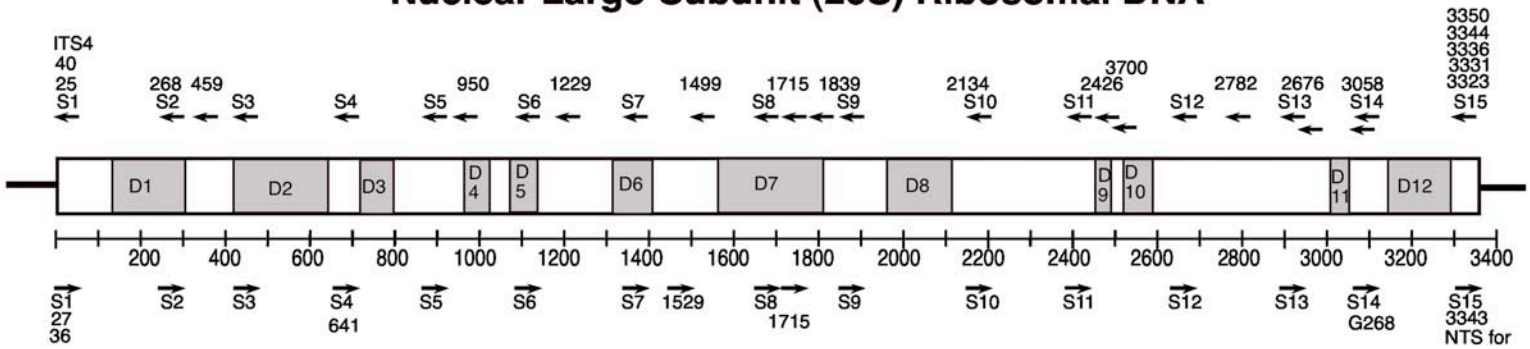
Forward Primers

Primer Sequence 5'-3'	Size	Position	Name
ATC TGG TTG ATC CTG CCA GT	20	2-21	I1a
TTG GTT GAT CCT GCC AGT AG	20	4-24	23 for
TCC TGC CAG TAS TCA TAT GC	20	12-21	12 for
GCA TCG ATG AAG AAC GTA GC	20	32-52	32 for
GCA GAA TCC CGT GAA CCA TC	20	32-52	79 for
GTC TGC CCT ATC AAC T	16	307-322	366 for
GTG CCA GCA GCC GCG G	16	566-581	530 for
GTG CCA GCA GCC GCG G	16	566-581	561 for
YAG AGG TGA AAT TCT TGG	18	901-918	700e for
GAA ACT TAA AKG AAT TG	17	1134-1150	922 for
TAA CGA ACG AGA CCT CAG CCT	21	1322-1342	1322 for
ARC TTC TTA GAG GGA C	16	1378-1393	1453 for
TGY ACA CAC CGC CCG T	16	1633-1648	1406 for
TTT GTA CAC ACY GCC CGT CG	20	1631-1650	1631 for
AAC AAG GTT TCC GTA GGT GA	20	1769-1787	1830 for
TCC GTA GGT GAA CCT GCG G	19		ITS1 for

Reverse Primers

Primer Sequence 5'-3'	Size	Position	Name
GAG ACA AGC ATA TGA CTA C	19	20-48	NTS-1 rev
GCT ACG TTC TTC ATC GAT GC	20	32-52	32 rev
GAT GGT TCA CGG GAT TCT GC	20	32-52	79 rev
CTA GYA TTR CTA CGG TTA TCC	21	141-161	145 rev
CCA TCG AAA GTT GAT AGG GCA	21	310-330	310 rev
TTT CTC AGG CTC CCT CTC CGG	21	385-405	428 rev
AAT TAC CGC GGC TGC TGG CA	20	567-586	567 rev
TCC AAC TAC GAG CTT	15	626-640	626 rev
ATC ATT ACT CCG ATC CCG	18	847-864	854 rev
AAT CCA AGA ATT TCA CCT CT	20	905-925	905 rev
CAA TTC CTT TAA GTT TCA GCC	21	1130-1150	1131 rev
AAG AAC GGC CAT GCA CCA CC	20	1269-1288	1324 rev
ATC TAA GGG CAT SAC AGA CC	20	1433-1452	1433rev
CTC GTT GAA GAC CAA CAA	18	1562-1579	1575 rev
CAC CTA CGG AAA CCT TGT T	19	1769-1787	1769 rev

Nuclear Large-Subunit (26S) Ribosomal DNA

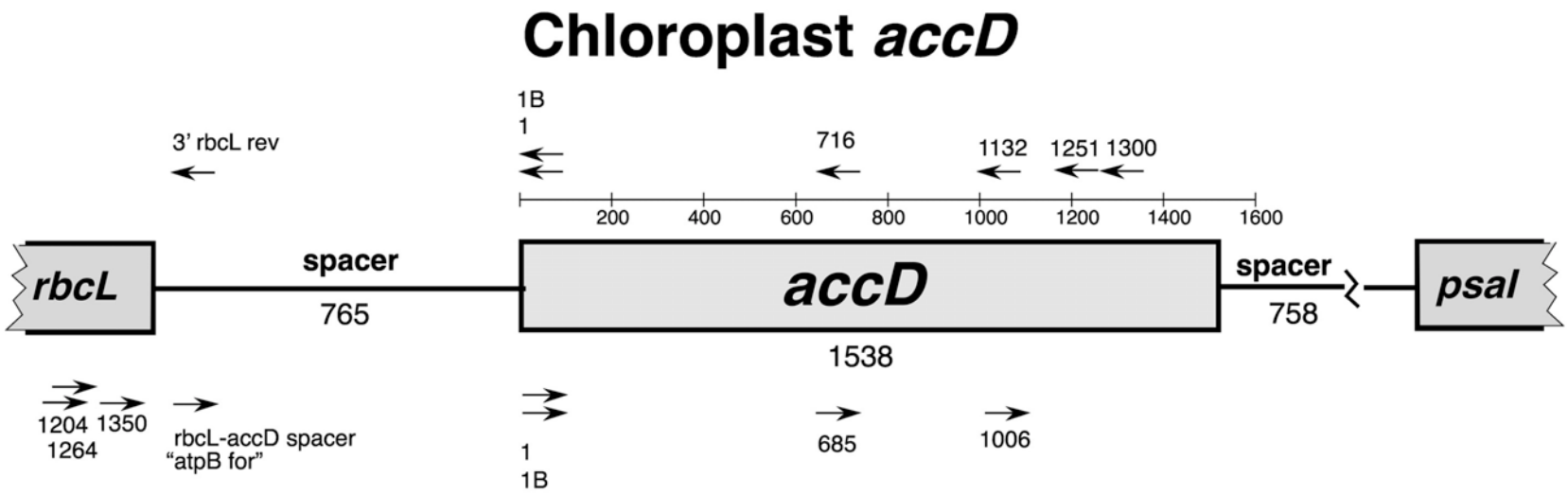


Forward Primers

Primer Sequence	Size	Position	Name
CGA CCC CAG GTC AGG CGG	18	3-21	N-S1 for
CCC GCT GAG TTT AAG CAT A	19	26-45	27 for
TTT AAG CAT ATC AAT AAG CGG AGG AAA	27	36-62	36 for
GAG TCG GGT TGT TTG GGA	18	264-282	N-S2 for
AGG GAA GCG GAC GGG GGC	18	416-433	N-S3 for
TTG AAA CAC GGA CCA AGG	18	657-674	N-S4 for
CGT GCA AAT CGT TCG TCT	18	889-906	N-S5 for
TGG TAA GCA GAA CTG GCG	18	1138-1155	N-S6 for
GAT GAG TAG GAG GGC GCG	18	1376-1393	N-S7 for
CAT GGG TTA GTC GAT CCT AAG	21	1526-1549	1529 for
ACG TTA GGG AGT CCG GAG	18	1643-1660	N-S8 for
AGC CGG AGG TAG GGT CCA GC	20	1729-1748	1715 for
AAT GTA GGC AAG GGA AGT	18	1893-1910	N-S9 for
TAA AAC AAA GCA TTG CGA	18	2146-2163	N-S10 for
AAT CAG CGG GGA AAG AAG	18	2390-2407	N-S11 for
GTC CTA AGA TGA GCT CAA	18	2658-2675	N-S12 for
CCT ATC ATT GTG AAG CAG	18	2891-2908	N-S13 for
TTA TGA CTG AAC GCC TCT	18	3110-3127	N-S14 for
CTG AAC GCC TCT AAG TCA G	19	3116-3134	G268 for
GGC CTT GCT GCC ACG ATC CAC TGA G	25	3343-3368	3343 for
TGC CAC GAT CCA CTG AGA	18	3351-3368	N-S15 for
CTG AGA TTA AGC CTT TGT	18	3362-3379	NTS for

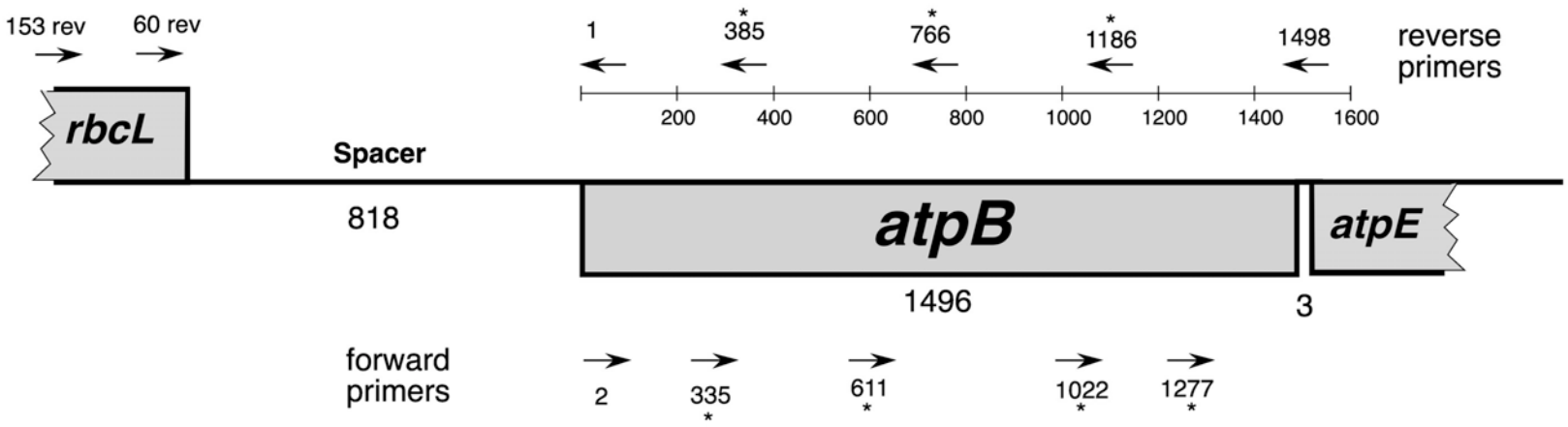
Reverse Primers

Primer Sequence	Size	Position	Name
CCG CCT GAC CTG GGG TCG	18	3 - 21	C-S1 rev
TAT GCT TAA AYT CAG CGG GT	20	26-45	25 rev.
TCC TCC GCT TAT TGA TAT GC	20	41-60	ITS-4 rev
TCC TCC GCT TAT TGA TAT GC	20	41-60	40 rev
TCC CAA ACA ACC CGA CTC	18	264-282	C-S2 rev
GCA TTC CCA AAC AAC CCG AC	20	267-285	268 rev
TTG GGC TGC ATT CCC A	16	278-293	307 rev
CTT TCC CTC ACG GTA	15	352-366	459 rev
GCC CCC GTC CGC TTC CCT	18	416-433	C-S3 rev
TTG GTC CGT GTT TCA AGA CG	20	653-673	641 rev
CCT TGG TCC GTG TTT CAA	18	657-674	C-S4 rev
TGT CGA ACG ATT TGC ACG	18	889-906	C-S5 rev
GCT ATC CTG AGG GAA ACT TC	20	962-981	950 rev
CGC CAG TTC TGC TTA CCA	18	1138-1155	C-S6 rev
ACT TCC ATG ACC ACC GTC CT	20	1239-1261	1229 rev
CGC GCC CTC CTA CTC ATC	18	1376-1393	C-S7 rev
ACC CAT GTG CAA GTG CCG TT	20	1516-1535	1499 rev
CTC CGG ACT CCC TAA CGT	18	1643-1660	C-S8 rev
GCT GGA CCC TAC CTC CGG CT	20	1729-1748	1715 rev
TTC ACC TTG GAG ACC TGA TG	20	1853-1872	1839 rev
ACT TCC CTT GCC TAC ATT	18	1893-1910	C-S9 rev
GGA CCA TCG CAA TGC TTT GT	20	2150-2168	2134 rev
TCG CAA TGC TTT GTT TTA	18	2146-2163	C-S10 rev
CTT CTT TCC CCG CTG ATT	18	2390-2407	C-S11 rev
MCT ACA CCT CTC AAG TCA T	19	2444-2562	2426 rev
AGA GTA GTG GTA TTT CA	17	2470-2487	3700 rev
TTG AGC TCA TCT TAG GAC	18	2658-2675	C-S12 rev
GGT AAC TTT TCT GAC ACC TC	20	2798-2817	2782 rev
CTG CTT CAC AAT GAT AGG	18	2891-2908	C-S13 rev
GGT CTA AAC CCA GCT C	16	2936-2951	2576a rev
TTC GCG CCA CTG GCT TTT CA	20	3074-3093	3058 rev
AGA GGC GTT CAG TCA TAA	18	3110-3127	C-S14 rev
ACT CYR CCA CTT ACA ATR CC	20	3323-3342	3323 rev
CTGAATCTCAGTGGATCGTGGCAGCAARRC	30	3344-3373	3344 rev
ATC TCA GTG GAT CGT GGC AG	20	3350-3369	3331 rev
TCT CAG TGG ATC GTG GCA	18	3351-3368	C-S15 rev
CTG GAT CTC AGT GGA TCG T	19	3355-3373	3336 rev



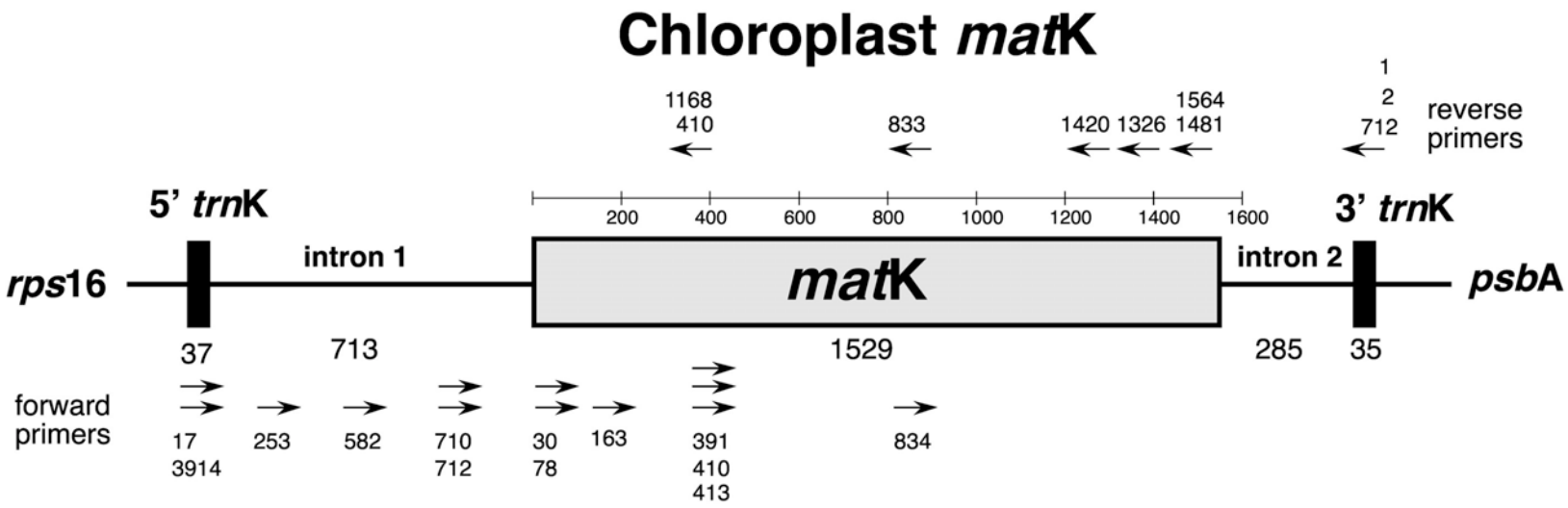
Primer Sequence 5'-3'	Size	Name
CTC GGC CCA ATC TTT TAC TA	20	rbcL-accD spacer for (misnamed atpB for)
TCT ATG GAA AGA TGG YGG TT	20	accD 1 for
ATG GAA AAA TGG YGG TTY AA	20	accD 1B for
CAA TGY GAA AAT TGT TAT GC	20	accD 685 for
GGT AGT ATG GGA TCC GTA GT	20	accD 1006 for
AAC CRC CAT CTT TCC ATA GA	20	accD 1 rev
TTR AAC CRC CAT TTT TCC AT	20	accD 1B rev
TCT GAA CTA CTC ATT TTC AA	20	accD 716 rev
ATT TTA GCC ATT TGC ATC A	19	accD 1132 rev
GCA ATA ATG ATA TCY CCC AAC	21	accD 1251 rev
TGY TCA ATT ACT CTT TTA CC	20	accD 1300 rev
TAG TAA AAG ATT GGG CCG AG	20	3' rbcL rev (in rbcL-accD spacer)

