Molecular Genetics Laboratory Procedures

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Table of Contents

Table of Figures

Table of Tables

INTRODUCTION TO RULES AND PROCEDURES

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Acknowledgments

This manual is the product of many years' experiences and presents the procedures which have been found most effective. As such, they represent the contributions of a number of persons from the laboratories of Dr. Donald J. Roufa, Kansas State University and Dr. Douglas D. Rhoads, University of Arkansas. Rather than attempt to list them all let us agree that there were a significant number. Most of the procedures are adapted from the work of others but have been found to work, possibly with some fiddling around on our part. Where procedures have been adopted or modified from published procedures we have tried to include literature references. You are encouraged to consult these, as well as more voluminous manuals on molecular biology such as the Molecular Cloning Manual (Cold Spring Harbor Press) for background information or detailed discussions. The more you understand the reasons for individual steps the better will be your ability to use the techniques described and to circumvent problems when a procedure fails to work for you. These procedures should work for you, but no procedure is `fool-proof' or static. There is almost always room for improvement. Furthermore, many of these procedures were developed or optimized empirically. Perhaps we and others have not envisaged all pitfalls, options, etc. Sometimes a very minor variation that seems harmless can have extreme consequences for the outcome. Your particular situation, implementation, or gravitational field strength may necessitate modification of procedures.

How to Use this Manual

Procedures described in this manual are broken down into three groups: General procedures, and then those specific to either Prokaryotes or Eukaryotes. Within each section there are chapters dealing with a particular method or activity. Thus the manual is not a chronological order of procedures but rather an encyclopedic collection of procedures. You need to know what you want to accomplish and then use the Contents Index at the front to locate the chapter and then the sub-index that applies. Therefore, it is important for you to peruse the chapter titles and headings so that you can rapidly find the procedure(s) you need. If it is your first time using a procedure, then you should read the entire procedure before you start. That way you can make sure that you have all the reagents, supplies and time that you need. Any beginners should first read and study the entire Introduction Chapters to familiarize themselves with general hints, rules, and safety. To save space and minimize repetition, not every little detail is given every time. For example, in some procedures you may be instructed to `perform an ethanol precipitation.' You need to familiarize yourself enough with the basic philosophy and techniques in this chapter to know what this means. To understand that you need to add salt, how much ethanol to add and how much is too much, how fast and long to spin, that you need to rinse with 70% ethanol and then dry the pellet. Furthermore, some standard (or not so standard) abbreviations are used such as ' for minutes (e.g., 10' means 10 min.), " for seconds (e.g., 15" means 15 sec.), EtOH for ethanol, and OAc for acetate.

General solutions and reagents used throughout the manual are listed in one of the early chapters. Some useful numbers and conversions are in a separate chapter. Finally, do not use the manual as your lab procedures notes. Each experiment should be recorded in your lab notebook with dates, volumes, etc.

General suggestions

1) Keep your work area clean and neat. Organization is the sign of a well-structured experiment. Leave your work area clean and things put away.
2) Most of the equipment is expensive, keep it clean and in good shape, it will break down less often that way. Many of the reagents are hazardous, clean your lab bench occasionally with a damp paper towel to reduce dust and remove spilled chemicals.
3) Leave common areas like electrophoresis areas or the darkroom clean, the way you would like to find them, clean and ready to use.
4) If you use the last of something don't put the tube or bottle back. Write a note and post it in the lab saying `We are out of *XXXXX*' and sign and date the note. That way you have warned everyone else and it may help you to remember to get it replaced.
5) Plan your experiment well:
   a) Read the procedure carefully and list solutions you will need. Before you start make sure you have all the solutions and equipment you will need.
   b) Write out as much of the experiment as possible before you start, this will help you envision the experiment and any special needs.
   c) Perform any calculations in advance, if you dry-lab your experiment you will determine whether the amounts of DNA, radioactivity, or reagents you plan to use is sufficient for the analysis.
   d) Place a note on any common equipment that you will need. This will warn your lab mates that you have reserved this apparatus and `should' guarantee that it is available when you need it. If it isn't then at least you have an excuse for "killing the inconsiderate jerk."

Safety

1) Molecular biology procedures involve manipulation of bacteria, and other microbes, as well the procedures all usually involve recombinant DNA. Therefore, proper biological safety procedures must be followed at all times. These include:
   a) NO FOOD OR DRINK IN THE LAB.
   b) NO MOUTH PIPETTING, USE A PUMP OR OTHER DEVICE
   c) WASH YOUR HANDS BEFORE AND AFTER ALL PROCEDURES
   d) CLEAN YOUR WORK AREA BEFORE YOU BEGIN AND WHEN YOU ARE DONE
   e) NO BARE FEET, NO PETS, NO SHOES-NO SERVICE
2) These procedures involve a number of carcinogens, caustic chemicals, and radioisotopes. Be careful and fully aware when handling these compounds so as not to contaminate yourself or others. If you spill something then be certain the spill is cleaned up correctly. If you don't know how then find out. You will rarely suffer much more than mild embarrassment from reporting a spill but you could be banished for life for trying to cover it up.
3) Wear the appropriate safety clothing. Lab coats are good. When dispensing strong acids, bases, or organic solvents protect your eyes by wearing safety goggles and wear latex gloves to prevent skin burns.
4) Visualization of gels often involves UV light. Avoid prolonged skin exposure and protect your eyes with thick glass or special UV rated goggles.
5) Know how to properly dispose of chemicals, solutions, and supplies.
   a) All glass should go into a glass disposal container. Never into the trash

b) "Sharps" are needles, razor blades, pins, etc. These must be placed into special containers and held for disposal as Biomedical Waste. NEVER IN THE TRASH.
c) Organic solvents and disposal are discussed in the General Procedures chapter (page 6).
d) Small quantities of strong acids and bases may be diluted substantially with water and flushed down the drain. For disposal of larger quantities contact your hazardous waste officer.

6) Know how to handle hazardous waste
   a) Never throw hazardous waste in garbage or pour waste down drain.
   b) All hazardous waste must be labeled as such immediately upon generation.
      1. Label with the **starting date** and the **specific chemicals** in the waste container
      2. Keep containers tightly sealed in a safe area (fume hood) and never leave a funnel in an unattended container.
   c) Keep chemical, radioactive and biohazard waste separate.
      1. Don't mix halogenated with non-halogenated wastes
   d) Do not mix incompatible chemicals or wastes together.
      1. Don't mix strong acids and bases
      2. Don't mix strong oxidizers with strong reducing agents
   e) Know what to do in case of a chemical spill.
   f) Recycle or reduce the volume and toxicity of your wastes whenever possible.
7) The most hazardous wastes in a Molecular Biology Lab are
   a) Radioactive compounds
   b) Organic solvents: phenol, Chloroform ($CHCl_3$), alcohols
   c) Strong acids and bases
   d) Carcinogens: ethidium bromide
   e) Toxins: acrylamide
   f) Biologicals: pathogens, bacteria (and their wastes) and viruses
8) Emergency procedures in case of a spill
   a) For eye contact with chemicals, flush with running water at least 15 minutes.
   b) For skin contact with chemicals, wash thoroughly with soap and water.
   c) Seek medical attention first, if necessary.
   d) Contact the Chemical Hygiene Officer at EH&S during business hours at 575-5448 (after hours, call UAPD at 575-2222) if spill is:
      1. Greater than 1 gallon
      2. Very Toxic
      3. Poses a Fire Hazard
      4. If You Need Assistance
      5. Laboratory spill kits should include: Absorbent materials, PPE, Clean-up materials (dust pan, scoop, plastic bags, etc.)