Chloroplast *atpB*



Primer Sequence 5'-3'	Size	Name
TAT GAG AAT CAA TCC TAC TAC TTC T	25	atpB 2 for
TCA GTA CAC AAA GAT TTA AGG TCA T	25	atpB 1498 rev
GAA GTA GTA GGA TTG ATT CTC AT	23	atpB 1 rev

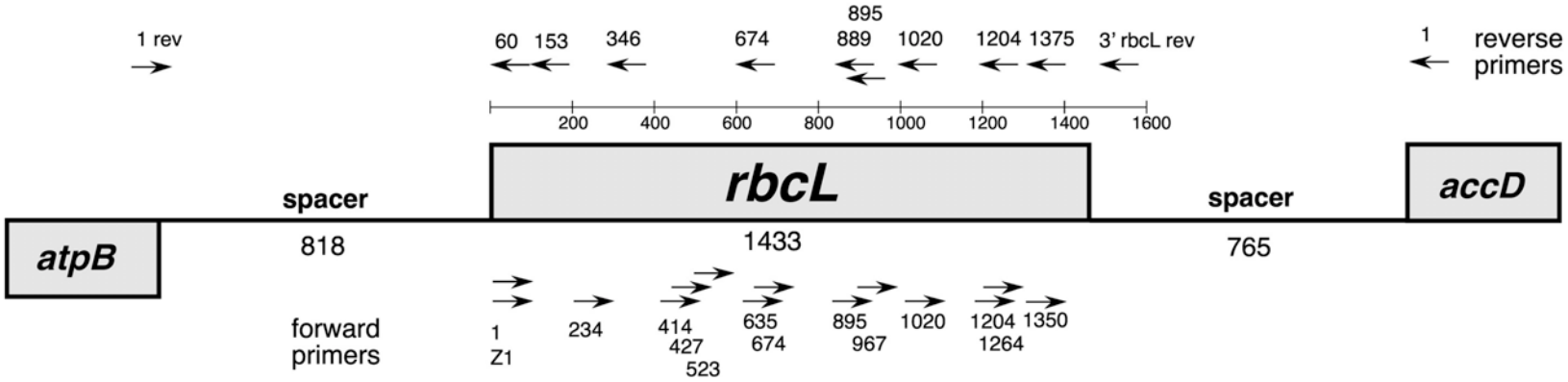
* = Sara Hoot primers (Hoot et al. 1995 Ann. Missouri Bot. Gard. 82:194)



Forward Primer Sequence 5'-3'	Size	Position	Name
GGG GTT GCT AAC TCA ACG G	19	5' trnK	5' trnK 3914 for
GGT AGA GTA GTC GGC TTT TAA	21	5' trnK	5' trnK 17 for
TTG GGT CGA GTC AAT AAA T	19	5' Intron	matKintron1 253f
CTA ACC ATC TTG TTA TCC T	19	5' Intron	matKintron1 582f
GTA TCG CAC TAT GTW TCA TTT GA	23	5' Intron	trnK 710 for
GTA TCG CAC TAT GTR TCA TTT GAT A	25	5' Intron	trnK 712 for
AGA TMT ARC TCG GAA ACA GG	20	30-43	30 for
CAG GAG TAT ATT TAT GCA CT	20	73-92	78for
AGG TTA CTA ATT GTG AAA CG	20	163-182	163 for
TAC GAT CAA TTC ATT CC	17	389-405	413 for
CGA TCA ATT CAT TCC AYA	18	391-408	410 for
CGA TCA AAT CAT TCM ATA TTT	21	391-411	391 for
GCA TTA TGT TAG GTA TCA AG	20	834-853	834 for

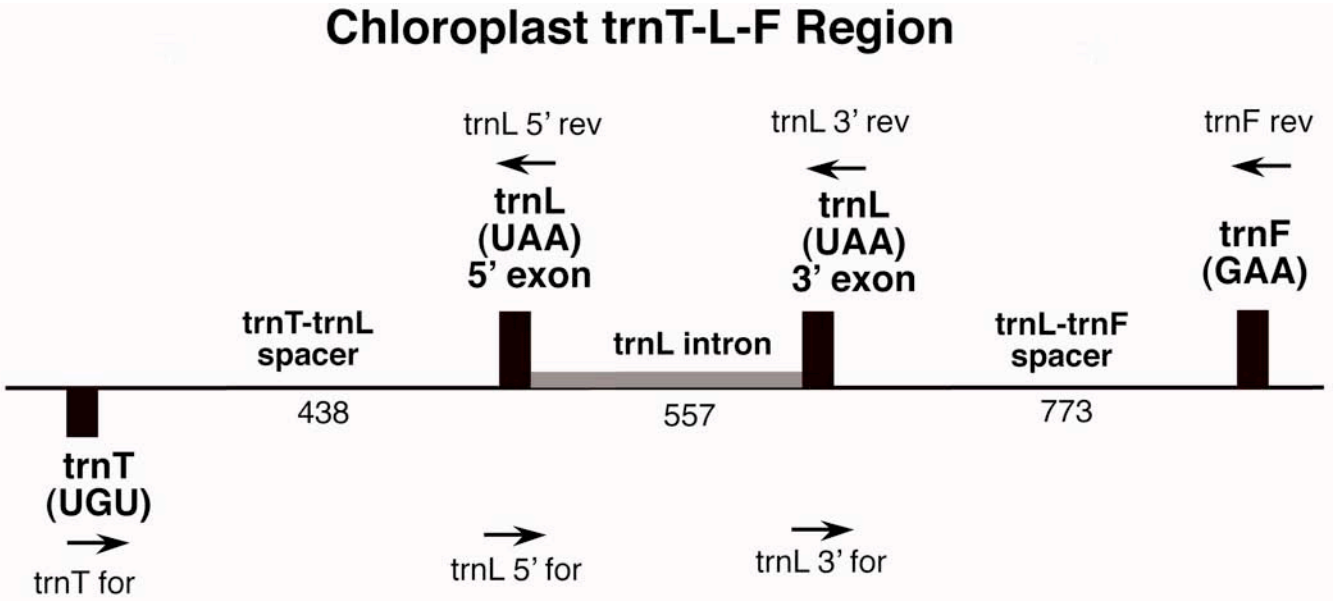
Reverse Primer Sequence 5'-3'	Size	Position	Name
ATT GAA TGA ATT GAT CGT A	19	389-407	1168 rev
TRT GGA ATG AAT TGA TCG	18	391-408	410 rev
CTT GAT ACC TAA CAT AAT GCA	21	833-853	833 rev
TCG AAG TAT ATA CTT TAT TCG	21	1291-1310	1420 rev
TGT TTA CGA GCT AAA GTT	18	1326-1343	1326 rev
CAA ATA ATA TCM AAA TAC CAA	21	1481-1502	1481 rev
ATG ATT RAC TAG ATC GTT GA	20	1504-1523	1564 rev
AAC TAG TCG GAT GGA GTA G	19	3' trnK	trnK 2 rev
AAC TAG TCG GAT GGA GTA GAT AA	23	3' trnK	3' trnK 1 rev
TAT CAA ATG AYA CAT AGT GCG ATA C	25	5' intron	psba rev

Choroplast *rbcL*



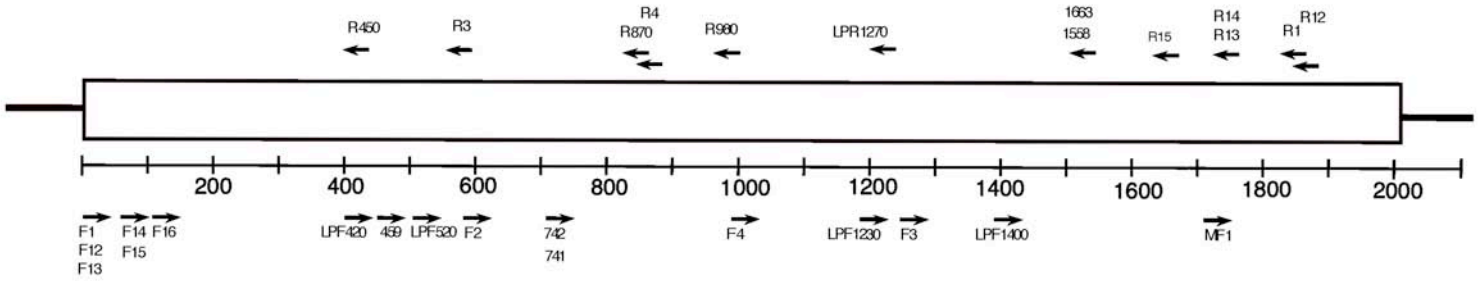
Primer Sequence 5'-3'	Size	Name
ATG TCA CCA CAA ACA GAR AC	20	1 for
ATG TCA CCA CAA ACA GAR ACT AAA GC	26	Z-1 for
CGT TAC AAA GGG CGA TGC TAC CGC ATC GAG	30	Z-234 for
CGA ATY CCW CCT GCT TAT	18	414 for
GCT TAT GTT AAA ACT TTC CAA GGT CCG CC	29	Z-427 for
TAA ACC TAA ATT GGG GTT ATC TGC TAA AAA CTA	33	Z-523 for
TGC GTT GGA GAG ACC GTT TC	20	635 for
TTT ATA AAG CAC AGG CTG AAA CAG GTG AAA TC	32	Z-674 for
GCa GTT ATT GAT AGA CAG AAA AAT CAT GGT	30	Z-895 for
GGA GAT CAT ATT CAC TCT GGT ACC GTA GTA	30	Z-967 for
ACT TTA GGT TTT GTT GAT TTA TTG CGC GAT GAT T	34	Z-1020 for
TTt GGT GGA GGA ACT TTA GGA CAc CCT TGG GG	32	Z-1204 for
GTA GCT AAT CGA GTA GCT CTA GAA GCA TGT GTA A	34	Z-1264 for
GGA GTC CTG AAC TAG CTG C	19	1350 for
CAG GAG TAT AAT AAG TCA ATT TG	23	60 rev
GAA GCA GGG GCC GCG GTA GCT GCC GAA TCT TCT A	34	Z-153 rev
ATG TTT ACT TCC ATT GTA GGT AAC GTA TTT GG	32	Z-346 rev
TTT ATA AAG CAC AGG CTG AAA CAG GTG AAA TC	32	Z-674 rev
CTA TCA ATA ACT GCA TGC AT	20	889 rev
ACC ATG ATT CTT CTG CCT ATC AAT AAC TGC	30	Z-895 rev
ATC ATC GCG CAA TAA ATC AAC AAA ACC TAA AGT	34	Z-1020 rev
CCC TAA GGG TGT CCT AAA GTT TCT CCA CC	32	Z-1204 rev
GTG AAG TAT GGA AAG AGA TCG TAT TTA ATT TTG	?	Z-1375 rev
TAG TAA AAG ATT GGG CCG AG	20	3' rbcL*

* 112 bp into rbcL-accD spacer



Primer Sequence 5' - 3'	Size	Name
CAT TAC AAA TGC GAT GCT CT	20	trnT for
CGA AAT CGG TAG ACG CTA CG	20	trnL 5' for
GGT TCA AGT CCC TCT ATC CC	20	trnL 3' for
ATT TGA ACT GGT GAC ACG AG	20	trnF rev
GGG GAT AGA GGG ACT TGA AC	20	trnL 3' rev
TCT ACC GAT TTC GCC ATA TC	20	trnL 5' rev

Mitochondrial matR



Forward Primers

Primer Sequence 5'- 3'	Size	position on Arabidopsis	
GAC CGC TNA CAG TAG TTC T	19	14-32	matR-F1 (ZP-1)
TTT AGA YCG CTY ACA GTA GT	20	10-29	matR-F12 (ZP-41)
TTT AGA YCG CTR ACA GTA GT	20	10-29	matR-F13 (ZP-42)
ATC CCG AGT TTC MRG ACA C	19	89-107	matR-F14 (ZP-46)
ATC CCG AGT TTC MRG ACG C	19	89-107	matR-F15 (ZP-47)
TCG GGT CGA GGC WGC CAC T	19	121-139	matR-F16 (ZP-59)
GAT CCG RCA RRA GCA CG	17	417-432	matR-LPF 420 (ZP 64)
GAT CGT TCA TGA CAG GTC GT	20	458-476	matR Raff459For
GAT CGG TTC TAT TAA GGG CAG	21	458-476	matR Tetra459For
CTC TRG AGA AGA RGC AAG C	19	504-522	matR-LPF 520 (ZP 103)
CAT TCC CTT RTT TCG TCG TG	20	595-614	matR-F2 (ZP-2)
CAT GGA ATC CGT GAC CCT TA	20	741-759	matR Raff742For
TCA TAG AAG CCG CCG GGT TGA	21	740-759	matR Tetra741For
ACG TAT CGC CCA CTT CCT AC	20	1023-1040	matR-F4 (ZP-7)
GAG YRA AGC ACC RTA TCC	18	1190-1207	matR-LPF 1230 (ZP 66)
CTA CGC TCC GCC ATC CAT T	19	1225-1243	matR-F3 (ZP-3)
ACC GCC AAG GAC ATM CRG C	19	1392-1410	matR-LPF 1400 (ZP 78)
GTG CCG CGA CAA CCT TTA CC	20	1767-1786	matR-MF1 (ZP 75)

Reverse Primers

Primer Sequence 5'- 3'	Size	position on Arabidopsis	
ATT TCG TRC TTC TGY CGG A	19	419-467	matR-R450 (ZP-51)
CAC GAC GAA AYA AGG GAA TG	20	595-614	matR-R3 (ZP-6)
CCG CCC AGC TCT ATM WG	17	850-866	matR-R870 (ZP-50)
TCT CTC TGA CCT GAT AAC TAG	21	877-897	matR-R4 (ZP-8)
AGT CGT CGG CAT ATC GCG C	19	955-973	matR-R980 (ZP-61)
GAA TKG ATG GCR GAR CG	17	1228-1244	matR-LPR 1270 (ZP 67)
AAT GGA TGG CGG AGC GTA G	19	1225-1243	matR-R2 (ZP-5)
TGG AAT GGA TGG CRG ARC G	19	1228-1244	matR-LPR 1250 (ZP 104)
TGA TAG ATC CCG CTC CTC CT	20	1557-1575	matR Raff1558Rev
ATA GAT CCC GCC CAG TGT C	19	1562-1579	matR Tetra1663Rev
TCT YCG GCT AAT GAK ACC TCG	21	1645-1665	matR-R15 (ZP-60)
TGR TAR AGG TTG TCG CGG CA	20	1768-1787	matR-R13 (ZP-48)
TGR TAR AGG TTG TCG CAG CA	20	1768-1787	matR-R14 (ZP-49)
TGC TTG TGG GCY RGG GTG AA	20	1831-1850	matR-R1 (ZP-4)
CGC GYC GAR GAT TTG TGC T	19	1847-1865	matR-R12 (ZP-19)

SECTION 8: PURIFICATION OF PCR PRODUCTS FOR USE IN SEQUENCING REACTIONS

There are many kits available to purify DNA products in sizes ranging from 100bp to 10kb. The procedures typically use ion-exchange columns to remove primers, nucleotides, polymerases and salts. These protocols are very easy – you only have to follow the manufacturer instructions. Example kits include QIAquick PCR Purification Kit (QIAgen), JETquick (GENOMED), and Wizard PCR Pre DNA purification System (Promega). We have found the EZNA Cycle-Pure kit to be comparable to more expensive ones.

Column Purification using EZNA Cycle-Pure Kit

1. Because you ran 4 μl of your PCR product on a gel, you have 21 μl of sample left. Add one volume of Buffer XP1. Vortex to mix.

2. Load all 42 μl of sample to the HiBind DNA spin column. Centrifuge at 10,000 x g for one minute. Discard the liquid from the collection tube (sink).

3. Add to column 700 μl of SPW buffer. ***Note: this buffer must be first diluted with ethanol for use***. Centrifuge at 10,000 x g for one minute. Discard the liquid from the collection tube (sink).

4. Repeat no. 3.

5. Centrifuge the column (without buffer) for two minutes to remove residual ethanol.

6. Add 30-40 μl of Elution buffer (lower amount for light PCR products) at 65-70˚ C (use heat block). Let stand for one minute. Centrifuge at 10,000 x g for one minute.

7. Run check gel to estimate quantity of purified PCR product.

Gel Purification of PCR Products using Qiaquick Gel Extraction Kit

Gel Isolation of PCR Product

1. Pour a TAE agarose gel with the wider comb. Use the graduated cylinder dedicated to ethidum bromide (red tape). When the agarose is cooled (but prior to solidification), add 0.5 μl of ethidium bromide. Use comb with wider teeth (10 lanes). Remember, you should skip a lane between each sample.

2. Load the entire contents of the PCR reaction (ca. 21 μl sample plus 5 μl of loading dye) into every other lane of the agarose gel. The gel typically runs at 75 volts (ca. 100 mA) for 40 minutes.

3. Use eye protection for the UV light. Remove the gel (on gel mold) and take it to the transilluminator. Slide off onto the surface. Keep the UV light setting at low. Visualize the band of interest and excise it using a sterile scalpel blade. Place band in a preweighed 1.7 ml microfuge tube. Determine the weight of the band using the analytical balance.

Column Purification

1. Add three volumes (remember, 100 mg = 100 μl) of buffer QG.

2. Incubate at 50˚ C for 10 minutes (in heat block). Vortex every 2 minutes to dissolve the agarose gel plug. This should be completely dissolved and yellow in color after 10 minutes. [note: the QG buffer has a pH indicator in it. If any color other than yellow, the pH must be adjusted by adding 10 μl of 3.0 M Na acetate].

3. Add one gel plug volume of isopropanol. Mix.

4. Place entire contents of tube in column and centrifuge for one minute at 10,000 g.

5. Discard the flow-through, the add 500 μl of buffer QG. Centrifuge for one minute.

6. Add 750 μl of buffer PE and let stand for 3 minutes. Centrifuge one minute.

7. Discard the flow-through. Centrifuge the empty column for two minutes.

8. Place column in a new labeled 1.7 ml tube. Elute the DNA with 40 μl of buffer EB at 65˚ C. Let stand for one minute. Centrifuge for one minute. Discard column – the purified DNA is now in your tube.

DEAE Nylon Membrane Protocol

1. Prepare a 1% acetate (TAE) or borate (TBE) agarose gel with eithidium bromide (ca. 10 μl per 100 ml gel). We generally use a large gel rig (21 X 7 X 1.3 cm) with a 16-place comb. The comb teeth are 13 X 11 X 1.5 mm. These lanes hold ca. 80-90 μl of sample + dye. The DEAE wicks are cut to 7.5 X 11 mm. Four lanes (400 μl) of PCR product provide sufficient DNA for sequencing the entire 18S rDNA.

2. Prepare the DEAE membrane by washing 10 min. in 10 mM EDTA pH 7.6-8.0 - enough to cover membranes (ca. 2 ml). Next, wash for 5 min. in 0.5 M NAOH. Finally, rinse the membrane several times in distilled water. Store the membrane in water at 4° C until needed. The membranes are best if prepared just prior to use.

3. Mix ca. 1 μl of Ficoll loading dye with every 10 μl of sample. Load the ds PCR product into the gel lanes being careful not to cross contaminate samples (skip lanes between different taxa). We have found that more sample can be loaded per lane by loading the gel lanes dry. For this, pour electrode buffer into each chamber until the buffer reaches ca. half way up the edge of the agarose gel. Load the samples into the gel lanes and begin electrophoresis. When the marker dye has migrated into the gel, add additional electrode buffer to cover the gel and continue electrophoresis.

4. When the band has migrated the desired distance (check progress with long wave UV lamp), turn off the power, remove the gel (on its support) from the electrode chamber, and using a new scalpel blade, make an incision just ahead of the band of interest. A single large incision can be made if all the products are the same size (but do not cut the gel completely in half). If more than one band exists in a lane, make the incision ahead of only the band of the correct size. Using forceps, place the prepared DEAE membranes in the incision, return the gel to the electrode chamber and continue electrophoresis. In approximately 10 minutes the band will completely run into the membrane (check with U.V. light).

<u>Elution of ds DNA from Membrane</u>

5. Remove DEAE membranes from the gel, briefly rinse in the gel electrode buffer to remove residual agarose. Place 2-3 membranes per sample in the same microfuge tube.

6. Add 350 μl of high salt NET buffer. Vortex to move the membranes under the buffer. Incubate in a heat block at 65 °C for 30-45 min. with occasional vortexing.

7. Move the buffer to another tube. Wash the membrane with another 75 μl of high salt NET buffer. Incubate at 65 °C for 10 min. Pool the buffers (discard membranes).

8. Extract the buffer with 3 volumes of water-saturated n-butanol (top phase in bottle!). Shake, centrifuge for ca. 5 minutes, and remove the top phase into a waste butanol container.

9. To the buffer add 2.5 volumes of 100% ethanol and precipitate at –20 °C for at least 1 hour

(overnight OK). Centrifuge for 15 minutes in a cold microfuge. Carefully discard the ethanol. Add ca. 1 ml of 70% ethanol. Invert several times and centrifuge again for 15 minutes. Discard ethanol and dry pellet in a SpeedVac.

10. Resuspend pellet in distilled water or TE, about 30 μl for every 100 μl of dsDNA loaded on the gel.

[If the DNA proves to be unsuitable for sequencing, try another ethanol precipitation. Resuspend the pellet in 100 μl TE. Add 10 μl 3 M NaOAc and 220 μl 100% ethanol. Precipitate at –20 ˚C for 30 min. (or more). Centrifuge, rinse in 70% ethanol, centrifuge, dry.]

Obtain DEAE Membrane from:

Schleicher & Schull
Keene, NH 03431
(800) 245-4024

Grade NA45, 3 X 51 cm, 0.45 micron
S. & S. order # 23400

The membrane is cut to appropriate size (e.g. 7.5 X 11 mm) using a clean razor blade.

Direct Precipitation of PCR Product using PEG

When only a single band is obtained following PCR amplification, the product can be quickly cleaned using this method. We have gotten excellent sequencing ladders using this approach, probably because it gives a higher yield than other methods.

1. For 100 μl of PCR product (ds DNA), extract in equal volume of phenol (Tris saturated, pH 8.0). Vortex briefly.

2. Centrifuge for 10 min at maximum velocity. Remove aqueous to new tube.

3. Extract again with equal volume of chloroform:isoamyl alcohol (24: 1)

4. Centrifuge for 10 min at maximum velocity. Remove aqueous to new tube.

5. Add equal volume of 13% PEG/Salt solution. Mix well.

6. Centrifuge for 10 min at maximum velocity. Pour off aqueous. Rinse pellet in 70% EthOH.

7. Dry pellet in desiccator (or vacuum drier). Rehydrate pellet in water or TE. For every 100 μl of
PCR product, use 20 μl of water or TE.

13% PEG/Salt Solution

13 g polyethylene glycol (MW 8000)
9.3 g NaCl
Bring up to 100 ml with dH_20

SECTION 9: TA CLONING AND SEQUENCING OF PCR PRODUCTS

Introduction

Methods will be described in this manual (Section 10) for directly sequencing PCR products. One may ask, then, "why would one ever want to clone and sequence a PCR product?" As it turns out, a PCR amplification may result in the production of heterogeneous products. Using gel purification as described above, one can usually select the band of the correct molecular weight and avoid products that are obviously smaller and larger. Situations occur, however, where products are formed that are the same (or nearly the same) length but differ in sequence. These products will migrate to the same position on an agarose gel and will all be obtained if this band is removed. We have encountered this situation when attempting to sequence ribosomal internal transcribed spacers (ITS) in some wild species of *Glycine* (soybean). Attempts to directly sequence such templates results in double peaks at some positions. In addition, PCR amplifications may result in too few products of the desired size to allow direct sequencing. An efficient way to overcome these problems is to clone and sequence these PCR products. Because of the way cloning works, a plasmid picks up only one insert and each colony derived from an *E. coli* cell that was transformed started with only one plasmid.

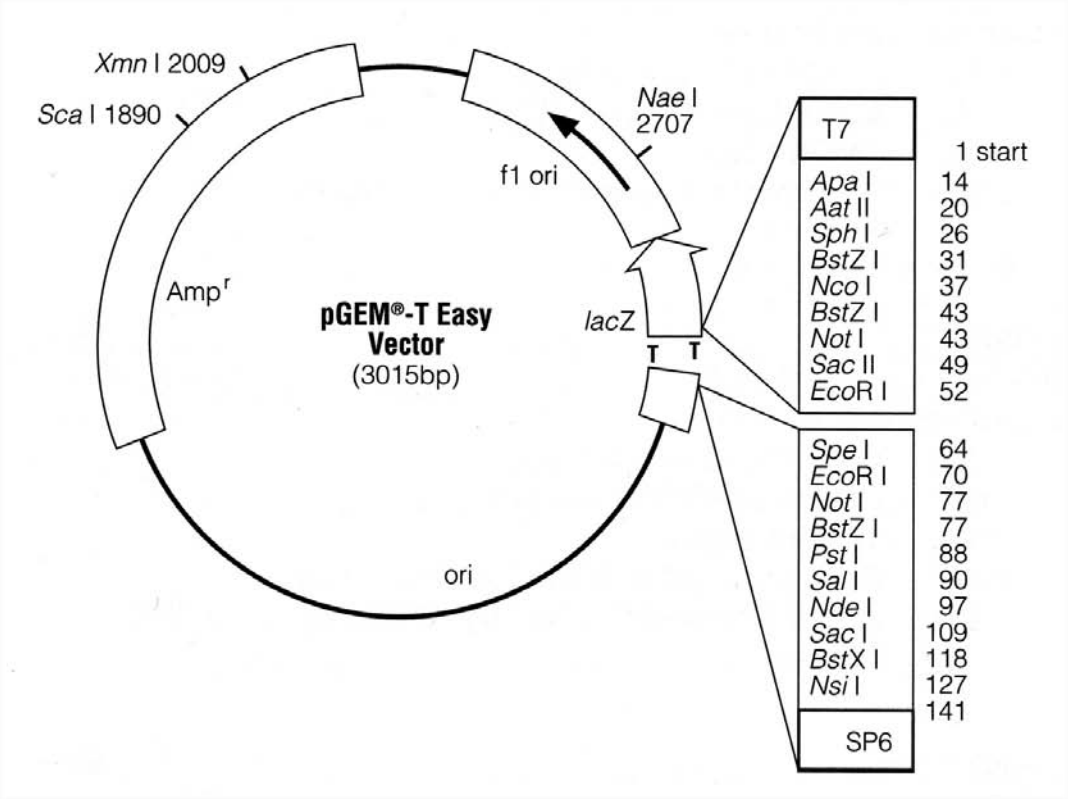
There are numerous cloning vectors available on the market, however, we have had good success using **TA Cloning** as described by Mead et al. (1991). This cloning method takes advantage of the fact that Taq polymerase has nontemplate-dependent terminal transferase activity which adds a single deoxyadenosine (A) to the 3' end of all PCR products. Linearized plasmid vectors are used which have deoxythymidine (T) ends that are complementary to the A overhangs of the PCR product.

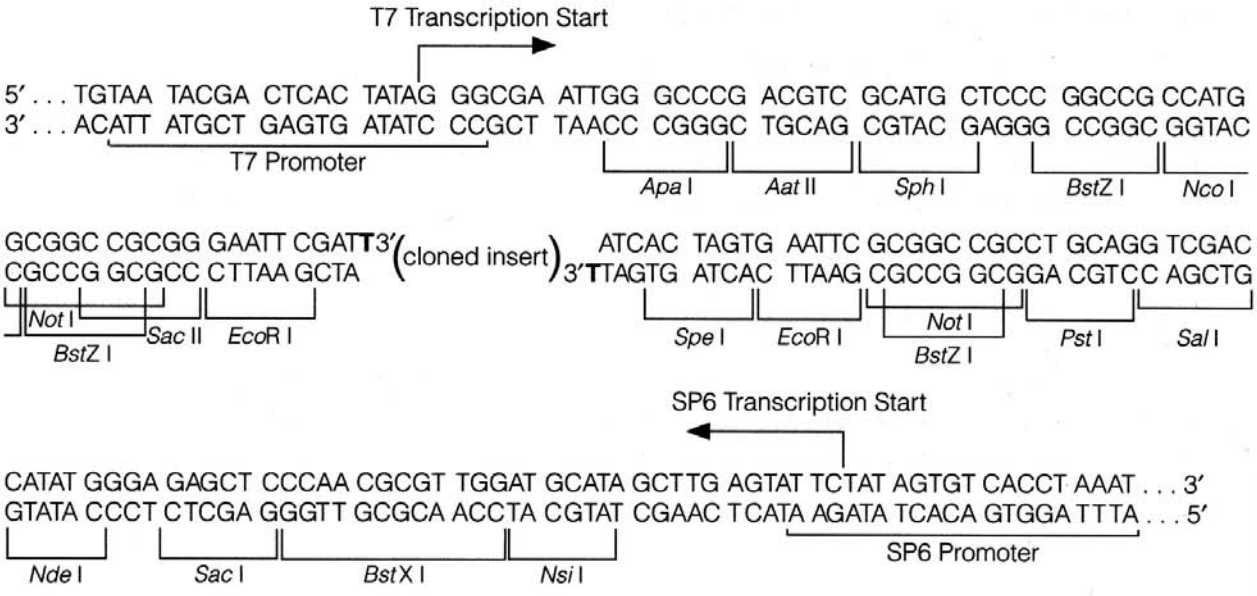
In the TOPO™ TA Cloning Kit (Invitrogen), the plasmid vector is supplied linearized with 3' thymidine (T) overhangs that are complementary to the A overhangs on PCR products. It also has a topoisomerase that is covalently bound to the vector in its activated form. This method allows ligation to occur at room temperature within five minutes. Although it works very well, the TOPO kit is rather expensive, so we use the Promega the pGEM®-T Easy Vector System. This kit requires the addition of DNA ligase and the reaction occurs in one hour at room temperature. Following the ligation reaction, competent *E. coli* cells are transformed with the vector. Supplied with the Promega pGEM-T kit are JM109 chemically competent cells. Transformation is accomplished using a heat shock method not by electroporation as works for other cell strains.