Emergency Procedures

Major Spill:

- Involves the release of a type or quantity of a chemical that poses an immediate risk to health or
- Involves a fire hazard / explosion risk

- Involves a highly dangerous chemical
- Involves unknown or highly reactive chemical(s)
- Involves a large quantity of chemical (generally over 1 liter of liquid or 1 kg of solid material).

1) Immediately activate the nearest fire alarm and evacuate the building.
2) Call 911 and provide details of the accident including:
3) location.
4) class of hazardous materials involved.
5) size of spill.
6) description of any personal injury.
7) control measures already taken.
8) Your name and phone number.
9) how you can be identified when emergency personnel arrive at the scene.
10) Call Environmental Health and Safety - 575-5448 (During Work Hours) 575-2222 (After Work Hours).
11) If the accident involves personal injury or chemical contamination, follow the above steps as appropriate, and at the same time:
12) Move the victim from the immediate area of fire, explosion, or spill (if this can be done without further injury to the victim or you).
13) Locate nearest emergency eyewash or safety shower.
14) Remove any contaminated clothing from the victim and flush all areas of the body contacted by chemicals with copious amounts of water for 15 minutes.
15) Administer first aid as appropriate and seek medical attention.

Minor Spill:

- Release of a chemical that does not pose an immediate risk to health and does not involve chemical contamination to the body:

1) Notify Environmental Health and Safety - 575-5448 (During Work Hours) 575-2222 (After Work Hours).
2) Notify lab personnel and neighbors of the accident.
3) Isolate the area, closing doors and evacuating the immediate area as necessary.
4) Remove ignition sources and unplug nearby electrical equipment.
5) Establish exhaust ventilation, if possible, by turning on fume hoods.
6) Locate the spill kit.
7) Choose appropriate personal protective equipment (goggles, face shield, impervious gloves, lab coat, apron, etc.). Note: All lab personnel MUST be properly fit tested before using a respirator. Contact EH&S (575-5448) for more information.
8) Confine and contain the spill by covering or surrounding it with appropriate absorbent material.
9) Neutralize acid and base spills prior to cleanup.
10) Sweep solid material into a plastic dust pan and place in a sealed 5 gallon container.
11) Wet mop spill area. Be sure to decontaminate broom, dustpan, etc.
12) Put all contaminated items (gloves, clothing, etc.) into a sealed 5 gallon container or plastic bag.
13) Request an EH&S Hazardous Waste Pickup.

Call 911 if the spill:

- Involves chemical(s) that poses an immediate risk to life or health
- Involves very toxic material
- Poses a fire hazard / explosion risk
- Is a large quantity of material, generally greater than 1 liter or 1 kg.

Spills that require special procedures:

- Acid Chlorides:
    - Use Oil-Dri, Zorb-All, or dry sand.
    - Avoid water and avoid sodium bicarbonate.
- Alkali Metals (lithium, sodium, magnesium, potassium):
    - Cover with contents from a Class "D" fire extinguisher.
    - Avoid contact with water.
- White or Yellow Phosphorus:
    - Blanket with wet sand or wet absorbent.
- Bromine:
    - Neutralize the spill with a 5% solution of sodium thiosulfate and
    - Absorb with inert absorbent material.
- Hydrofluoric Acid:
    - Contain using an HF compatable spill pillow or inert absorbent material.
    - Neutralize with calcium carbonate (limestone) or calcium oxide (lime). DO NOT use sodium bicarbonate!
- Mercury:
    - Use aspirator bulb or suction device to collect mercury beads (Do not use a vacuum cleaner).
    - Mop up with mercury decontaminating powder.
- Label waste with a Hazardous Chemical Waste Tag and request an EH&S Hazardous Waste Pickup.

Reportable Quantities:

1) The Reportable Quantity (RQ) of a spilled hazardous material is one (1) pound for many chemicals.
2) The university is legally required to report certain spills to the U.S. Environmental Protection Agency and to the Arkansas Department of Environmental Quality within twenty-four (24) hours of the spill occurance.
3) In order for the university to comply with this requirement, you must make sure that EH&S (575-5448) is notified immediately of any hazardous material spill over one (1) pound.
4) There can be legal consequences for failure to report a spill involving a Reportable Quantity of hazardous material.

Laboratory Safety OSHA Laboratory Standard

OSHA's Occupational Exposure to Hazardous Chemicals in Laboratories standard (29 CFR 1910.1450), referred to as the Laboratory standard, covers laboratories where chemical manipulation generally involves small amounts of a limited variety of chemicals. This standard applies to all hazardous chemicals meeting the definition of "laboratory use" and having the

potential for worker exposure.

Hazardous chemicals present physical and/or health threats to workers in clinical, industrial, and academic laboratories. Hazardous laboratory chemicals include cancer-causing agents (carcinogens), toxins that may affect the liver, kidney, or nervous system, irritants, corrosives, and sensitizers, as well as agents that act on the blood system or damage the lungs, skin, eyes, or mucous membranes. OSHA rules limit all industry exposures to approximately 400 substances.

Elements of the Laboratory Standard

1) This standard applies to employers engaged in laboratory use of hazardous chemicals.
   a) The scope of the Formaldehyde standard (29 CFR 1910.1048) is not affected in most cases by the Laboratory standard. The Laboratory standard specifically does not apply to formaldehyde use in histology, pathology, and human or animal anatomy laboratories; however, if formaldehyde is used in other types of laboratories which are covered by the Laboratory standard, the employer must comply with 29 CFR 1910.1450.
2) "Laboratory" means a facility where the "laboratory use of hazardous chemicals" occurs. It is a workplace where relatively small quantities of hazardous chemicals are used on a non-production basis.
3) "Laboratory use of hazardous chemicals" means handling or use of such chemicals in which all of the following conditions are met:
   a) Chemical manipulations are carried out on a "laboratory scale" (i.e., work with substances in which the containers used for reactions, transfers, and other handling of substances is designed to be easily handled by one person);
   b) Multiple chemical procedures or chemicals are used;
   c) The procedures involved are not part of a production process, nor do they in any way simulate a production process; and
   d) "Protective laboratory practices and equipment" are available and in common use to minimize the potential for worker exposure to hazardous chemicals.
4) Any hazardous chemical use which does not meet this definition is regulated under other standards. This includes other hazardous chemical use within a laboratory. For instance:
   a) Chemicals used in building maintenance of a laboratory are not covered under the Laboratory standard.
   b) The production of a chemical for commercial sale, even in small quantities, is not covered by the Laboratory standard.
   c) Quality control testing of a product is not covered under the Laboratory standard.
5) If the Laboratory standard applies, employers must develop a Chemical Hygiene Plan (CHP). A CHP is the laboratory's program which ad- dresses all aspects of the Laboratory standard.
   a) The employer is required to develop and carry out the provisions of a written CHP.
   b) A CHP must address virtually every aspect of the procurement, storage, handling, and disposal of chemicals in use in a facility.
6) Primary elements of a CHP include the following:
   a) Minimizing exposure to chemicals by establishing standard operating procedures, requirements for personal protective equipment, engineering controls (e.g., chemical fume hoods, air handlers, etc.) and waste disposal procedures.
   b) For some chemicals, the work environment must be monitored for levels that require action or medical attention.
   c) Procedures to obtain free medical care for work-related exposures must be stated.

d) The means to administer the plan must be specified.
e) Responsible persons must be designated for procurement and handling of Material Safety Data Sheets, organizing training sessions, monitoring employee work practices, and annual revision of the CHP.