Following transformation, the JM109 cells are grown in SOC medium, plated onto LB (Luria-Bertani) agar plates that contain the antibiotic ampicillin, IPTG as well as X-Gal (5-bromo-4-chloro-3indolyl-ß-D-galactoside). *E. coli* cells containing the pGEM-T plasmids are resistant to ampicillin, hence only those transformed cells can grow on the plates containing this antibiotic. The plasmid also has a multiple cloning site intercalated between the *lac* promoter and the *lac*Zα fragment which codes for the first 146 amino acids of the ß-galactosidase enzyme.

X-Gal is the substrate for ß-galactosidase and its breakdown product results in a blue color. Since active ß-galactosidase is only produced when the *lac* promoter is on (and the *lac*Zα fragment is complemented in trans with the Ω fragment of ß-galactosidase), disruption of the promoter by an insert at the multiple cloning site results in no breakdown of X-Gal. Thus, cells that contain a transformed plasmid will appear as white colonies on the plate and cells that were not transformed appear as blue colonies (hence the name blue-white screening). Several white colonies are picked and grown to sufficient density to harvest the plasmid DNA. This DNA can then be used for additional PCR reactions or sequencing.

Note: The JM109 cells express the *lac* repressor (*lac*I^{q}) which will repress transcription from the *lac* promoter. Thus to perform blue-white screening for inserts, the IPTG must be added to your plates to express LacZα. Remember that competent cells are especially sensitive to temperature and mechanical lysis so keep them on ice and avoid excessive pipetting.


Xmn I 2009
Sca I 1890
Nae I 2707
f1 ori
Amp^r
pGEM®-T Easy Vector (3015bp)
lacZ
T T
ori
T7
1 start
Apa I 14
Aat II 20
Sph I 26
BstZ I 31
Nco I 37
BstZ I 43
Not I 43
Sac II 49
EcoR I 52
Spe I 64
EcoR I 70
Not I 77
BstZ I 77
Pst I 88
Sal I 90
Nde I 97
Sac I 109
BstX I 118
Nsi I 127
141
SP6


T7 Transcription Start
5′ . . . TGTAA TACGA CTCAC TATAG GGCGA ATTGG GCCCG ACGTC GCATG CTCCC GGCCG CCATG
3′ . . . ACATT ATGCT GAGTG ATATC CCGCT TAACC CGGGC TGCAG CGTAC GAGGG CCGGC GGTAC
T7 Promoter
Apa I
Aat II
Sph I
BstZ I
Nco I
GCGGC CGCGG GAATT CGATT 3′
CGCCG GCGCC CTTAA GCTA
(cloned insert)
ATCAC TAGTG AATTC GCGGC CGCCT GCAGG TCGAC
3′TTAGTG ATCAC TTAAG CGCCG GCGGA CGTCC AGCTG
Not I
Sac II
EcoR I
BstZ I
Spe I
EcoR I
Not I
BstZ I
Pst I
Sal I
SP6 Transcription Start
CATAT GGGA GAGCT CCCAA CGCGT TGGAT GCATA GCTTG AGTAT TCTAT AGTGT CACCT AAAT . . . 3′
GTATA CCCT CTCGA GGGTT GCGCA ACCTA CGTAT CGAAC TCATA AGATA TCACA GTGGA TTTA . . . 5′
SP6 Promoter
Nde I
Sac I
BstX I
Nsi I

Ligation and Cloning of PCR Products

Typical double-stranded PCR products are generally suitable for cloning. The quantity and quality of the amplification is usually checked on an agarose gel. If a single, clear band is obtained, the PCR reaction can be used directly for cloning. Most PCR reactions, however, result in at least a few nonspecific bands that can interfere with cloning a specific fragment. Ideally, one should optimize the PCR reaction, but this is not always possible. In this case we use gel purification to isolate the fragment of interest. It should also be kept in mind that the 3' A overhangs on the PCR products are removed over time and with excessive manipulation such as electrophoresis and precipitation.

Cloning Reaction

1. Fill out the cloning sheet, which is located in the cloning binder, with the species being cloned, collection number, and either the PCR or purification number.

2. Label the proper number of 1.7 ml tubes for the PCR products you will be cloning.

3. From the Promega kit located in the –20° C freezer, remove the pGEM-T plasmid and 2X ligase buffer and thaw in your ice bucket

4. Thaw your PCR products (or purifications) and then place them on ice.

5. On the cloning sheet calculate volumes needed given the number of cloning reactions and prepare a master mix (coctail) with everything except the PCR product (of course).

PCR product	1.5-3.0 μl	X _____ tubes
2X ligation buffer	2.5 μl	_____ μl
pGEM-T vector	0.5 μl	_____ μl
T4 DNA ligase	0.5 μl	_____ μl
Final Volume	5.0 μl	

6. Once you have prepared the master mix, vortex the tube and distribute 3.5 μl into each labeled sample tube.

7. Add 1.5 μl of the PCR product to each tube. Incubate at room temperature for one hour or overnight at 4° C (for low concentration PCR products).

Transformation of Competent Cells: Chemical (Heat Shock) Method

Before engaging in the transformation, one should have already prepared sufficient LB medium and LB agar plates. Competent cells are very sensitive to temperature and mechanical lysis, hence one should aim to treat them gently. When removing the cells from the ultralow freezer, always place immediately on ice to thaw.

1. Thaw on ice one vial of JM109 *E. coli* cells This MUST be done on ice! One tube of cells from Promega can be divided into 8 separate reactions (to save money!). Transfer 25 μl of cells to each of 7 tubes and leave the remainder in the original tube. This last tube can be considered a control since even gentle pipetting can disrupt the cells.

2. Add 2 μl of the ligation reaction (previous page) to the cell tubes and mix gently with the pipet tip by stirring, not pipetting up and down.

3. Incubate on ice for 20 minutes. During this time, turn on the heat block set at 42° C.

4. Heat shock the cells for 45-50 seconds in a heat block. Do not mix or shake. Remove and place immediately on ice for 2 minutes.

5. Add 475 μl of LB (or SOC) medium. Tape the tubes horizontally on a lidded tube rack and add the lid. Take the setup to the floor model shaker/incubator in the Clark lab. Tape the entire setup to the shaker platform. Incubate at 37° C for at least 2 hours with shaking (150 rpm).

Recovery and Plating

1. Dry LB agar plates in incubator (upside down, with lid slightly ajar) for 30 minutes.

2. Centrifuge the tubes briefly (short pulse) in the room temperature centrifuge.

3. Eliminate (down sink) 200 μl of the SOC medium and resuspend the cells by gently pipeting up and down. Spread 100 μl of the transformation onto LB agar plates (containing X-Gal, IPTG and ampicillin) using a sterilized bent glass rod.

4. Incubate overnight at 37° C.

Colony Selection

1. Remove the LB agar plates from the incubator and place in dairy case for at least one hour. This helps the development of the blue/white color of the colonies.

2. Examine the colonies (through the bottom of the petri dish) using the stereo microscope in room 1052. Mark at least five positive (pure white) colonies with a Sharpie pen. Do not pick colonies that superficially appear white but have a small amount of blue in the center. Try to mark white colonies that are well-separated from others to make picking easier.

3. Back in the lab, prepare the proper number of 15 ml sterile plastic tubes (with blue or orange caps) by pipetting 2 ml of LB medium with ampicillin. Use the electronic pipetter with a sterile 10 ml disposable pipet.

4. Using a sterile toothpick, select a marked white colony by simply touching it. Drop the toothpick inside the LB tube. Continue for all desired colonies. Usually we do two colonies per plate.

5. Incubate with shaking (Clark lab shaker/incubator) for 18 hours at 37° C.

Plasmid Mini-Prep: EZNA Column Method

We have found that the spin column plasmid purification methodologies developed and marketed by various companies (such as EZNA and QIAGEN) are extremely useful because they yield high-quality and quantity of DNA in less time than the "old-fashioned" alkaline lysis method (below). The method actually does use an alkaline lysis methodology, but the purification is accomplished with a silica-gel membrane inside a column.

1. Swirl the 15 ml LB tube and pour its contents into a 2 ml microfuge tube. Centrifuge the tubes for 2 minutes, pour off medium, and invert tubes on a paper towel for ca. 5 mins. to drain.

2. Add 250 μl of **Solution 1** (kept refrigerated) and resuspend pelleted bacterial cells by vortexing.

3. Add 250 μl of **Solution 2** and gently mix by inverting the tubes 4-6 times to mix to obtain a clear lysate. A 2 minute room temperature incubation may be necessary. Avoid vigorous mixing as this will shear chromosomal DNA and lower plasmid purity.

4. Add 350 μl of **Solution 3** and invert the tube immediately but gently 4-6 times until a floculent white precipitate forms.

5. Centrifuge for 10 minutes at room temperature. During centrifugation, label the HiBind minprep columns and their collection tubes.

6. Pour the supernatants from step 5 into the HiBind columns. Centrifuge one minute at room temperataure. Discard the flow-through.

7. Wash HiBind spin column by adding 500 μl of **Buffer HB** and centrifuge one minute. Discard the flow through.

8. Wash HiBind spin column by adding 750 μl of **Wash Buffer** (must be diluted with ethanol) and centrifuge for one minute. Discard the flow-through.

9. Repeat step 8.

10. Centrifuge the empty column for an additional two minutes to remove residual wash buffer.

11. Place HiBind column in clean, labeled 1.7 ml microcentrifuge tube. To elute the DNA, add 50 μl of **Buffer EB** (10 mM Tris-HCl, pH 8.5; from Qiagen) to the center of each HiBind column and let stand for one minute. Place the tubes in the centrifuge rotor with their caps facing out. Centrifuge for one minute. Discard the columns and proceed to restriction digestion to check which plasmids have inserts.

Plasmid Mini- Preparation and Insert Confirmation: Alkaline Lysis Method

This protocol is essentially as described in "Molecular Cloning" (Sambrook et al. 1989).

1. Place 1.5 ml of LB medium in 17 X 100 mm sterile, snap-cap polypropylene tube (Fisher 14-956-1J). Using a sterile (autoclaved) wooden stick, pick up a single white bacterial colony and inoculate the medium. Incubate at 37 ˚C overnight with vigorous shaking.
2. Pour into a 2.0 ml microfuge tube and centrifuge for 2 min.
3. Pour off the supernatant and drain the pellet for 5 min. with the tube upside down.
4. Resuspend the pellet completely in 100 μl of Lysis Solution I. Vortex.
5. Let stand at room temperature for 5 min.
6. Add 200 μl of freshly-made Lysis Solution II. Mix by inverting the tube several times.
7. Store on ice for 5 min.
8. Add 150 μl of Lysis Solution III. Mix by shaking the tube several times.
9. Store on ice for 5 min.
10. Centrifuge 1 – 5 min.
11. Transfer the supernatant to a fresh tube.
12. Add 450 μl of phenol/chloroform. Vortex. Spin 5 min. Transfer the upper aqueous phase to a fresh tube.
13. Add 900 μl of 100% ethanol. Vortex.
14. Centrifuge 1 minute, pour off ethanol, rinse pellet with 70% EtOH, dry pellet in SpeedVac. OR: Precipitate 20 min. at –70˚C. Centrifuge 20 min, rinse with 70% EtOH, dry pellet.
15. Resuspend in 20-40 μl TE with 20 μg/ml RNase A (= 2 μl of 10 mg/ml RNase A plus 1 ml TE)
16. Digest 2-4 μl with appropriate restriction enzyme and run on an agarose gel.

A mini-prep shortcut for purifying plasmids was published by Zhou et al. (Biotechniques 8:172-173, 1990), however, we have found that the result is less pure than the alkaline lysis method.

Restriction Digest of Plasmid DNA

Once it is determined which plasmids contain inserts, this DNA can be used for sequencing. The plasmid must first be linearized by conducting a restriction digest. A typical digest is:

Restriction Digest	**X1**	**X _____**
Sterile H_2O	15 μl	_____ μl
10X buffer	2 μl	_____ μl
Enzyme (EcoRI)	1 μl	_____ μl
BSA	x μl	_____ μl
DNA	2 μl	
Total	20 μl	

Incubate reaction in 37 ˚C water bath for at least 1 hr.
Run samples on 1% TAE agarose gel

Colony PCR: An Alternative to the Mini-Prep

In the main lab, prepare and label 1.7 ml microfuge tubes with 50 µl of distilled water for each colony.

1. With a sterile toothpick touch a colony from the LB-agar plate and resuspend it (by spinning the toothpick between your fingers) in the corresponding tube from above (one colony per tube).
2. Heat tubes with the resuspended colony at 85-95°C for 5 minutes in heating block. This produces the **lysate**.
3. In PCR room, prepare PCR mix using appropriate primers for each reaction (e.g. T7 & SP6 or the M13 forward and reverse):

Reagent	Amount	Final concentration
PCR buffer 10X	2.5 μl	1X
$MgCl_2$ 25mM	1.5 μl	1.5 mM
dNTPs 2.5mM each	0.5 μl	50 μM
forward primer 10µM	1.0 μl	0.4 μM
reverse primer 10µM	1.0 μl	0.4 μM
Taq (1/5)	0.2 μl	~0.5 units
DMSO	1.25 μl	5%
dH_2O	15.05 µl	

4. Bring PCR reaction to the main lab, add **lysate**

Lysate	2 μl
Final volume	25 μl

5. PCR parameters (user jfc, program colony):
   Denaturation: 94°C for 5 minutes
   30 cycles: 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 2 minutes
   Final extension: 72°C for 10 minutes
   Hold (indefinite time): 4° C.

6. Run an agarose check gel

	Expected sizes
if negative	85 bp + 116 bp = 201 bp
if positive	201 bp + INSERT

7. For purification run another gel. Cut out the agarose band that corresponds to the insert and follow the protocol from the QIAGEN gel extraction purification kit.

Note: Colony PCR may not work if, for example, plastid 16S rDNA has been cloned. This is because the primers in the reaction will also anneal to *E. coli* chromosomal 16S rRNA genes. One can also use internal primers (from the PCR reaction) as opposed to universal primers.

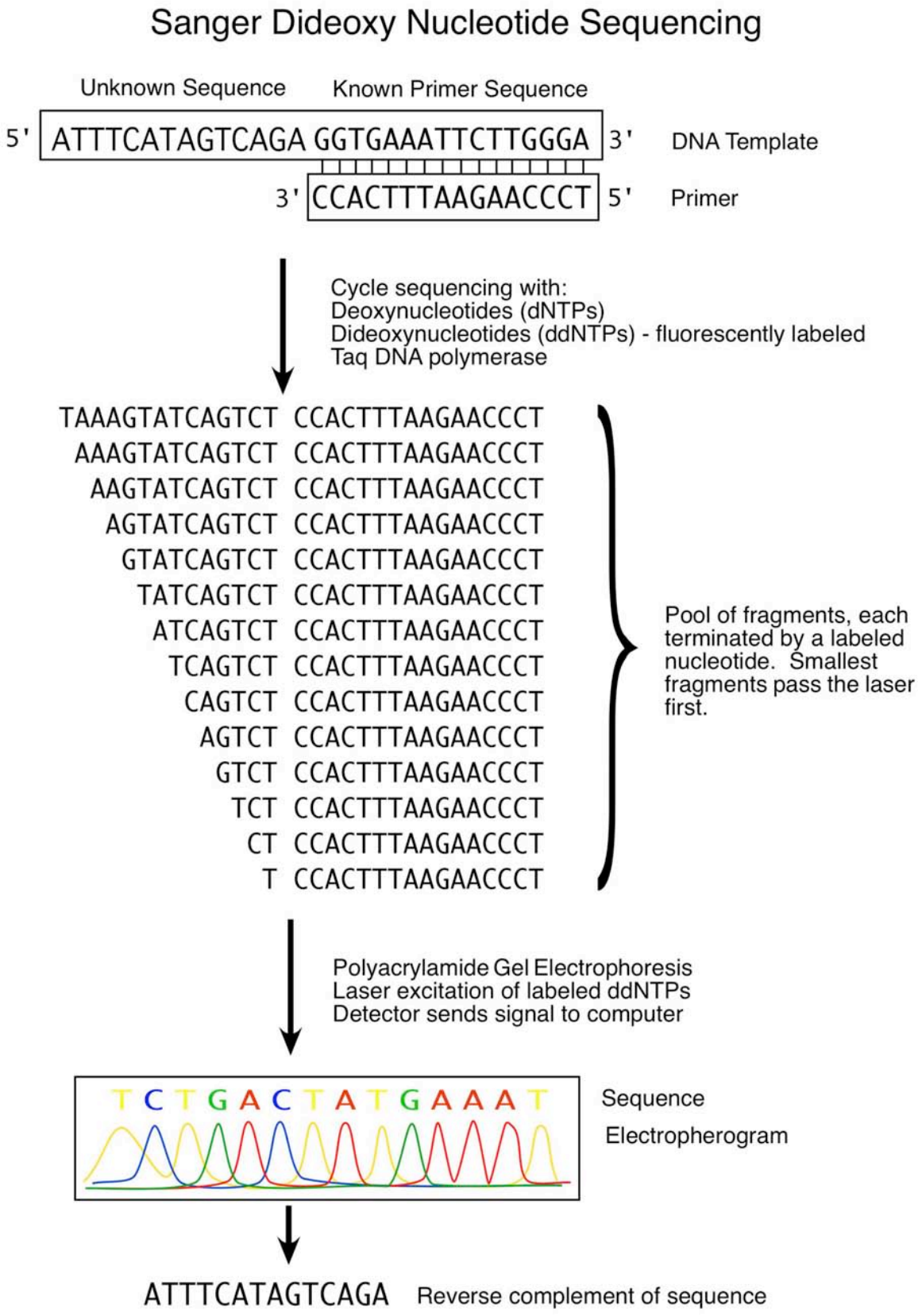
SECTION 10: DNA SEQUENCING REACTIONS

How Sequencing Reactions Work

The sequencing protocol described below utilizes the dideoxynucleotide terminated chain elongation method (Sanger et al. 1977). Since the original description of this method, various modifications have been made such as the choice of polymerase and the method in which the DNA fragments are visualized. Several different polymerases have been used with this method, such as Klenow Fragment, modified T7 DNA polymerases, and *Taq* DNA polymerase. The latter is used almost exclusively now because cycle sequencing requires an enzyme that can tolerate high temperatures without denaturing.

For cycle sequencing, the DNA template is first denatured at 94˚ C. Next the temperature is lowered and the primer is annealed to the template DNA (figure, next page). A labeling reaction then extends from the growing nucleotide strand at the 5' end of the primer by incorporating deoxyadenosine triphosphate nucleotides that are fluorescently labeled. Each nucleotide (A, C, G, and T) has a different fluorescent moity that emits a different color when excited with a laser. The growing strands are terminated because the reaction mixture also contains a small proportion of dideoxynucleotides (ddNTPs) which lack the 3' -OH group necessary for continued chain elongation. When one of these nucleotide analogs is incorporated, chain elongation is terminated. The result is that the reaction solution now contains a mixture of DNA strands of all possible lengths. All those that terminate in A (for example), have one type of fluorescent label, all those with C another, etc. When these fragments are run on an acrylamide sequencing gel, all the fragments are separated based upon their molecular weight (shorter fragments migrate farther). During electrophoresis, the labeled fragments migrate to the bottom of the gel where a laser excites them and causes them to fluoresce. A detector then records whether this fragment carried an A, C, G, or T. As the electrophoresis proceeds, larger and larger fragments pass, one at a time, before the laser. In the end, the linear order of the labeled fragments and their colors is recorded in a computer. This DNA sequence is output as a graph (electropherogram) where peak height corresponds to the intensity of the fluorescent signal and peak order the DNA sequence.

For sequencing plasmids containing inserts, gene-specific internal primers can be used or universal primers. Universal priming regions were purposely designed so that they flank the multiple cloning site of the plasmid vector. The figure of the pGEM-T plasmid (Section 9) shows the T7 and SP6 transcription sites. Primers are available that anneal at these sites.


Sanger Dideoxy Nucleotide Sequencing
Unknown Sequence
Known Primer Sequence
5' ATTTCATAGTCAGA GGTGAAATTCTTGGGA 3'
DNA Template
3' CCACTTTAAGAACCCT 5'
Primer
Cycle sequencing with:
Deoxynucleotides (dNTPs)
Dideoxynucleotides (ddNTPs) - fluorescently labeled
Taq DNA polymerase
TAAAGTATCAGTCT CCACTTTAAGAACCCT
AAAGTATCAGTCT CCACTTTAAGAACCCT
AAGTATCAGTCT CCACTTTAAGAACCCT
AGTATCAGTCT CCACTTTAAGAACCCT
GTATCAGTCT CCACTTTAAGAACCCT
TATCAGTCT CCACTTTAAGAACCCT
ATCAGTCT CCACTTTAAGAACCCT
TCAGTCT CCACTTTAAGAACCCT
CAGTCT CCACTTTAAGAACCCT
AGTCT CCACTTTAAGAACCCT
GTCT CCACTTTAAGAACCCT
TCT CCACTTTAAGAACCCT
CT CCACTTTAAGAACCCT
T CCACTTTAAGAACCCT
Pool of fragments, each terminated by a labeled nucleotide. Smallest fragments pass the laser first.
Polyacrylamide Gel Electrophoresis
Laser excitation of labeled ddNTPs
Detector sends signal to computer
T C T G A C T A T G A A A T
Sequence
Electropherogram
ATTTCATAGTCAGA
Reverse complement of sequence

Cycle Sequencing

This protocol has been optimized for all Applied Biosystems thermal cyclers. In addition to your primers (10 μM) and samples, you will need:

• ABI Prism™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase [Applied Biosystem].
• Perkin Elmer GeneAmp PCR System 9700.
• 0.2 ml PCR reaction tubes (no mineral oil is required). Applied Biosystems, MicroAmp reaction tube with cap (N801-0540).

Method

All tubes to be kept on ice throughout these procedures.

1. Prepare primers:

Mastermix Seq. Reac.	Initial concentration	µl/sample	x _____
sterile Nanopure H_2O		2.92	
Primer	10 µM	0.08	
Total volume		3.0	

2. Dilute BigDye with BetterBuffer (The Gel Company, DAF-5). For each reaction, 3.0 µl of BetterBuffer and 1.0 of BigDye Terminator (Final vol. 4.0 µl).

3. Distribute 4.0 μl of the above mix into the 0.2 ml reaction tubes. Add 3.0 μl diluted primer to each tube, mixing upon addition.

4. Using new tips for each sample, add 4 µl of purified product (20-30 ng/µl) to each sequencing reaction. Mix gently with the pipet.

5. Use the BigDye program under user "jfc" and then the "bigdye" run (below). Keep tubes on ice. When thermocycler reaches 96° C, hit Pause, add tubes, secure caps with rolling instrument, close heated lid. Press Resume and begin run.

Denaturation	Denaturation	Annealing	Extension	Store
94° C	96°C	50°C	60°C	4°C
3 min.	10 sec.	5 sec.	4.0 min.	∞
	25 cycles			

These cycle sequencing products can be purified using either CENTRI SEP Columns with Sephadex or by means of an ethanol precipitation.

Purifying Cycle Sequencing Products using Sephadex/CENTRI SEP Columns

A. Preparation of Sephadex

1. In a 250 ml flask weight 5 g of Sephadex and add 125 ml of distilled water and stir bar.

2. Stir VERY slowly on the stirring hotplate (no heat) for 20 minutes.

3. Allow to stand for 15 minutes.

4. Decant the water on the top of the Sephadex - pour slowly so as not to disturb the Sephadex while decanting.

5. Add 50 ml of distilled water.

6. Stir for 20 minutes.

7. Allow to stand 15 minutes and then decant water.

8. Add fresh water until is 75% Sephadex and 25% water. This Sephadex can be used directly. For storage (a week or more), the Sephadex must be autoclaved.

9. Cover flask with foil. Autoclave (on liquid setting) for 15-20 minutes.

10. Store at 4˚ C.

B. Purification

1. Using a cut-off 1.0 ml tip, add 800 μl of Sephadex (move pipet through suspension while pipeting) to each column.

2. Centrifuge 5 minutes at 3400 rpm (note position of the column).

3. Remove water.

4. Centrifuge 5 minutes at 3400 rpm and remove water.

5. Place column into a clean 1.7 ml tube.

6. Transfer the sequence reaction directly onto the center of the gel without disturbing the gel surface. Do not touch the sides of the column.

7. Centrifuge 15 minutes at 3400 rpm (keep proper orientation of column).

8. Dry sample in SpeedVac for 15 minutes at medium heat.

Purifying Cycle Sequencing Products via Ethanol Precipitation

Solutions

3 M sodium acetate (NaOAc), pH 5.2
95% ethanol (EtOH)
70% EtOH

Method

1. For each sequencing reaction, prepare a 1.5 ml microfuge tube containing:
   2.0 µl of 3M NaOAc
   50 µl of 95% EtOH

2. Pipette the entire content of the extension reaction into the above tube containing the NaOAc/EtOH mixture. Mix thoroughly.

3. Vortex the tube and leave at room temperature for 15 min in dark (drawer) to precipitate the extension product.

4. Centrifuge the tube at room temperature for 20 min at maximum speed.

5. Carefully decant the supernatant with a pipette and discard.

6. Rinse the pellet with 250 µl of 70% EtOH.

7. Vortex briefly.

8. Centrifuge for 5 min at room T at maximum speed.

9. Carefully decant the supernatant and discard.

10. Dry pellet in the SpeedVac for 15 min, or until dry. Do not overdry.

At this point, the reaction is stable for up to a year at -20°C. **Just prior to electrophoresis** the sample is resuspended in a 1.5 – 2.0 μl of a formamide gel loading buffer, heated to 90° C (2 minutes) to denature the DNA, and then chilled on ice.

SECTION 11: AUTOMATED DNA SEQUENCING: THE ABI 377

The ABI 377 automated DNA sequencer is located in the Sipes lab, LS II room 473. Because Dr. Sipes has kindly made her lab available to us, we must be extremely considerate of her time and equipment. The DNA sequencer is an expensive instrument, therefore nobody should attempt to use it without proper training and supervision. Additionally, there are serious safety concerns associated with using the sequencer. First, the gel contains acrylamide which is a neurotoxin. Second, when the sequencer is running, it uses very high voltage, thus presenting the potential for electrocution. Schedule your use of the machine bearing in mind that Dr. Sipes is "first in line" and we must adjust our usage accordingly. Communicate with other workers using the machine to work out the best times for a run. Typically, one package of acrylamide is sufficient to pour two gels, thus you may wish to coordinate with others if you cannot use both. These two gels are run back-to-back because the poured gel cannot be stored for more than one day.

Before the Gel Run

1. 10X or 5X TBE Buffer. Always use fresh TBE buffer. This buffer only lasts up to about 2 weeks before borate precipitates out of solution. Even if the buffer is heated to dissolve the borate, it will not work!

2. Formamide Aliquots. ***WARNING! Formamide is a tetrogen and is harmful by inhalation, skin contact, and ingestion***. The deionized formamide is stored in the lab freezer. Thaw the bottle on ice or in refrigerator. To reduce the exposure of formamide to oxygen, make several aliquots of 150 μl (this will be plenty for resuspending 96 samples) using a *filter tip*. Store aliquots in the –20˚ C freezer on the top shelf.

3. Coolant. Check the water reservoir level (located on right side of ABI 377 sequencer). Refill the reservoir when it is between 1/3 and 1/2 full. See user's manual page 8-5 "Refilling the Water Reservoir".

Cleaning and Assembling the Plates and Pouring the Gel

A. Cleaning the Glass Plates

1. Glass plates are most conveniently cleaned in the SteamScrubber (dishwasher).
2. Use the "science" cycle with both "steam" and "pure" options chosen
3. Place glass plates in the special plate rack
4. Fill soap reservoir with Alconox powder (1 1/2 spoonfuls).
5. Fill the Nalgene container on top with Nanopure water (use autofill option for 20 liters)
6. Connect the SteamScrubber to the sink and turn on all the way only the *hot* water.

7. Close the door, plug the machine into an electrical outlet, and press the start button. The steam and pure light should be on.

B. Plate Assembly

1. Wear powder-free vinyl gloves (in drawer next to freezer).
2. Make sure plates, combs, and spacers are completely clean and dry.
3. Make sure plates are oriented correctly!! Each plate has an etched mark on the outside, though the mark is faint on the bottom plate ("hipped" plate). The outside of the top plate ("eared" plate) can also be recognized by the two hydrophobic lines caused by the top clamp and top buffer chamber gaskets.
4. Make sure the cassette is clean and dry.
5. Place the bottom plate ("hipped) on the frame with the outside of the plate facing down.
6. Place the spacers on each side of the plate so that the open notched ends are at the top and open towards the inside of the plate. Make sure spacer bottom is flush with the bottom edge of the plate. Once spacers are in the correct position, you may "stick" the spacers in place by touching a dampened finger to the spacer and then sticking the spacer to the glass plate.
7. Place the top plate (the one with the "ears") on. Make sure the plate is oriented correctly! If the top plate gets assembled inside-out, the gel *will not* pour correctly, as the gel will pull away from the hydrophobic lines. Also, if you use the comb clamp or upper buffer chamber with the top plate inside out, you will put another hydrophobic mark on the real inside of the plate and it won't work in either direction until the marks are cleaned off with 10% hydrochloric acid.
8. Slide the plates to the bottom of the grey cassette until the notches of the rear plate catch on the plate stops of the cassette (you can not push the plate any further).
9. Make sure the bottoms of the top and rear plates are flush.
10. Put top clamp on the gel and lock it into place.
11. Put the bottom gel injection device on and clamp it into place. Be sure the hole orange gasket is exactly aligned with the hole in the plastic device.
12. Lock the plates in place by turning all the cassette clamps to the closed position.
13. If you are not using the plates the same day you wash them, you may want lightly cover the ends with dry Kimwipes or plastic wrap, or at least place the straight edge of the comb between the top of the plates (helps to keep dust, etc. away from the exposed areas of the plates).

WARNING: Unpolymerized acrylamide is highly toxic. It is a strong neurotoxin and carcinogen. People have died a slow painful death from mishandling this stuff. Wear gloves, wear a lab coat, and eye protection!

C. Pouring the Gel

Before pouring the gel, make sure that you have combs with flat edges that are not damaged!! Have a pair of scissors, a syringe and beaker on hand. Since each Long Ranger Singel packs cost about $12.50 each, we pour two gels from one pack.

1. Place both assembled gel plates/cassettes on a platform of plastic tip boxes.
2. Remove combs from loading region and unclip the top clamping device, but have the combs ready for use. Lift the laser beam safety bar (so that it is easier to view the gel while pouring).
3. For gel preparation, follow protocol on Singel package: remove the BLACK clip and mix contents of the compartments by hand thoroughly but gently for 12 minutes.
4. Remove the RED clip and mix content of compartments well by hand for 1 minute. Now proceed without hesitation through the rest of the steps. Your gel is already in the process of polymerizing, and you only have a couple of minutes to complete the gel pouring.
5. Remove the WHITE clip (filter). Hold the bag so that contents drain into the filter end. Roll or fold over pouch to the indicated line.
6. With scissors, cut the pouch on the embossed line across the filter port. *Avoid introducing air bubbles as you squeeze out the contents by letting the gel solution trickle down the side of the beaker*. Squeeze out all of the gel solution.
7. Suck up about 30 ml of the gel contents from the beaker with the syringe. Carefully remove excess air from syringe (don't push with such force that the unpolymerized gel squirts all over the ceiling!).
8. Insert the syringe tip tightly into the gel injection device at the bottom of the plates. Begin pushing the gel into the plates and don't stop. Be prepared to knock on the plates if the gel front begins to get uneven. Push in the gel until it begins to come out the top edge.
9. Reload the syringe and repeat with other gel.
10. Quickly check for any air bubbles that may have formed at either end of the plate. You may be able to fish these out with the "bubble hook".
11. Insert the flat end of the comb into the loading region and reclamp the top clamping device.
12. Remove the bottom gel injection device. Place the laser beam safety bar back down and make sure all 6 cassette clamps are closed (keeping the plates pressed together).
13. 15 min to 1/2 hour after the gel is poured, make sure to place 1X TBE moistened Kimwipes along the bottom edge of the gels, then plastic wrap. Allow 2 hours for gel polymerization.