7) The following OSHA Interpretations of the Laboratory standard provide additional information:
   a) Labeling Requirements under the HAZCOM and Laboratory standards; use of safe needle devices. (2001, January 11). Available at: http://www.osha.gov/pls/oshaweb/owadisp.show_document?p_table=INTERPRETATIONS&p_id=23781. Also, for labeling information, refer to the Laboratory Safety QuickCard.
   b) Coverage of various types of laboratories by the Laboratory standard. (1991, February 8). Available at: http://www.osha.gov/pls/oshaweb/owadisp.show_document?p_table=INTERPRETATIONS&p_id=20190 .
   c) The Laboratory standard does not apply to a pharmacy operation mixing cytotoxic drugs. (1990, June 22). Available at: http://www.osha.gov/pls/oshaweb/owadisp.show_document?p_table=INTERPRETATIONS&p_id=20025.
   d) OSHA's Safety and Health Topics Page entitled Laboratories, provides more detailed information about the Laboratory standard and is available at: http://www.osha.gov/SLTC/laboratories/index.html.

Laboratory Safety Chemical Hygiene Plan (CHP)

OSHA's Occupational Exposure to Hazardous Chemicals in Laboratories standard (29 CFR 1910.1450), referred to as the Laboratory standard, specifies the mandatory requirements of a Chemical Hygiene Plan (CHP) to protect laboratory workers from harm due to hazardous chemicals. The CHP is a written program stating the policies, procedures and responsibilities that protect workers from the health hazards associ- ated with the hazardous chemicals used in that particular workplace.

Required CHP Elements

1) Standard operating procedures relevant to safe- ty and health considerations for each activity involving the use of hazardous chemicals.
2) Criteria that the employer will use to determine and implement control measures to reduce exposure to hazardous materials [i.e., engineering controls, the use of personal protective equipment (PPE), and hygiene practices] with particular attention given to selecting control measures for extremely hazardous materials.
3) A requirement to ensure that fume hoods and other protective equipment are functioning properly and identify the specific measures the employer will take to ensure proper and adequate performance of such equipment.
4) Information to be provided to lab personnel working with hazardous substances include:
   a) The contents of the Laboratory standard and its appendices.
   b) The location and availability of the employer's CHP.
   c) The permissible exposure limits (PELs) for OSHA regulated substances or recommend- ed exposure limits for other hazardous chemicals where there is no applicable OSHA standard.
   d) The signs and symptoms associated with exposures to hazardous chemicals used in the

laboratory.

e) The location and availability of known reference materials on the hazards, safe handling, storage and disposal of hazardous chemicals found in the laboratory including, but not limited to, the Material Safety Data Sheets received from the chemical supplier.

5) The circumstances under which a particular laboratory operation, procedure or activity requires prior approval from the employer or the employer's designee before being implemented.
6) Designation of personnel responsible for imple- menting the CHP, including the assignment of a Chemical Hygiene Officer and, if appropriate, establishment of a Chemical Hygiene Committee.
7) Provisions for additional worker protection for work with particularly hazardous substances. These include "select carcinogens," reproduc- tive toxins and substances that have a high degree of acute toxicity. Specific consideration must be given to the following provisions and shall be included where appropriate:
   a) Establishment of a designated area.
   b) Use of containment devices such as fume hoods or glove boxes.
   c) Procedures for safe removal of contaminated waste.
   d) Decontamination procedures.
8) The employer must review and evaluate the effectiveness of the CHP at least annually and update it as necessary.

Worker Training Must Include:

1) Methods and observations that may be used to detect the presence or release of a hazardous chemical (such as monitoring conducted by the employer, continuous monitoring devices, visual appearance or odor of hazardous chemicals when being released, etc.).
2) The physical and health hazards of chemicals in the work area.
3) The measures workers can take to protect themselves from these hazards, including spe- cific procedures the employer has implemented to protect workers from exposure to hazardous chemicals, such as appropriate work practices, emergency procedures, and personal protective equipment to be used.
4) The applicable details of the employer's written CHP.

Medical Exams and Consultation.

The employer must provide all personnel who work with hazardous chemicals an opportunity to receive medical attention, including any follow-up examinations which the examining physician determines to be necessary, under the following circumstances:

1) Whenever a worker develops signs or symptoms associated with a hazardous chemical to which the worker may have been exposed in the laboratory, the worker must be provided an opportunity to receive an appropriate medical examination.
2) Where exposure monitoring reveals an expo- sure level routinely above the action level (or in the absence of an action level, the PEL) for an OSHA regulated substance for which there are exposure monitoring and medical surveillance requirements, medical surveillance must be established for the affected worker(s) as prescribed by the particular standard.
3) Whenever an event takes place in the work area such as a spill, leak, explosion or other occurrence resulting in the likelihood of a hazardous exposure, the affected worker(s) must be provided an opportunity for a medical consultation to determine the need for a medical examination.
4) All medical examinations and consultations must be performed by or under the direct supervision of a licensed physician and be provided without cost to the worker, without loss of

pay and at a reasonable time and place.

Using a Micropipettor

Using a micropipettor properly is essential to molecular biology. Most solutions are measured and dispensed using these marvels of invention. The micropipettor is an expensive unit costing usually between $150 and $250. Disposable autoclavable tips are fitted onto the end of the barrel for handling liquids. Just as these devices are essential they are also a great source of problems. Inaccuracy from abuse or misuse can lead to gross errors and lost experiments. They can be easily contaminated if you don't pay attention or don't use them properly. Watch what you are doing and be careful. Most of the major mistakes that people make when learning molecular biology are in the proper use and care of micropipettors. So read this section very carefully and pay heed.

1) Use the appropriate micropipettor. Usually these come in different size ranges: 0.5 to 10 µl (P10), 2 to 20 µl (P20), 10 to 50 µl (P50), 20 to 200 µl (P200), and 200 to 1000 µl (P1000). Use the smallest size range that will dispense the needed volume. Never use a P1000 to dispense 50 µl, that volume is for a P50 or P200 depending on which you have. The P1000 would be very inaccurate for this volume and your accuracy would be highest using the P50.
2) Accuracy is affected by the speed with which the `plunger' or actuator is released. Depress the actuator, place the tip of the pipet tip just into the solution and then slowly allow the actuator to raise.
3) If you allow the actuator to `snap' up the amount of solution drawn into the tip will likely be inaccurate and additionally you will create aerosols within the tip which will contaminate the pipettor barrel.
4) To expel the solution either place the tip into the accepting solution or onto the wall of the receiving container. Gently depress the actuator. If the solution you are delivering is viscous (as for enzyme solutions in 50% glycerol) you should rinse the tip by gently drawing solution back into the tip and then repeat the expelling process.
5) Accuracy in pipetting is very important. Learn what volumes look right in the pipet tip and tube. Most micropipettors have an accuracy of only ± 5 to 10% but measurments less than about 2 µl are much more inaccurate. Carefully observe how much volume is apparent in the pipet tip and judge whether it looks right.
6) To minimize contamination possibilities **all tips and pipets used MUST be sterilized to degrade contaminating biopolymers such as proteins and nucleases and the sources of these nasty biopolymers, micro-organisms.** Keep tip boxes and pipet cans closed when not in use. Never have a tip or pipet out for more than seconds before you use it.
7) The barrel of the micro-pipettor is NOT sterile and can be a major source of contamination. Do not touch the inside of a solution container with the barrel or tip ejector. Instead, tip the solution bottle so the solution is near the lip for easier access.
8) **Never allow the plunger to snap up as this will create aerosols in the tip that will get sucked into the barrel of the pipettor and contaminate it. With a tip of solution never bring the pipettor to horizontal as the solution will run into the barrel.** If you contaminate a pipet barrel the pipettor needs to be disassembled and cleaned thoroughly.
9) Check the accuracy of your micropipettor. Most of the time you can tell by observation whether the micropipettor is delivering the correct volume. For accuracy checks the easiest method is to use a balance that measures to an accuracy of 1 milligram.
    a) Start with the highest acceptable volume of the pipettor, a clean tip, and a beaker of H_2O.
    b) Using your best pipetting technique carefully pipet the water onto the pan and then take the reading.