Jobs to do While the Gel is Polymerizing

The following jobs can begin ~ 1 hr before polymerization is complete

A. Computer Stuff

1. If the computer is on, shut it down.
2. Turn on the ABI 377 DNA sequencer. Wait a moment (after the red light goes off) and then turn on the Macintosh computer. The program must now be restarted.
3. Under "ABI Prism 377" choose "File", "New" and then click on "Sequence Run"
4. Again, select "File", "New" and then click on "Sequence Sample". This is your sample sheet.
5. Fill in the empty boxes under sample name. Leave unused or skipped lanes blank. Close this file and save.
6. Back to the Run sheet. You can now select your sample sheet from the menu. Make sure all other options are filled in correctly as in the attached photo. Most importantly, make sure the Run Module reads Seq Run 36E-1200 and run time 9 hours (default is 7).

B. Wet Lab Stuff

1. Mix up the 1X TBE. You'll need approx. 1300 ml (260 ml of 5X TBE + sufficient ddH_2O to reach the 1300 ml mark). There is a large plastic graduated cylinder for this purpose.
2. If your sequencing reactions are in individual tubes, make sure that the heat block is turned on. It takes a while for it to warm up.
3. Rinse/wash plates. In the sink wash the outside of the plates with dH_2O.
4. Remove and wash comb with ddH_2O.
5. Wearing powder-free gloves, rub off any polymerized gel on the plate surface and edges. Make sure loading region gets rinsed well.
6. Stand plates up in the gel pouring region to dry. Let air dry for a few minutes and then you can use a folded Kimwipe to wipe off excess beaded water.
7. Wash off cassette by using a dampened paper towel.
8. Insert the paper comb. (Skip this step if using a membrane comb). Place the dry glass plates into the cassette and close clamps. If using a disposable paper comb, you must dry the well of water using a Kimwipe. Hold plates/cassette upright on lab bench. Center comb in the loading region with teeth facing down. Slowly and EVENLY insert the comb until it is about 1-2 mm into the gel.

C. Plate Check

1. Place the bottom reservoir in the sequencer and plug in the electrode.
2. Carefully insert the plates/cassette into the "woodstove" and close the four clamps - one hand should always be on the plate/cassette. IMPORTANT: Make sure that the amber colored thingies on the sequencer are pressing firmly against the back plate (visible below the "hips"). Sipes' machine is weird and sometimes you have to fiddle with (jiggle) the gel cassette to get it in properly. Close door making sure that no wires get pinched in the door.
3. The "Plate Check Module" should be selected to "Plate Check E". Click the "Plate Check" icon. Then under "Window" select "Status". Make sure there is a 30 second countdown. If the computer does not do this, click on the "Cancel" icon, wait a few seconds, then select "Plate Check" again. There is a software bug and sometimes it does not countdown correctly. Under the status window, there should be NO ELECTROPHORESIS POWER. If there is, cancel immediately!
4. After a few minutes, you should observe red, black, green and blue lines in the Scan Window. If you have any large peaks (baseline should be smooth), Cancel the Plate Check, remove and wash plates again. Otherwise Cancel the "Plate Check" and proceed to next step.
5. Record the position of the baseline in the log sheet.

D. PreRun (Skip this step if using a membrane comb)

1. Place top buffer chamber on the plates and make sure it is securely clamped. Plug in.
2. Mix 1X TBE and add to top buffer chamber to the fill line. Make sure there are no leaks. Do not fill past the max. fill line. Wipe up any splashes.
3. Fill bottom reservoir until full.

4. Place heating plate on front of plates and clamp in. (Careful not to drop the heating plate!) Plug in electrical ground wire and the two coolant tubes. Check that the coolant tubes don't leak. [note: when worn out, the rubber rims must be replaced – locally at True Value].
5. Place the lid on the top buffer chamber and close door making sure not to pinch any wires.
6. Select the PreRun icon (this should be set for "Seq PR 36E - 1200").
7. Go to Window and select "Status". Make sure the 30 second count-down is complete otherwise cancel and try again.
8. During the PreRun, you should see the following on the status window:

Electrophoresis Voltage kV	1.01 / 1.00
Electrophoresis Current mA	~12 / 35.0
Electrophoresis Power W	10 / 50
Gel Temp. ˚C	/ 51
Laser Power W	40.0 / 40.0

(the numbers correspond to the green and grey boxes respectively)

9. Record the prerun mA in the log sheet.
10. Reuspend your samples. To your 150 μl aliquot of fomamide, add 30 μl the blue dye (blue Dextran/EDTA). Mix well.
11. Add 1.2 μl of the formamide/dye mix to each of your sequencing reactions. Mix by stirring with the pipette tip and/or gently vortex (or flick mix).
12. Centrifuge the samples briefly to move all liquids to the bottoms of their tubes.

Gel Loading and Running

Skip this step if using a membrane comb – see alternate procedure below

A. Gel Loading

1. Denature the sequencing reactions when the gel temperature reaches approximately 35 to 40˚C. Denature at 90˚C in the heat block for 2 min. then place the samples on ice. (Get ice from Andrew Wood's Lab).
2. Push the "Pause" icon and wait for the message saying it is OK to open the instrument panel.
3. Rinse the loading wells with 1X TBE using the syringe and needle or with a 1.0 ml pipetter.
4. Using a Hamilton gel-loading syringe, load the gel by loading every other lane. Load the odd lanes, rinsing the needle twice or thrice with water between loading each lane. Load plain formamide/dye mix to the flanking lanes.
5. Close the door and select "Continue". Allow electrophoresis to continue for 4 to 5 minutes.
6. Select "Pause", wait for the OK, then repeat loading procedure with the even lanes. Once lanes are loaded, select continue and allow electrophoresis for another 2 minutes.

B. Gel Running

1. Stop the Prerun by selecting "Terminate".
2. If you haven't chosen your sample sheet on the run window, do so now. Put your name in the user window and choose 9 hours run time.

3. Wait a few seconds then select "Run" (Again, this should be run module: "Seq Run 36E-1200")
4. Save your gel file at the prompt.
5. Make sure the 30 second countdown is complete (viewed under the Status window).
6. The status window should display something similar to:

Electrophoresis Voltage kV	1.70 / 1.68
Electrophoresis Current mA	26.1 / 50.0
Electrophoresis Power W	44 / 150
Gel Temp. ˚C	51 / 51
Laser Power W	40.0 / 40.0

(the numbers correspond to the green and grey boxes respectively)

7. Make sure you also have the correct run time.
8. Close the status and scan windows, and flip the "computer in use" sign down. Turn off the monitor.

C. Clean-up

1. The 2 liter graduated cylinder for the 1X TBE should be rinsed well with dH_2O and then ddH_2O, as do the combs and spacers. Everything else gets washed with Alconox and rinsed well with dH_2O and then ddH_2O
2. Make sure all lab benches are wiped clean. TBE is very sticky – clean up all puddles and drops!
3. Make sure the heat block or thermal cycler is turned off.
4. If you poured two gels, make sure that the second gel gets covered. Place a 1X TBE-soaked Kimwipes on both ends of the gel (you can leave the comb in or not). Then wrap both ends in plastic wrap. Place in refrigerator.

Post Run

A. Clean-up

1. Turn off power switch to the sequencer.
2. Open the door and disconnect the three electrical plug-ins from the upper and lower buffer chambers and the heating plate.
3. Disconnect the coolant tubes and immediately wipe off any residual coolant using Kimwipes.
4. Remove the heating plate. Make sure the bottom surface is clean, otherwise, wipe it off with ddH_2O-dampened Kimwipe. Return the plate to its designated drawer. Make sure the clamps from the heating plate get clamped to hold glass plates in place.
5. Use a small beaker to remove the buffer from upper chamber into a plastic beaker.
6. Use Kimwipes to dry the gasket area of the upper buffer chamber. Remove the upper chamber and wipe off any remaining drops of buffer.
7. With one hand holding the cassette/plates, release the four cassette clamps. Remove the cassette/plates.

8. Remove the lower buffer chamber.
9. Make sure the inside of the sequencer is CLEAN. You may use a ddH_2O-dampened Kimwipe to GENTLY clean any surfaces. Do NOT scrub surface of heating pad - just gently wipe from side to side. The upper and lower buffer chambers get cleaned with dH_2O only.
10. Remove glass plates from cassette and place in sink. CAREFULLY use the wedge to pry the plates apart. Have patience! Place dry Kimwipes across the gel then roll the gel off. Discard in designated container.
11. Wash the combs and spacers with dH_2O only. Final rinse with ddH_2O.
12. Wash plates as described above.
13. Wipe off all lab surfaces.
14. Make sure there is enough ddH_2O for the next user!

B. Post Run Computer Stuff

1. Open the Gel File in the Run Folder. The computer will create the gel image.
2. Track lanes. You can track lanes under "Gel" menu. Select "Track Lanes" and then "Autotrack." After the autotrack is finished, click on the triangles at the top of the gel to manually adjust and correct any mistracking.
3. From the "Gel" menu, choose "Extract Lanes" and "OK." A grid will appear, select column "A" for all your samples, click on "Start" button. When finsihed, close all windows. Your sequences will be saved in a folder that has a name such as "gelrun+date".
4. Send your gel run folder to the Nickrent lab computer (Curupira) via AppleTalk.
5. After some time (several weeks), remove your run folder(s) from the sequencer computer hard drive. The computer needs as much disk space as possible.
6. Turn off the monitor.

Protocol Modifications when using "Membrane Combs"

1. After pouring your gels, DO NOT use the binder clamps to clamp the area around the casting comb. Later on, when inserting the membrane comb, the gap between plates should be as loose as possible.
2. Do not perform a pre-run. It's unnecessary when using the membrane comb. But, make sure the heating plate is on.
3. Make sure that excess water gets removed from the well area after performing the final rinse. You may need to invert the gel or use a Kimwipe to facilitate this.
4. Put the bottom buffer chamber into the machine and clamp the gel cassette and top buffer chamber in place.
5. Denature your samples for 2 minutes at 90° C and then place immediately on ice.
6. Using either a single-channel or an 8-channel pipettor, pipette approximately 1 μl of each sample into a well of the loading tray. Note that if you are using an 8-channel pipettor to transfer one column of a 96-well plate at a time, samples will end up in every third well of the loading tray. MAKE SURE THAT THE SAMPLE SHEET REFLECTS THIS!
7. According to the manufacturer of the membrane combs, it is important that steps 8 through 12 (below) are done as quickly as possible. Ideally, the Ficoll solution should remain on top of the gel for as little time as possible. Likewise, try to minimize the amount of time that your samples are bound to the membrane comb.

8. Using a 1 ml pipettor, inject approximately 700-1000 μl of Ficoll loading buffer (20% Ficoll) between the plates at the top of the gel.
9. Dip the tips of the membrane comb into the loading tray. Allow your samples to wick into the comb for 15 seconds.
10. Carefully insert the membrane comb (with your samples) between the plates at the top of the gel. You will easily be able to feel when the comb teeth make contact with the gel itself.
11. Fill both buffer chambers with 1X TBE buffer.
12. Immediately start the sequencing run. When prompted, save the file listed to the default location. Allow your samples to run into the gel for 60 seconds after electrophoresis power is applied.
13. Pause the run, open the instrument door (there should be no electrophoresis current at this point), and remove the membrane comb from the gel. Use a syringe full of 1X TBE to gently flush the Ficoll solution from between the two glass plates.
14. Close the instrument door and resume the sequencing run.

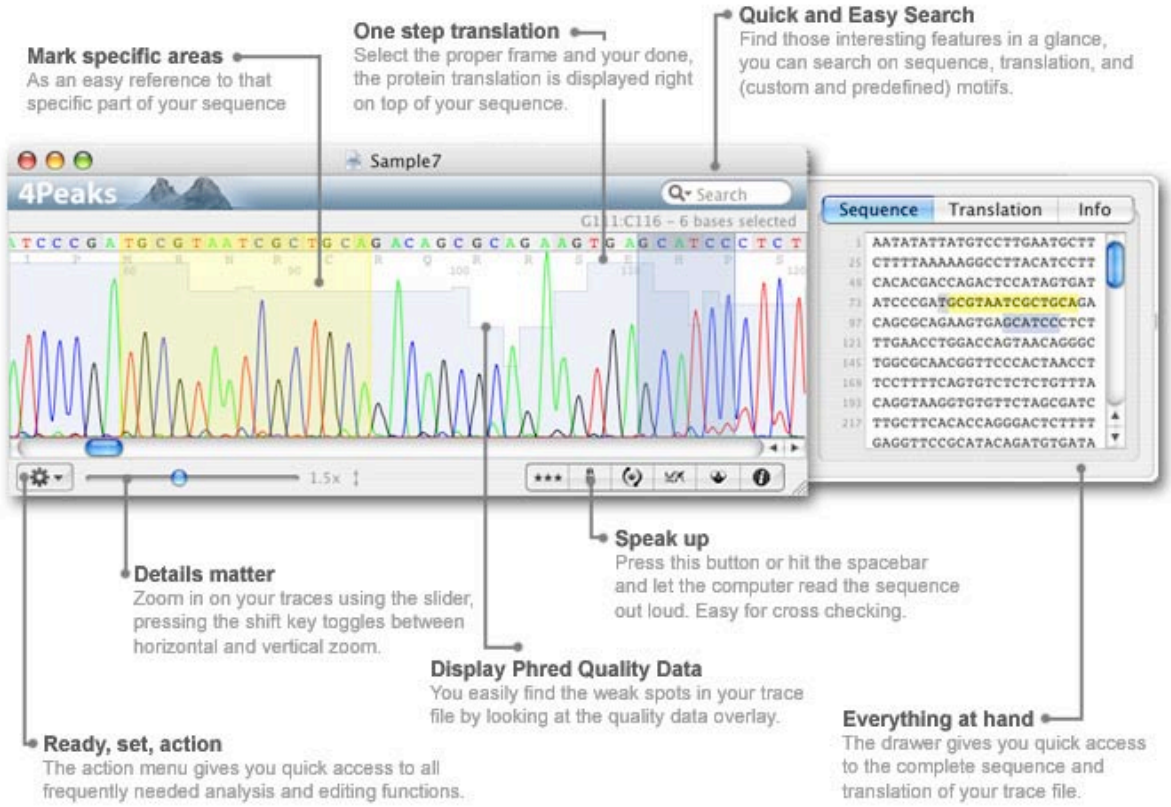
Exporting Data to Other Computers

Your gel run file can now be ported to another computer to allow editing without using the Sipes sequencing computer. The latter is considered dedicated to the ABI sequencer and is to be used only for data collection and post-run operations (e.g. lane tracking). The Nickrent lab numbers gel runs sequentially with file names such as: 100DN, 101DN, etc. The file folder will contain one file for each sequence (lane) and each will be numbered such as 23•100DN 1for. The folder will also contain a gel file, a run file, and a log file. We typically use AppleTalk (via COS-PlantNet) to connect to the Macintosh computers (servers) in the Nickrent student lab (Curupira). If you have problems connecting to Curupira, you can place the folder in LabAccess (also via AppleTalk). See Dr. Nickrent or a graduate student regarding how to use file sharing.

When planning a sequencing run, we fill in a blank form composed in MS Excel called "Sequencing Run Data Sheet". This form has the following information: reaction date, operator name(s), gel run number, gel run date, taxon and collection number, sample number (e.g. purification number), primer, and run notes. After the gel run is complete, these data are tranferred to another Excel file called "Autoruns Compilation". The Autoruns Compilation file should reflect what was actually loaded on the sequencing gel. This file serves as the database for permanently archiving information on all gel runs. The file is extremely useful because it allows one to search and sort for all the information listed above. This file is present on my (DLN's) computer (Rafflesia) and aliases are located on Curupira and Nuytsia. Login with password is required to modify the file.

Editing Sequence Data

We open the sequence files in a program called 4Peaks (http://www.mekentosj.com/4peaks/). The program is very intuitive and allows one to easily perform a number of operations on your sequence such as search, reverse-complement, translate to amino acids, adjust peak height and width, etc. We typically copy and paste the "good" sequence (usually the 5' and 3' ends are not used) into our multiple sequence alignment program SeAl version 2.0 (Rambaut 2004) and keep this window open at the same time as the 4Peaks window. We leave the 4Peaks file intact and make all editing changes to the version in SeAl.



Interpretation of Sequence Data

When the sequence is pasted into SeAl, one must be aware of whether the sequence was generated from a forward or reverse primer. The reverse primer sequence must be "flipped," i.e. turned into the reverse complement to allow alignment with existing sequences. For genes that were cloned and sequenced using a universal primer present on the plasmid, the orientation of the fragment is not known. A convenient way to quickly find the orientation is to copy the sequence and paste it into the window at NCBI BLAST (http://www.ncbi.nlm.nih.gov:80/BLAST/) and conduct a blastn (nucleotide) search. This program will align your sequence with others like it, thus giving you the proper orientation.

For genes longer than 700 bp, we usually have to sequence it in parts. For example, *rbcL* is ca. 1400 bp in length. We usually get good sequence reads for 700 bp, so a primer at the 5' end will generate enough sequence to overlap sequence generated from the primer at the 3' end. When assembling these contiguous fragments ("contigs"), I paste the separate contigs into different windows. Once the sequences are moved to the proper position (aligned) and edited, they can be concatenated. This is done simply by copying and pasting from one sequence line into the other. Any conflicts between the two sequences on the parts that overlap must be resolved. Generally, one places less confidence in sequence generated near the 5' and 3' ends. In some instances, it is not possible to unambiguously assign a base to A (adenine), C (cytosine), G (guanine) or T (thymine). In these instances, ambiguous bases can be used. The following table gives the accepted convention for nomenclature of incompletely specified bases.

Symbol	Meaning	Origination
R	G or A	pu**R**ine
Y	T or C	p**Y**rimidine
M	A or C	a**M**ino
K	G or T	**K**eto
S	G or C	**S**trong interaction (3 H bonds)
W	A or T	**W**eak interaction (2 H bonds)
H	A or C or T	**not G** (H follows G in alphabet)
B	G or T or C	**not A** (B follows A in alphabet)
V	G or C or A	**not T** (not U, V follows U)
D	G or A or T	**not** C (D follows C in alphabet)
N	G or A or T or C	a**N**y

Gel artifacts may occur for a number of different reasons. One of the most common ones involves band compression (2 or more peaks occurring at the same position). This usually occurs because of secondary structural features of the DNA template that cause the polymerase to pause. Also, DNA that is unusually rich in G and C may not fully denature during cycle sequencing, thus interferring with the polymerase. For templates with high A and T, primer binding is less stable and this may cause artifacts. False priming can also occur during PCR and sequencing when two or more sites exist for primer annealing. In PCR, this results in more than one product; in sequencing, "double peaks" may appear. In some cases this reflects an actual biological phenomenon (e.g. a hybrid organism with different alleles). Ultimately, cloning may be necessary to properly sequence such alleles.

SECTION 12: RESTRICTION DIGESTS AND SOUTHERN BLOTTING

Restriction enzymes cleave DNA every 4^n base pairs where 4 is the number of different nucleotides (A,C,G,T) and n is the recognition sequence for the particular enzyme. For EcoRI, the recognition sequence is GAATTC. The probability of cleavage is therefore 46, or 4,096 bases for this enzyme. The average chloroplast genome contains 156,000 base pairs, thus one would predict the enzyme to cut the circular molecule into 38 fragments. The size of the nuclear genome in plants is much larger, ranging from 5×10^7 to 1.25×10^{11}. Even with the smallest genome, one would predict this enzyme to produce over 12,000 fragments, thus bands are not resolvable on a gel and the multiple fragments would appear as a brightly staining smear. To pick out individual bands, one can transfer the DNA from the gel to a charged membrane (nitrocellulose or nylon) and then hybridize with a DNA probe specific to a gene or group of genes that are complementary to the DNA bound to the membrane. This procedure was first developed in 1975 by E. M. Southern and the method is now called a Southern blot.

Restriction Digestion of Plant DNA

A typical restriction digest is shown below:

2 μl	DNA (plasmid)
15 μl	ddH_2O
2 μl	10X buffer (use buffer associated with restriction enzyme)
1 μl	Enzyme (ca. 1 unit per microgram of DNA)
20 μl	Total

Digest at 37° C for 1.5 hours or longer.

Agarose Gel Electrophoresis of Restriction Digests

1. Prepare a TAE agarose gel (see Section 6). The electrode buffer will be the same as the type of gel. The final purpose for the gel will determine the percentages of agarose to use, e.g.:

0.8% -- for examining chloroplast DNA digests
0.9% -- for conducting Southern blots
1.0% -- for examining ribosomal DNA

2. Add glycerol loading dye to tubes (ca. 4 μl sample, 10 μl of dye). Load into gel lanes. Load molecular weight marker. Begin electrophoresis. Run times vary depending upon the thickness

and size of the gel, the buffer used, and the current applied to the gel (from 1 hour to overnight).

3. After electrophoresis, stain gel with EtBr (and de-stain with water). The gel should then be marked (notch on left side) and photographed with fluorescent rulers on each side.

Southern Blotting - Wet Transfer Method

1. Cut off the unused portions of the gel (to minimize use of expensive membrane) and measure the gel dimensions. Photograph the gel with fluorescent rulers on each side.

> Note - Sometimes the DNA is depurinated with an acid wash in 0.25 M HCI for 8 minutes at this time. This step does not seem to be necessary unless blotting very large (> 15 kb) DNA fragments. If the gel is left in the HCl too long it may be cleaved into tiny fragments that cannot stick to the membrane. For these reasons, we normally do not do this step.

2. Denature gel in 1.5 M NaCl and 0.5 M NaOH for 15 min.

3. Neutralize gel in 0.5 M Tris-Cl, pH 7.0 and 3 M NaCl for 10 minutes. Pour off solution and repeat.

4. Using a paper cutter, cut 10 pieces of Whatmann 3 MM filter paper, slightly smaller than the gel and soak in 2X SSC.

5. Wearing gloves and using clean scissors dedicated for this purpose, cut the nylon membrane exactly the same size as the gel. Mark the filter (top right) with a pen to identify your samples. It will be assumed that the side without writing is the side in contact with the gel. Soak the membrane in distilled water for 2-3 min.

6. Place a large (10 cm diameter or larger) cover of a Petri dish on a support (e.g. top of a plastic buchner funnel). This "platform" is then placed in an 8 inch Pyrex baking dish. Wrap the Petri dish cover with a large piece of 3 MM filter paper and place back in the baking dish. This filter paper will act to wick the buffer upward through the gel, the membrane, stack of filter paper, and finally the paper towels. Fill container almost to Petri dish with transfer buffer (10X SSC). Make sure there are no air bubbles between the glass and the wet paper.

7. Place the gel (well-side down) on filter paper saturated with 10X SSC buffer. Make sure there are no air bubbles between the gel and the paper.

8. Carefully place the nylon filter on the gel, lining up the upper edge of the filter exactly with the bottom edge of the wells. Again check for air bubbles.

9. Place the 2X SSC saturated 3 MM paper sheets on the filter, one at a time for the first three, being careful not to introduce air bubbles between the paper.

10. Cut a stack of paper towels to the size of the 3MM filter paper. Stack the toweling onto of

the filter paper to a height of a few inches. Wrap the entire baking dish in plastic wrap. Place a weight (brick, Sigma catalog, etc.) on top of the stack. Let the transfer proceed for 12-24 hours. The rate of transfer varies depending upon the size of the fragments. DNA less than 1 kb transfers within an hour whereas fragments > 15 kb takes 15 hours or more.

11. Peel off filter from gel and papers and soak in 5X SSC for 5 min.

12. Allow filter to air-dry on a sheet of Whatmann 3MM paper. Sandwich filter between 1 MM paper (not 3 MM or heavier) and dry at 80 ˚C under vacuum (use gel dryer) for 1 hour.

13. Store filter in a sealed bag or use directly in hybridization experiment.

SECTION 13: NON-RADIOACTIVE HYBRIDIZATION

Non-radioactive hybridization techniques offer the advantages of stable probes, inexpensive disposal, and convenience. The use of digoxigenin as a non-radioactive label has been shown to be as sensitive as ^{32}P for detecting picogram amounts of homologous DNA. The following procedures were written by Laurie Achenbach (SIUC, Dept. Microbiology) and are modifications of Höltke et al. (1992) and Lanzillo (1990).

Probe Construction Using Digoxigenin in a PCR Amplification

Reagents:

DIG-dNTP mix:

- 13.2 μl 1 mM digoxigenin- 11-dUTP (Boehringer Mannheim cat #1093-088)
- 2.7 μl 10 mM dTTP (i.e. Perkin Elmer cat. #N808-0007)
- 4.0 μl 10 mM dATP
- 4.0 μl 10 mM dCTP
- 4.0 μl 10 mM dGTP
- 12.1 μl dH_20

_____

40 μl total

Method:

1. Amplify the gene of interest in the usual manner to determine the appropriate conditions for proper symmetric amplification prior to probe construction.

2. PCR mix for probe construction:

- 15.5 μl dH_20
- 2.5 μl 10X PCR buffer
- 2.5 μl DIG-dNTP mix
- 1.5 μl 25 mM $MgCl_2$
- 1.0 μl forward primer at 10 μM
- 1.0 μl reverse primer at 10 μM
- 0.5 μl DNA (PCR product – read Section 7 on considerations)
- 0.5 μl Taq DNA polymerase

_____

25 μl total

3. Amplify using the parameters determined in step 1. Use a thermocycler with a heated lid.

4. The probe is ready to denature and use in a hybridization experiment (5 μl of probe per ml of hybridization solution). No purification is necessary. The probe is stable for over one year when stored at –20 ˚C.

Hybridization with Digoxigenin-Labeled Probe

Reagents:

- Casein Hybridization Solution: 5X SSC; 0.5% casein (Sigma C-5890); 0.1% N-lauroyl-sarcosine sodium salt; 0.02% SDS. Heat to 50-70 ˚C to dissolve. Store in 50 ml aliquots at -20 ˚C.
- LP Buffer 1: 0.1 M maleic acid; 0.15 M NaCl; pH to 7.5 with solid or concentrated NaOH; autoclave and store at 4 ˚C.
- Blocking Reagent: 10% casein (Sigma C-5890) in LP Buffer 1. Heat to 70 ˚C to put into solution; autoclave and store at 4 ˚C.
- 25% Formamide Hybridization Solution (100 ml):

  25 ml formamide,
  25 μl 20X SSC
  20 ml blocking reagent
  1 ml 10% N-lauroyl-sarcosine sodium salt
  200 μl 10% SDS
  H_20 to 100 ml
  Store in 50 ml aliquots at -20 ˚C

Method:

1. Perform Southern blots using charged nylon membranes (MagnaGraph works well).

2. Place the membranes in a heat-sealable bag that has been rinsed with water. Add 20 ml of hybridization solution per 100 cm^2 of filter; seal. Prehybridize 1 hour at the hybridization temperature.

Hybridization conditions:

Stringent: Use Casein Hybridization Solution at 68 ˚C for homologous probes **OR**: 50% Formamide Hybridization Solution at 42 ˚C. Decrease temperature as necessary. Background is usually decreased when using formamide solution.

Less stringent: Use Casein Hybridization Solution at 55 ˚C **OR**: 25% Formamide Hybridization Solution at 42˚ C. Decrease temperature as necessary. Background is usually decreased when using formamide solution.

3. Denature probe at 95 ˚C in a heat block for 10 min. right before use. Use 5 μl of digoxigenin-labeled probe per ml of hybridization solution. Mix well.

4. Cut off a corner of the bag and squeeze out the prehybridization solution. Immediately add 2.5 ml of hybridization solution (with denatured probe) per 100 cm^2 of filter; seal. Hybridize overnight at the hybridization temperature in the hot shaker.

5. Cut off a corner of the bag. Squeeze the hybridization solution into a tube and store at -20 ˚C. The hybridization solution is stable for over one year at -20 ˚C and may be reused multiple times.

6. Wash the membrane 2 X 15 minutes in 20 ml of 2X SSC, 0.1% SDS at the hybridization temperature. Store in distilled water at 4 ˚C if not performing the detection immediately.

Chemiluminescent Detection of Digoxigenin-Labeled Probe Hybridization

Reagents:

- Washing Buffer: LP Buffer 1 + 0.3% Tween 20. Make only as needed; doesn't store well.
- LP Buffer 2: Blocking Reagent diluted 1: 10 in LP Buffer 1. Make only as needed.
- LP Buffer 3: 0.1 M Tris; 0.1 M NaCl; 50 mM $MgCl_2$; pH to 9.5. Store at 4 ˚C.
- Anti-digoxigenin - alkaline phosphatase conjugate (Boehringer Mannheim cat. #1093-274)
- Lumi-Phos™ 530 (Boehringer Mannheim cat. #1275-470)

Method:

Volumes are for 100 cm^2 of membrane. All steps are done with gentle shaking at room temperature except as noted. Never allow the membrane to dry or it cannot be stripped effectively.

1. Wash briefly (1 min) in 20 ml of Washing Buffer.

2. Incubate membrane 30 min in 20 ml of LP Buffer 2.

3. Dilute 2 μl of anti-DIG alkaline phosphatase conjugate (at 75 mU/ml) in 20 ml LP Buffer 2. Incubate membrane 30 min in 20 ml of dilute anti-DIG solution.

4. Wash 2 X 15 min in 50 ml Washing Buffer.

5. Wash briefly (1 min) in 20 ml LP Buffer 3.

6. Dilute 100 μl of Lumi-Phos (at 0.1 mg/ml) in 10 ml LP Buffer 3. Diluted Lumi-Phos may be reused multiple times and is usually good for I week to 1 month, depending on usage. Store in a dark plastic bottle at 4 ˚C.

7. Incubate membrane 5 min in 10 ml of diluted Lumi-Phos solution.

8. Briefly let excess liquid drain off of the membrane.

9. Seal the damp membrane in a heat-sealable bag that has been rinsed with water.

10. Incubate the sealed membrane for 15 min at 37 ˚C.

11. Expose to X-ray film at room temperature. Start with a 1-4 hour exposure and increase time as necessary. When using homologous probes, a 20 min. exposure may be sufficient. When using heterologous probes, you may need to expose overnight. Multiple exposures may be taken as luminescence continues for at least 24 hours.

If the membrane will be used for another hybridization, perform the following steps:

12. Immediately after exposure to film slit the bag, remove the membrane and wash at least 5 min. in distilled water. The membrane may be stored in water for several days before stripping.

13. Wash 2 X 15 min in 0.2M NaOH, 0. 1 % SDS at 37° C

14. Wash 2 min in 2X SSC.

15. Prehybridize and hybridize as usual ***OR*** store in a sealed bag with sterile water at 4 °C.

SECTION 14: SOLUTION RECIPES

Ammonium acetate, **4.0** M- for precipitating nucleic acids 154.18 g of NH_4OAc
Bring up to 500 ml with dH_20
Filter sterilize, do not autoclave.