c) Since 1 µl = 1 mg at room temp you can compute how close the dispensed weight agrees with the volume set on the pipettor.

d) Don't expect the readings to be in absolute agreement since most measuring devices have an accuracy of only about ± 5%. Thus, for a setting of 200 µl on a 20 to 200 µl micropipettor the weight delivered should be between 190 and 210. 1000 µl pipettors are usually less accurate. The smaller the volume the more the inaccuracy. For example, a setting of 1 µl on a 0.5 to 10 µl micropipettor usually delivers somewhere between 0.5 and 2 µl.

e) If the readings don't agree very well, repeat the check several times to determine whether it is consistently off or variable.

f) Next adjust down to a lower setting (about 1/4th of max. setting) and repeat your accuracy check.

g) If both high and low settings are off by the same amount then the pipettor is out of adjustment. Adjustment involves loosening the upper screws holding the volume adjustment knob and turning the central shaft. **NOT RECOMMENDED FOR THE TECHNICALLY CHALLENGED OR THOSE WITH NO PATIENCE**. If you don't have a skilled expert around then consider sending the pipettor in to one of the repair companies.

h) Variable readings usually result from operator error or from failing seals inside the micropipettor.

10) Learn how to disassemble and reassemble your micropipettors. Most manufacturers provide an exploded diagram of the pipettor and most follow a general structure.

a) Failure to keep your pipettors clean will result in a fouled barrel, gummed up seals, or even a ruined plunger assembly that can cost as much as one hundred dollars to replace. Not to mention the ruined experiments from contamination or poor measurement.

b) Routine cleaning of the pipet barrel can reduce contamination of experiments and solutions. A simple technique is to rinse down the pipet barrel before starting each experiment

1. Hold the pipettor(s) upright with the plunger button up and the pipet tip barrel down
2. Over a sink, rinse the pipet tip barrel with a stream of dilute (~10%) bleach in a squirt bottle.
3. Wipe off the excess liquid with a clean paper towel and be certain to wide the bottom of the pipettor (bottom opening on the end of the pipet tip barrel).
4. Poor pipetting technique or working with bacterial solutions can lead to contamination of the pipet tip barrel and internal contamination of the pipet tip barrel. This will lead to contamination of your stocks, and reagents. This usually results in bands in your negative control PCRs!

Handling Solutions

1) Treat all solutions with great care when handling. Most solutions are steam sterilized. Contamination of solutions with micro-organisms, ions, chemicals, or nucleic acids is the easiest way to ruin your experiments. If you have any reason to question a solution or reagent it is best to **throw it out** and make a new one rather than lose your precious time from a bad reagent. Check solutions for precipitates, cloudiness or other signs of microbial contaminations.

2) Accuracy in pipetting is very important. Learn what volumes look right in the pipet tip and tube. Most micropipettors have an accuracy of only ± 5 to 10% but measurments less than about 2 µl are much more inaccurate. Carefully observe how much volume is apparent in the pipet tip and judge whether it looks right.

3) To minimize contamination possibilities **all tips and pipets used MUST be sterilized to degrade contaminating biopolymers such as proteins and nucleases and the sources of these nasty biopolymers, micro-organisms.** Keep tip boxes and pipet cans closed when not in use. Never have a tip or pipet out for more than seconds before you use it.

4) The barrel of the micro-pipettor is NOT sterile and can be a major source of contamination. Do not touch the inside of a solution container with the barrel or tip ejector. Instead, tip the solution bottle so the solution is near the lip for easier access.

5) One of the best ways to minimize contamination of stock solutions is to first dispense solutions into small "working" solutions in sterile slip cap tubes or microfuge tubes. These are easier to take aliquots from and then you throw away the unused portion. This is especially true for microbiological reagents such as L broth, $CaCl_2$ for making competent cells, or the 80% glycerol solution for making frozen culture stocks. These solutions should NEVER be pipetted from as this represents too great a risk of contamination with molds or bacteria which can grow in the refrigerated stock. For these solutions pour more than what you need into a sterile container, dispense by pipetting from this "working" stock and then throw the remainder away. Of course you can't be this wasteful with very expensive solutions like radioisotopes and most enzymes. Know what the costs of various solutions and stocks are.

6) **Never return unused solutions or chemicals to the bottle**, discard the excess. Never `double-dip' (using the same tip or pipet for another aliquot of the solution). If you need to dispense multiple samples of a solution, pour out what you need into a sterile tube, dispense your samples and then discard the excess.

7) Because molecular biology involves the detection of minute quantities of nucleic acids or proteins be very careful when preparing large quantities of plasmids or other nucleic acids so that other solutions do not become contaminated with your sample. Nucleic acids or proteins are often prepared in µg (10^{-6} g) or even mg (10^{-3} g) quantities. Southern blots detect nucleic acids in the pg (10^{-12} g) range and PCR can amplify contaminating nucleic acids when only a few molecules are present. There are numerous instances in the literature where whole publications have had to be retracted because contaminants led to erroneous conclusions. MAKE SURE THIS DOESN'T HAPPEN TO YOU.

8) **Never take the lid off of a bottle and set it down**. Unscrew the lid, lift it off and keep it in your hand, remove what you need and replace the lid immediately. Never set the lid down or invert the lid (bottle side facing up). Setting the lid on the counter will contaminate the lip of the lid and thus contaminating the lip of the bottle when you replace it. Inverting the lid will allow debris and microorganisms in the lab air to settle into the lid. When you replace the lid the contents of the lid will fall into the bottle.

Hazardous Chemicals

1) **Organic solvents**- (also see the section on **Safety**, above) common hazardous chemicals are phenol and $CHCl_3$. Phenol can cause severe burns and $CHCl_3$ is a carcinogen. Protect your eyes, clean up spills immediately, and rinse contaminated skin quickly with soap and water. All wastes should be collected in a well-marked suitable container (glass is best). For disposal contact your institutional hazardous waste office for pickup and disposal.

2) Carcinogens- ethidium bromide is often used to detect nucleic acids in gels. Handle

solutions and gels with gloved hands and clean all contaminated chambers and work areas by rinsing well with water when through. Disposal of ethidium bromide wastes is a common problem, no truly effective method has been developed so consult your hazardous waste officer on the recommended procedure in your area.

Radioactivity

Many of the procedures in molecular biology require use of radioisotopes, although a number of colorimetric, fluorescent, or chemiluminescent methods have recently been introduced.

1) Handling of isotopes requires certification, contact your institutional radiation officer about certification courses, and radiation monitors (badges and meters).
2) Use extreme care in use and disposal of isotopes. Always check equipment, your hands, and work area after radiation use. Mark spill areas or contaminated equipment IMMEDIATELY and notify your supervisor or co-workers. Don't coverup, NOTIFY. Mistakes and accidents do occur. Two wrongs don't make a right.
3) Always be ready for the worst, anticipate spills and know what you need to do when one occurs. Tubes and closures will leak, use positive seals such as screw-cap tubes with o-ring seals when possible.
4) Make sure that people around you know you are using isotopes. Work over spill paper and mark waste containers CLEARLY. Keep wastes for different isotopes separate, disposal procedures differ for different isotopes. Consult with your supervisor or radiation office for the appropriate methods for disposal.
5) Biological molecules are sensitive to ionizing radiations. Do not allow labeled material to sit dry as the molecules will be subject to radiolysis. Re-solvation will minimize this degradation.

Handling Enzymes

1) Most enzymes are very unstable. Most are supplied in 50% glycerol (prevents freezing) containing specific buffers for storage at -20^{o}C. The major cause of loss of activity is improper temperature of storage or repeated warming and cooling. To avoid warming the enzyme never remove it from the freezer (or -20^{O}C freezer tray) and never handle the bottom of the tube with your warm little fingers. Remember all the cautions about handling solutions, they apply doubly to expensive enzymes.
2) Enzymes have optimal buffer concentrations for storage and for activity (which are often not the same). Optima for salt, ions and temperature are empirically determined but often can be flexible. Many enzymes will work well under a variety of conditions while others are quite restricted. Improper conditions can lead to inactivity or aberrant behavior.
3) Aberrant behavior for restriction enzymes is called `star' activity. Star activity usually means a lack or loss of specificity in restriction site recognition. Primary causes of star activity are:
   A) inappropriate ions (Mn^{+2} instead of Mg^{+2} or K^{+} instead of Na^{+})
   B) glycerol concentrations greater than 5%
   C) ethanol concentrations over 5%.
4) Most enzymes have a restricted temperature range of activity. Below that temperature they work inefficiently and above that temperature they may denature. The Klenow fragment of DNA Polymerase I *works* over a broad range of temperatures (25 to 50^{o}C) but this is a multifunctional enzyme and particular enzymatic activities are favored at different

temperatures. Most restriction enzymes work best at 37ºC but there are a few that must be used at 30ºC or even at 65ºC. **Know your enzymes**.

5) The best guidance on the flexibility of buffer and digestion conditions is the appendices of the catalog for the enzyme supplier. **Before you use an enzyme read the manufacturer's guidelines**. You will also benefit from reading the appendices of catalogs from suppliers such as Gibco-BRL, New England Biolabs, and Promega Corp. There is a wealth of information there that can save you time, and lost experiments.

6) Most enzymes that act on DNA require Mg^{+2} ions from 1 to 10 mM. Most of these enzyme activities can be inhibited by sequestering (chelating) the Mg^{+2}. This is usually achieved with EDTA which chelates up to 4 molecules of Mg^{+2} per molecule of EDTA.

7) Most DNAs are stored in buffered EDTA solutions. If too much EDTA is added as part of the DNA to a reaction then insufficient Mg^{+2} may remain for enzyme activity.
   A) If you add 5 µl of a TE solution into a final reaction volume of 10 µl containing 2 mM $MgCl_2$ (the optimum Mg^{+2} concentration for the enzyme) then the reaction will probably not work. Since the final EDTA concentration is 0.5 mM this will chelate approximately 2 mM equivalents of Mg^{+2} leaving little or no Mg^{+2} to act as a cofactor for the enzyme.
   B) To rectify this problem you can make up for the chelated Mg^{+2} by addition of $MgCl_2$ to bring the final `working' concentration back to the optimum.

Handling DNA and RNA

Storage

1) Nucleic acids (DNA and RNA) should be stored in a low salt solution (10-20 mM total salts) buffered to a near neutral pH in the presence of EDTA. Typically, this would be in the form of TE (10 mM TrisCl pH 7.5, 1 mM EDTA) for large quantities.
   A) EDTA is typically included in DNA storage solutions to chelate (bind up) any residual divalent cations. This is particularly true for Mg^{+2} which is a necessary cofactor for most polymerases and nucleases. Therefore, if there is no free Mg^{+2} (because the EDTA has chelated it) then nucleases can not “chew up” your DNA or RNA.
   B) However, too much carry over EDTA in enzyme reactions can inhibit a restriction endonuclease
   C) Each molecule of EDTA can bind up to 4 divalent cations so 1 mM EDTA can bind up 4 mM Mg^{+2}. Typically, polymerases or endonucleases have Mg^{+2} optima of 1 to 5 mM, provided in the reaction buffer. Therefore, never add so much of a TE solution of DNA solution to bind up all the Mg^{+2} and block the reaction.
   D) For the reasons above, we recommend that for “clean” DNA preparations such as genomic or plasmid DNAs purified by some of the protocols in this manual, or for dilute DNAs plasmid preparations you should reduce the amount of EDTA in your buffer and use something we call **Te (10 mM TrisCl pH 7.5, 0.1 mM EDTA)**. If you use Te for your pure nucleic acid storage and dilution solution this will reduce carryover of EDTA into enzyme reactions while still maintaining a level of protection against trace contamination with divalent cations.

2) DNA (and RNA) is most stable when stored at -20ºC (or -70ºC) in 66% EtOH as a salt precipitate.
   A) Working stocks of **plasmids** and small (<50 kbp) DNAs should be stored frozen at -20ºC and can be thawed and frozen many times with little degradation.

B) Purified **high molecular weight DNAs** (>50 kbp) should never be frozen as the shear forces of ice crystallization can break these longer molecules.

C) **RNAs** are much more labile and should only be frozen and thawed a few times before they have degraded considerably. If you purify RNAs you should aliquot them and store them long term as ethanol precipitates. Shorter term storage is fine at -20ºC, but remember, after a few freeze-thaw cycles it will be of much lower quality.

3) **Do not over purify or over handle RNAs**. For RT-PCR or cDNA library synthesis you should purify only the minimum (as described in the manual).

pH and Ions

1) The double stranded structure of DNA is held together by hydrogen bonds and electrostatic-hydrophobic interactions. These bonds are disrupted either when the pH rises above around pH 9 or the concentration of ions drops to 0. Both of these effects are accentuated by heat.
2) Most double stranded DNAs of >100 bp will be stable at neutral pH and 10 mM salt to around 45-50ºC. Higher temperatures will require higher salt concentrations to stabilize the DNA.
3) Acid pH (pH ≤2.0) can lead to depurination (electrophilic attack of the purine ribose bond) and eventually to cleavage of the phosphate-sugar backbone. Most DNA strands, although denatured, are not broken by alkaline pH even at temperatures of 65ºC or even 80ºC (unless they have been depurinated).
4) **RNAs** are extremely labile at alkaline pH, just a few minutes at room temperature at >pH 9 or 9.5 can lead to strand scission and fragmentation of the RNA (this is a fundamental aspect of RNA removal in Alkaline-lysis isolation of plasmid DNAs).