Ammonium persulfate, 10% (w/v) - for acrylamide gels 5 g ammonium persulfate
Bring up to 50 ml with dH20
Store as 1.5 ml aliquots in freezer

Borate Extraction Buffer - for RNA extraction.
Final conc. 0.2 M borate, 30 mM EDTA, 5 mM dithiothreitol, 1 % SDS.

For 50 ml of extraction buffer:
7.5 ml 0.2 M EDTA stock soln
0.5 ml 0.5 M dithiothreitol (DTT)
Bring up to 50 ml with 0.2 M Na borate (pH 9.0 with NaOH). Make only as much as you will need (do not store). To minimize foaming during extraction, it is best to add the SDS (2.5 ml of 20% SDS solution) after the plant tissue is homogenized.

Bromophenol blue 6X Dye
0.26% bromophenol blue
30% glycerol in H_20
Store at 4° C.

Bromophenol blue 1X Dye - for agarose gels
25 nil 10X TE
10 ml of 6X glycerol dye (above)
For loading samples, add 4 0 of sample to 10 μl of 1 X dye.

Bromophenol Blue Agarose Gel Loading Dye

	25 ml	50 ml
25% Ficoll	6.25 g	12.5 g
40 mM Tris pH 8	1 ml	2 ml
20 mM EDTA	2.5 ml	5 ml
0.05% BPB	12.5 mg	25 mg

Add SDS (to 2%) for use with single-stranded DNA/RNA

Cesium Chloride Solution - for ultracentrifugation of nucleic acids
For 100 ml:
83.33 g CsCl
8 1.1 ml of Tris (1 0 mM, pH 7.5)
2.23 ml of ethidium bromide (10 mg/ml)

Chloroform/Isoamyl Alcohol - for DNA and RNA extractions.
A mixture of 24:1 by volume
Store in a dark bottle under the hood

2X CTAB Buffer for DNA extractions
Cetyltrimethylammonium bromide (Aldrich 85,582-0)
Also called hexadecyltrimethyl ammonium bromide (Fisher O3042-500)
100 mM Tris, 1.4 M NaCl, 30 mM EDTA, 2 % (w/v) CTAB.

	100 ml	500 ml	1.0 liter
Tris base	1.214 g	6.05 g	12.1 g
NaCl	8.18 g	40.9 g	81.81 g
EDTA (disodium)	1.11 g	5.5 8 g	11.16 g
CTAB *	2.0 g	10.0 g	20.0 g

* cetyltrimethylammonium bromide (Aldrich 85,582-0)
Takes awhile to go into solution (use heat). The buffer is usually ca. pH 8.0 without adjustment.
Do not autoclave CTAB buffer!

Dithiothreitol (DTT), 0.5 M (FW = 154.25 g/mole) for DNA and RNA extractions

	10 ml	25 ml	50 ml	100 ml
DTT	0.771 g	1.928 g	3.856 g	7.712 g

Store solutions as frozen 1.0 ml aliquots in microfuge tubes.

EDTA, 0.5 M, pH 8.0
Different amounts depending upon how many water molecules on the EDTA

	50 ml	100 ml	500 ml
EDTA, Na_2, anhydrous (FW = 336.2)	8.4 g	16.8 g	84.05 g
EDTA, $Na_2 \bullet H_20$ (FW = 354.2)	8.85 g	17.7 g	88.55 g
EDTA, $Na_2 \bullet 2\ H_20$ (FW = 372.24)	9.3 g	18.6 g	93.05g

For 500 ml, start w/ ca. 430 ml of water, add 8 g of NaOH pellets and let go into solution (takes time). Correct pH to ca. 8.0 with 6.0 N NaOH solution (plastic bottle). The EDTA will go into solution well only when the pH is near 8.0. Autoclave.

Ethidium Bromide - for visualizing DNA and RNA
10 mg/ml
Light Sensitive! Store in dark bottle in refrigerator. See Section 14 for disposal.

Ficoll Loading Dye - for high sample/low dye ratio agarose gels.
25% Ficoll (Type 400, Sigma 4375 or Pharmacia 17-0400-01), 40 mM Tris, pH 8.0, 20 mM EDTA, 0.05% Bromophenol Blue.

For use with single-stranded DNA, use 2% SDS

	20 ml	100 ml
Ficoll	5.0 g	25.0 g
1.0 M Tris	800 μl	4.0 ml
0.2 M EDTA	2.0 ml	10.0 ml
Bromophenol blue	10.0 mg	50.0 mg
dH_20	17.0 ml	85.0 ml

Glycerol Loading Dye, 6X
0.26% bromophenol blue
30% glycerol in H_20
Store at 4 ˚C.

Glycerol Solution - for storing cells
Make a 50% glycerol solution, autoclave. For use, add 300 μl of 50% glycerol and 700 μl of bacterial cells. Mix well and store at -70 ˚C.

IPTG (isopropyl-ß-D-thiogalactoside)
Make 1 M stock by dissolving 0.238 g in 1.0 ml of water. Filter sterilize using Nalgene 0.2 μm cellulose acetate, disposable filter fitted to a 3 or 5 ml syringe. Store frozen in the –20 ˚C freezer. Add 250 μl of the 1M stock to 500 ml of LB medium for an 0.5 mM final concentration.

Kanamycin
Stock solution of 50 mg/ml. Filter sterilize using Nalgene 0.2 μm cellulose acetate, disposable filter fitted to a 3 or 5 ml syringe. Aliquot and store frozen in the –20 ˚C freezer. Add 500 μl to 500 ml of LB medium for a 1 mM final concentration.

LB Medium - Homemade
1.0% tryptone
0.5% yeast extract
1.0% NaCl

For 1.0 liter, dissolve 10 g of bacto-tryptone, 5 g bacto-yeast extract, and 10 g NaCl in 950 ml deionized water. Adjust pH to 7.0 with NaOH and bring volume up to 1.0 liter. Autoclave on liquid cycle for 20 minutes and allow to cool (then add antibiotic if desired). Store at 4 ˚C.

LB Medium - Purchased
Use Fisher LB Broth, (BP1426-500). Results in 2% tryptone.

LB Agar Plate Preparation

1. Weigh out 6.25g of LB broth medium (powder) and place in a 500ml Pyrex bottle.
2. Add 3.75 g granulated agar (final 1.5%).
3. Add 250ml of distilled water, stir bar and then stir on the hot plate, without heat.
4. Remove from hot plate and remove the stir bar.
5. Autoclave for 45minutes under liquid setting (program 2). Let cool to no less than 50 ˚C (should be able to touch the bottle).
6. While the medium is cooling, get IPTG, AMP, XGAL out of the freezer and let thaw on ice or inside the dairy case.
7. Label 10 petri dishes and note which have IPTG, AMP, XGAL.
8. To the 250ml solution add:

Stock sol.	Final Conc.	Volume of stock to add
IPTG (100mM)	0.5mM	1250μl
Amp (50mg/ml	50μg/ml	250μl
Xgal (50mg/ml)	80μg/ml	400μl

9. Put the petri dishes around the Bunsen burner. The burner sterilizes the air around the dishes, thus helping keep down contamination.
10. Pour ca. 25 ml of the medium into each petri dish (ca. 1/2 full). The petri dishes furthest from burner should be closed to avoid contamination.
11. Let the medium cool and solidify. Place the petri dishes in a plastic bag and store in the dairy case (in box, in dark).
12. As an alternative, 100 μl of 100 mM IPTG and 20 μl of 50 mg/ml X-Gal may be spread over the surface of an LB ampicillin plate. Allow to absorb for 30 minutes at 37˚ C prior to use.

Lysis Solution I: (100 ml)
5 ml 1 M glucose
2 ml 0.5 M EDTA
2.5 ml 1 M Tris-Cl (pH 8.0)
dH_2O to 100 ml

Lysis Solution II: (100 ml) (Make fresh weekly)
2 ml 10 N NaOH
88 ml dH_2O
10 ml 10% SDS

Lysis Solution III: (100 ml)
60 ml 5 M KOAc
11.5 ml glacial acetic acid
28.5 ml dH_2O
(Resulting solution is 3 M with respect to potassium and 5 M with respect to acetate.)

Magnesium Chloride, 1 M. (FW = 203.3 g/mole)
101.7 g of $MgCl_2 \bullet 6H_20$
Bring up to 500 ml with dH_20

NET Buffer - for DEAE gel purification of DNA
20 mM Tris pH 8.0, 0.15 M NaCl, 0. 1 mM EDTA

2.00 ml	1 M Tris pH 8.0	1.00 ml	2 M Tris pH 8.0
3.75 ml	4 M NaCl	3.75 ml	4 M NaCl
50.00 μl	0.2 M EDTA	50.00 μl	0.2 M EDTA
94.20 ml	dH_20	95.20 ml	dH_20
100 ml total		100 ml total	

High Salt NET Buffer - for DEAE gel purification of DNA
20 mM Tris pH 8.0, 1 M NaCl, 0. 1 mM EDTA

2.00 ml	1 M Tris pH 8.0	1.00 ml	2 M Tris pH 8.0
25.00 ml	4 M NaCl	25.00 ml	4 M NaCl
50.00 μl	0.2 M EDTA	50.00 μl	0.2 M EDTA
72.95 ml	dH_20	73.95 ml	dH_20
100 ml total		100 ml total	

10X PCR Buffer
An alternative to the Promega 10X buffer, for PCR amplifications
0.5 M KCl, 0. 1 M Tris, I % Triton X- 100

For 100 ml of 10X buffer:
20 ml of 2.5 M KC1
10 ml of 1 M Tris, pH 8.8
10 ml of 10% Triton X-100
Fill to 100 ml with autoclaved dH_20

Phenol Saturation Protocol

1. Melt the phenol crystals at 68 ˚C in a waterbath.
2. When melted, add an equal volume of 1.0 M Tris (pH 8.0) and a stirbar. Stir the solutions until the phenol dissolves.
3. Let the solution sit long enough for the phases to separate. Remove the upper phase with a pipet attached to a hose and the lab vacuum.
4. Add one volume of 0.1 M Tris, pH 8.0 and stir.
5. Test pH and if not at 8.0, repeat steps 3 and 4 (1 to 3 times may be needed).
6. Store solution in dark bottle in the refrigerator with a layer of buffer on the top. Draw the phenol off the bottom for use.
7. If the antioxidant 8-hydroxyquinoline is added(to 0.1%, final concentration, e.g. 0.1 g per 100 ml phenol), the shelf life of the phenol will be extended.

Potassium Acetate, 2 M (MW = 98.148 g/mole) - for RNA extraction
19.63 gm of KAc
Bring up to 100 ml with DEPC treated H_20 pH to 5.5 with glacial acetic acid.
Note: the acetate concentration will be higher than 2 M (see Maniatis 1982, p. 447).

Potassium Acetate, 5 M (MW = 98.148 g/mole)
245.4 g of KAc
Bring up to 500 ml with dH_20
pH to 5.5 with glacial acetic acid

Potassium Chloride, 2.0 M (MW = 74.56 g/mole)- for RNA extraction
74.56 g of KCl
Bring up to 500 ml with DEPC treated dH_20.

Potassium Chloride, 3M (MW = 74.56 g/mole) - for RNA extraction
11 1.8 g of KCl
Bring up to 500 ml with DEPC treated dH_20

Proteinase (Protease) K Solution - for nucleic acid extractions
(Sigma, P-2143)
This particular type has 1 unit/mg. We want 50 units in each tube that contains 1 ml of water.
So, for 10 tubes you need 500 mg. For 20 tubes 1 g.
Recalculate if using another type of protease.
Dissolve in a small beaker and aliquot into microfuge tubes.

Ribonuclease T1 - to remove RNA from DNA samples
1900 units/μl, total of 65.7 μl (125,000 units).
Add 1.0 μl stock for every ml of sample.

Ribonuclease A - to remove RNA from DNA samples
Add enough salt buffer (1 0 mM Tris, 1 0 mM NaCl, pH 7.5) to get 10 mg/ml. Heat to 100 ˚C, for 10 minutes. Slow cool to room temp. Dispense into aliquots. Store at -20 ˚C for short term, -75 ˚C for longer term. For RNAase digestion, add 10μl/ml of sample in TE.

Sarcosyl Stock, 20% w/v - for RNA extractions.
You may need to heat to get into solution.
pH to 7.0 - 7.2 with 1.0 M HCl.

Sarcosyl Rinse Solution - for RNA extractions.
To one liter of dH_20, add 5.0 ml of 20% Sarcosyl and 1.0 ml of 0.2 M EDTA. [0.1% Sarcosyl, 0.2 mM EDTA]. Use this solution to rinse the Polytron generator and mortar and pestle (early stages of extraction).

SOC Medium

2.0% tryptone	2.5 mM KCl
0.5% yeast extract	10 mM MgCl
10 mM NaCl	20 mM glucose

For 250 ml:
5 g bacto-tryptone
1.25 g yeast extract
0.125 g NaCl
2.5 ml 250 mM KCl
Dissolve in 200 ml of deionized water
Adjust pH to 7.0 with 5N NaOH
Autoclave solution. Let cool to 55 ˚C.
Add 2.5 ml of 1 M MgCl (autoclaved solution)
Add 2.5 ml of 2 M glucose with sterile water
Aliquot and store at 4˚C

Sodium Acetate, 3M (MW = 82.03 g/mole)
123 g NaAc
Bring up to 500 ml with dH_20
pH to 5.2 with glacial acetic acid

Sodium Borate, 0.2 M [= 200 mM] (MW = 381.37 g/mole) - for RNA extraction

38.13 g of $Na_2B_40_7 \bullet 10\ H_20$
Bring up to 500 ml with dH_20
pH should be at or near 9.0.

Sodium Borate, 0.02 M [= 20 mM] (MW = 381.37 g/mole) - for RNA extraction
Dilute 2.0 M sodium borate 1:9 --or --
3.813 g of $Na_2B_4O_7 \bullet 10\ H_20$
Bring up to 500 ml with DEPC treated H_20
pH should be at or near 9.0.

Sodium Chloride, 5.0 M
146 g of NaCl
Bring up to 500 ml with DEPC treated H_20.

Sodium Dodecyl Sulfate (SDS), 20%- for nucleic acid extractions
[= Lauryl Sulfate Sodium Salt] (FW 288.4)
10 g of SDS, bring up to 50 ml with dH_20.
Gentle heating OK to get to go into solution.

STE Buffer (= Salt, Tris, EDTA) - for RNA extraction
10 mM Tris HCl, 10 mM NaCl, 1 mM EDTA

1.0 ml of 1 M Tris
200 μl of 5 M NaCl
0.5 ml of 0.2 M EDTA (or 0.2 ml of 0.5 M EDTA).
Bring up to 100 ml with DEPC treated H_20 and pH to 7.5 with HCl.

TAE Buffer - 50X
0.04 M Tris acetate
0.001 M EDTA

	500 ml	1.0 liter
Tris Base	121 g	242 g
Glacial Acetic Acid	28.55 ml	57.1 ml
0.5 M EDTA pH 8.0	50 ml	100 ml

TE Buffer 10X
10 mM Tris, 1 mM EDTA

0.605 g Tris base
0.168 g EDTA, disodium, dihydrate (= • 2 H_20)
Bring up to 500 ml. pH to 7.5 with HCl. Autoclave before use.

TE Buffer 1X
1 mM Tris HCl, 0.1 mM EDTA
Dilute 5X TE (above) 1:4 with sterile water.

Tris, 1 M (MW = 121.1 g/mole)
60.55 g of Tris
Bring up to 500 ml with DEPC treated H_20 pH to 7.5 with conc. HCl.

Tris, 2 M (MW = 121.1 g/mole)
121.1 g Tris
Bring up to 500 ml with dH_20
pH to 7.5 with conc. HCl.

X-Gal (5-bromo-4-chloro-3-indolyl-ß-D-galactoside)
Make solution to 40 mg/ml in dimethylformamide. Store in dark container in the freezer. Add 40 μl of X-Gal to plates and spread. Let dry in 37° C incubator prior to use.

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