Alcohol Precipitation

1) DNA and RNA are weak acids due to the negatively charged phosphates in their backbone. Salts of nucleic acids are insoluble in cold alcohols. The degree of INsolubility is proportional to the length of the nucleic acid and influenced by the cations present. The least soluble salts are those with mono-valent cations (H^+, K^+, Na^+, NH_4^+, Li^+) but the pH of the solution will obviously affect the degree to which the phosphate charges (check the pKa of phosphates) have coordinated with a cation.
2) When recovering nucleic acids by alcohol precipitation one MUST add a sufficient amount of monovalent cations (generally around 300 mM) and lower the pH (the closer to pH 5 the better). The higher the pH the more monovalent cations you will need to drive the nucleic acid towards its non-ionized form.
3) **Addition of monovalent cations must be done prior to addition of the alcohol** otherwise the nucleic acid will not be in salt (uncharged) form and thus will not precipitate (the negative charges of the phosphates will repel each other).
4) **Which Monovalent Cation?** There is some disagreement in the community as to the efficacy of different cations for precipitation, the most common selections are NaOAc (OAc=acetate) and NH_4OAc. OAc^- is preferred because it is a volatile ion. Na^+ is preferred because most enzymes prefer Na ions over K^+ (with some exceptions) but the advantage of NH_4 is that this ion, like the OAc, is volatile. Recommendations for precipitations are either addition of NaOAc to 300 mM or NH_4OAc to 2.5 M. Stocks are usually 3 M NaOAc pH 5 to 5.3 (add 0.1 volumes to DNA solution), and 10 M NH_4OAc (add 1/4 to 1/3 volume to DNA solution).

A) There have been those that suggest that DNA precipitation with NH_4OAc will help to "clean up" a dirty DNA. However there has been no definitive proof of this concept. Generally, you are just as well off using NaOAc and making sure that you do an adequate 70% EtOH rinse to remove the residual salts, before drying and redissolving the DNA. There is one exception during plasmid preparations where large RNAs can be selectively precipitated with cold 2.5 M NH_4OAc.

5) **Which Alcohol?** Although many will work the most popular are those miscible with water: such as isopropanol and ethanol. There has been some debate as to the efficiencies of each for precipitation of large vs. small nucleic acids.

A) Isopropanol is used at a final concentration of 50%; add 1 volume of 100% isopropyl alcohol to 1 volume of DNA solution.

a) Generally, isopropanol precipitations are done at room temperature. Use of lower temperatures may cause some salts to precipitate from the 50% isopropanol

b) Isopropanol precipitations take very little time (a few minutes at room temperature).

c) After centrifugation to pellet the DNA then the pellet and inner walls of the tube are rinsed with cold 70% ethanol. Then the pellet is dried *in vacuo*.

d) Isopropanol precipitations are usually used for quick precipitations where contamination of the pellet with small oligos and salts are not a concern.

B) Ethanol (EtOH) is used at a final concentration of 66 to 70%; which means you add 2.5 to 3 volumes of 95% EtOH to 1 volume of DNA solution. You can use higher final concentrations of EtOH (4-5 volumes of 95% EtOH) to speed up the precipitation.

a) EtOH precipitations are 4 ºC or -20 ºC.

b) For longer length nucleic acids (vs. short nucleic acid fragments) less time is required to precipitate and the more readily they will precipitate in lower alcohol concentrations. By longer we mean >1000 bp and short means when you get down under 500 bp.

c) Everything is relative, though. The concentration of the DNA in the solution you are precipitating is also critical as 1 ng/µl solutions precipitate much less efficiently than 1 µg/µl solutions.

d) After the DNA is pelleted by centrifugation the tube is drained and then the pellet and inner walls of the tube are rinsed with cold 70% EtOH. Then dry *in vacuo*

6) **Which Temperature?** EtOH precipitation is usually performed by incubation at -20ºC for 30 minutes to one hour, while isopropanol precipitation is generally performed at room temperature.

A) There is some evidence that precipitations actually proceed faster at 0ºC (ice water) so that incubation for 15 to 30 minutes in ice water may be the fastest to precipitate even small nucleic acids

B) Some typical contaminating salts will be less soluble in isopropanol than ethanol, or -20ºC vs 0ºC) so be careful. As a practice, if you have the room (in your container) and time then use EtOH to perform your precipitation. JUST REMEMBER TO ADD MONOVALENT CATIONS AND MIX BEFORE ADDING THE ALCOHOL OR THERE WILL BE LITTLE OR NO RECOVERY OF NUCLEIC ACIDS.

7) **Centrifugation- How fast for how long?** Nucleic acid precipitates are generally collected by centrifugation (>8000 x g) at 4ºC for 5-10 min. The time and speed are proportional to

the quantity (concentration) of the nucleic acids and the length of the tube.

A) For microfuges it is best to use 13000 to 15000 rpm for 5-10 min. Generally, larger (longer) nucleic acids will sediment much faster than shorter nucleic acids.

B) For preparative centrifuges (15 to 40 ml tubes) 10-15 minutes at 10,000 rpm should be sufficient. However, large volumes (400 ml) may require 20 minutes at 8-9000 rpm.

8) **The Rinse** After pelleting the nucleic acid, the supernate is decanted (poured) off and then the pellet and tube are rinsed by addition of cold 70% EtOH to rinse away excess salts and aqueous contaminants.

A) If the pellet is small or invisible you should re-spin the tube with the 70% rinse solution in it for a few minutes to make sure the pellet has not been dislodged by the addition of the 70% rinse.

B) The rinse solution is decanted off, the tube is usually inverted on a paper towel or tissue for a few minutes to drain and then placed in a vacuum to remove all traces of alcohol.

C) Finally, new aqueous buffer is added to redissolve the pellet.

9) **Minute Samples** One of the biggest problems for the beginner is dealing with precipitation of samples that are invisible. Many procedures involve precipitation of sub microgram quantities of nucleic acids. A milligram is barely a small fleck or crystal but we often deal with precipitating 1/1000 or 1/10000 of this amount. During early stages of DNA purification you often get very visible precipitates and sizable pellets but these pellets are mostly contaminating proteins, or RNA. Once these contaminants are removed then the pellet becomes invisible. When you reach this point in your procedure you must rely on your confidence in your technique that the pellet is really there. For these small samples there are several things to recommend:

A) Remember to add sufficient monovalent cation before the alcohol.

B) Use a conical bottom tube if possible.

C) If you have room in the tube add 4 or 5 volumes of ethanol rather than the minimum 2.5 volumes.

D) Mix the alcohol into the aqueous sample by several inversions to make sure of thorough mixing.

E) Incubate the precipitation at -20ºC for >30 minutes, or freeze at -70ºC, or 30 minutes in an ice water bath.

F) Spin the tube(s) in a horizontal rotor or swinging bucket rotor to make a single pellet in the very bottom of the tube. Angle rotors tend to form a pellet that is `smeared' along one side of the tube. If you must use a angle rotor then be sure and note the orientation of the tube so that you know which side of the tube has the pellet smeared on it. Be sure this same side is on the outside when you spin the rinse. Otherwise you will be spinning the pellet off of the tube during the rinse.

G) Rules of thumb I like to use are

a) For microfuge tubes put the hinge tab for the lid to the outside so that the pellet is smeared along that same side.

b) For bigger tubes put the center of the writing on the tube label to the outside so that the pellet is on the side right below the name of the sample.

H) Spin for 15 to 20 minutes at 9 to 12000 x g.

I) Remove and decant the tube IMMEDIATELY when the rotor stops. If you let the tube sit there the pellet will tend to dislodge.

J) Add the cold 70% ethanol rinse by gently letting the solution run down the side of the tube so as not to disturb or dislodge the pellet.

K) Don't let the rinse sit around, put the tube back in the centrifuge and spin again for a few minutes. This makes sure the pellet is still stuck down and moves all salt-bearing solutions from the walls down into the rinse solution. Remember that for angle rotors you need to put the same tube face to the outside of the rotor during the rinse spin.

L) Again, remove and decant the tube right when the rotor stops.

M) After decanting you should invert the tube on a paper towel to drain for a second or two and then make sure to touch the entire tube lip (and lid) to the paper towel to blot off most of the residual rinse solution. This will mean less salt carry over and less residual solution to evaporate during drying.

N) Use a speedvac or other centrifugal evaporation system to dry the sample. Be sure to start the rotor spinning before opening the chamber to the vacuum. Centrifugal force will be all that keeps the sample in the tube.

O) For most centrifugal evaporators with only a few `well decanted' samples they should be dry in 4-5 minutes. Be sure to release the chamber vacuum before stopping the centrifuge rotor, otherwise the surge of air into the chamber will blow your sample out of its tube.

P) NEVER invert the dried tube. Cap it and rack it until ready to add the next aqueous solution. True story: *I watched as another person in the lab, two days into a DNA isolation, removed a microfuge tube from a centrifugal evaporator, inverted it to look for the pellet, and I saw a small fleck (the entire DNA sample) drift out of the tube and flutter gracefully to disappear amongst the flecks on the floor.*

Q) When you do rehydrate your sample add the solution, flick it down to the bottom, thip the tube to rinse the walls and dislodge the pellet, incubate at 37ºC for 5 to 10 minutes with occasional thipping of the tube to distribute the sample. Most small quantities will be completely redissolved in 5 to 10 minutes at 37ºC. Only large quantities (>100 µg), high molecular weight DNA or those with contaminating denatured proteins should or will take longer. Usually, for those samples, you can see the pellet and visual inspection can tell you when the pellet is completely dispersed and dissolved.

USEFUL NUMBERS & CONVERSIONS

Metric prefixes

Mega	M	10^{6}
kilo	k	10^{3}
milli	m	10^{-3}
micro	µ	10^{-6}
nano	n	10^{-9}
pico	p	10^{-12}
femto	f	10^{-15}
atto	a	10^{-18}

Isotopes

1 µCi = 2.2 x 10^{6} dpm = 37 kBq (for ^{32}P: 1 dpm=1 cpm)
1 Bq = 1 dps = 2.7 x 10^{-5} µCi Bequerel (Bq)
1 Gray (Gy) = 100 rad
1 Sievert (Sv) = 100 rem

Halflives:

β-emitters	^{32}P	14.29 days	^{35}S	87.44 days
	^{14}C	5730 years	^{3}H	12.43 years
	^{33}P	25.5 days		
γ-emitters	^{125}I	60 days	^{131}I	8.06 days

label	sp. act. Ci/mmole	µCi per µl	pmole per µl	% molecules labeled
^{32}P	800	10	12.5	10.7
^{32}P	3000	10	3.33	40.2
^{32}P	6000	100	16.6	80
^{35}S	1500	12.5	8.3	69

DNA Conversion Factors

1 bp = 660 g/mole
1 µg DNA = 1.515 nmoles bp
(nmoles bp)/(bp per molecule) = nmoles of DNA molecule
1 µg plasmid DNA= [1.515/(bp per plasmid)] nmoles of plasmid

Genome Sizes

Human	3 x 10^{9} bp
Yeast	2 x 10^{7} bp
E. coli	3 x 10^{6} bp

DNA Molecular Weight Markers

λ·*Hind*III- 23150, 9420, 6560, 4380, 2320, 2020, 560, 125
λ·*Bam*HI- 16840, 7230, 6785, 6530, 5620, 5530
λ·*Eco*RI- 21240,7420, 5810, 5650, 4880, 3540
λ·HBE (*Hind*III,*Bam*HI,*Eco*RI)- 15721, 5505+3530, 5505, 3775, 3530, 2752, 2564, 2409,

2396, 2027, 1584, 1375, 1120, 947, 831, 784, 564, 493, 125

M13·*Hpa*II- 1596, 829, 818, 652, 559, 545, 472, 454, 357, 205, 176, 156, 129, 123, 78, 60, 19, 18, 7

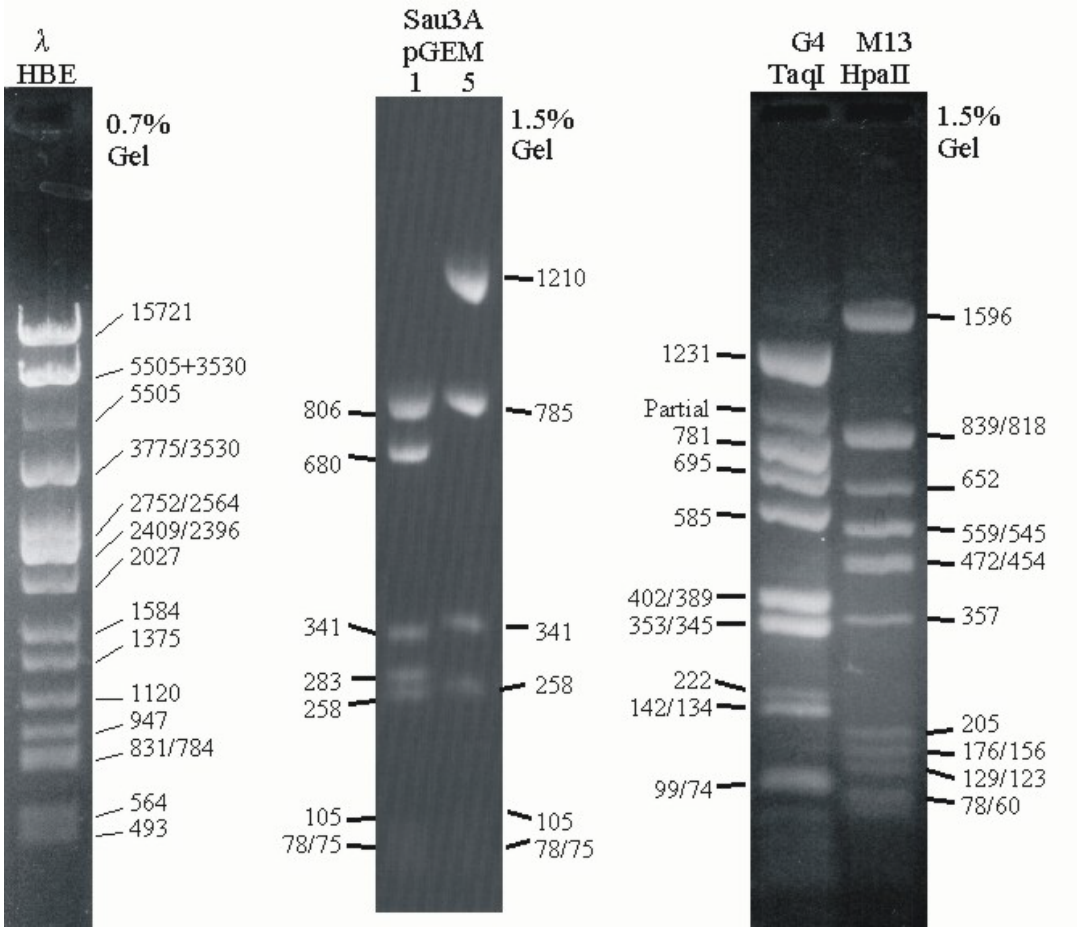
G4·*Taq* I- 1231, 781, 695, 585, 402, 389, 353, 345, 222, 142, 134, 99, 74, 53, 51, 21

pGEM1 Sau3A- 806, 680, 341, 283, 258, 105, 91, 78, 75, 46, 36, 18, 17, 12, 11, 8

pGEM5 Sau3A- 1210, 785, 341, 258, 105, 78, 75, 46, 36, 18, 17, 12, 11, 8

Figure 1. Representative DNA Size Markers for Agarose Gels

Representative gel pictures for λHBE, pGEM1 Sau3A, pGEM5 Sau3A, G4 TaqI, and M13 HpaI. For each gel image the agarose % and sizes of visible bands are indicated



Approximations for Chinese Hamster Cells (CHO & CHL)

Number of cells/gm wet weight: 6.2×10^{8} cells/g

Amount of DNA/cell	Nuclear	8.8×10^{-12} gm	8×10^{9} b.p.
	Non-nuclear	1.7×10^{-12} gm	
	TOTAL	9.5×10^{-12} gm	
Amount of Protein/cell:	Cytoplasmic	1.3×10^{-10} gm	(30,000 x g supernate)

	Nuclear	3.1×10^{-11} gm
	TOTAL	1.6×10^{-10} gm
Cells Per Confluent Culture:	25 cm^2 flask	5×10^6 cells
	75 cm^2 flask	1×10^7 cells
	150 cm^2 flask	2×10^7 cells
	roller bottle	1×10^8 cells

Approximations for Yeast Cells

1 ml packed cells = 10^{10} cells = 1 g wet weight
1 loopful of yeast cells = 10^6 cells
A 99 ml milk dilution of water where the turbidity is just visible is app. 10^6 cells/ml
A 15 mm glass tube where the turbidity is just visible is app. 10^7 cells/ml

Approximations for Bacterial Cells

1 ml packed cells = $10^{11 \text{ to } 12}$ cells = 1 g wet weight
A milk dilution of water where the turbidity is just visible is app. 10^7 cells/ml
A 15 mm glass tube where the turbidity is just visible is app. 10^8 cells/ml

STANDARD SOLUTIONS & REAGENTS

Gel Solutions

5xFicoll Loading Buffer 15% Ficoll MW 400,000, 20 mM Na_2EDTA and 0.1% each bromophenol blue and xylene cyanol (autoclave each component separately made up in H_2O 25% Ficoll, 250 mM Na_2EDTA, 2% bromophenol blue and 2% xylene cyanol then mix. Store at room temp.

20x TAE 800 mM Tris-Acetate pH 7.8, 40 mM EDTA

10x TBE 1 M Tris Borate pH 8.3, 20 mM EDTA
for 1 L- 121.1 g Tris, 7.45 g Na_2EDTA, ≈57.5 g Boric Acid, pH 8.3 with boric acid.
0.5xTBE= 50 mM Tris, 1 mM Na_2EDTA, ~25 mM Borate pH 8.3
To make 20x TAE or 10x TBE, add appropriate amount of Tris (free base) and Na_2EDTA then adjust pH with counter ion (HOAc or Boric Acid) then bring to volume.
1x TAE or 0.5x TBE with or without EthBr is made up in distilled H_2O.

20x TEB 2 M Tris 200 mM Borate 40 mM EDTA
To make 1L 20x TEB, dissolve 242 g Tris (free base) and 11.7 g EDTA (free acid, NOT disodium form) in <1 L H_2O, adjust pH to 9.2 with boric acid, then bring to 1 L. **NOTE**: use of Na_2EDTA will result in smearing and distortions in bands on sequence gels. Be sure to calibrate the pH meter before preparing this solution. If the pH is >9.2 the gels may not polymerize. **1x TEB**=100 mM Tris 10 mM Boric Acid 2 mM EDTA (pH 9.2)

Standard Solutions

$CHCl_3$-IAA 24 volumes of $CHCl_3$ with 1 volume of isoamyl alcohol, mix and store at room temp in a ground glass stoppered bottle or screw cap bottle with teflon lined cap. **Caution:** isoamyl alcohol fumes can be harmful to your lung epithelium and vapors will settle in your lungs leading to shortness of breath, suffocation or internal bleeding; work with pure isoamyl alcohol in a well ventilated area or in a fume hood.

Church Buffer 7% SDS, 1% BSA, 0.5 M $NaHPO_4$ pH 7.2, 1 mM Na_2EDTA. Dissolve 70 g SDS in 650 ml H_2O and autoclave. Dissolve 10 g fraction V BSA in 100 ml sterile H_2O. With all solutions at **ROOM TEMPERATURE** add 250 ml 2 M NaPB, 4 ml 250 mM EDTA, and the 100 ml 10% BSA solution to the SDS solution while stirring. Bring to volume and then incubate one hour at 65ºC. Store at room temperature. Heat to 65ºC and mix well before use. reference: Church & Gilbert (1984) PNAS 81:1991-1995

Denhardt's Solution, 50x 0.5 g Ficoll, 0.5 g Polyvinylpyrolidone for 100 ml (make up sterile in millipore filtered autoclaved H_2O)

DNA, Sonicated Salmon Sperm 5 mg/ml- (Dissolve in TE, Sonicate, Phenol extract 1-2 times, $CHCl_3$-IAA extract, EtOH ppt, dissolve in TE, and store frozen) just prior to use-boil 10' to denature

Dextran Sulfate, 50% made up in millipore autoclaved H_2O

DNAseI 10 mg/ml DNAseI Bovine Pancreas type II (Boehringer Manheim #104159) dissolved in 1x BRL React #2 enzyme buffer, or 10 mM Tris pH 7.5, 5 mM $MgCl_2$, 100 mM NaCl; aliquot to microfuge tubes, store frozen at -20ºC. May be frozen and thawed a

few times.

DTT 100 mM Dithiothreitol in H_2O. Dispense powder into pre-weighed sterile tube. Determine weight of powder. Add sterile ultrapure H_2O to generate 100 mM stock. Aliquote in microfuge tubes and store frozen at -20ºC. Concentrated stocks may be frozen and thawed multiple times but ONLY at room temp. Thawing at higher temperatures leads to oxidation of the DTT and loss of anti-oxidative properties.

NTP or dNTPs Nucleotides are purchased either as individual 100 mM stock solutions or purified lyophilized powders. Dilutions or rehydration should be in 10 mM TrisCl pH 7.5, 1 mM DTT. GTP and dGTP stocks above 10 mM should be made in 50 mM TrisCl pH 7.5, 1 mM DTT to protect against acidification. Working stocks are best at around 10 mM to protect concentrated stocks from repeated freeze-thaw cycles. The phospho-diester linkages in the triphosphate are subject to hydrolysis as well as the linkage between the sugar and base. Therefore, nucleotide solutions, especially those with low nucleotide concentration, should be thawed at room temperature and NOT at 37ºC.

ET 50 mM Na_2EDTA, 10 mM TrisCl pH 7.5

250 mM EDTA Na_2EDTA in H_2O pH adjusted to app. 7.5 with NaOH to dissolve. Autoclave and store at room temp.

GTE (Glucose-Tris-EDTA) 25 mM TrisCl pH 8.0, 50 mM Glucose, 10 mM EDTA. Mix 12.5 ml 1 M TrisCl pH 8.0, 9 ml autoclaved 50% glucose, 20 ml 250 mM EDTA, 458.5 ml sterile H_2O; store @ 4ºC

5 M KOAc pH ~5 294 g KOAc (3 M) in 885 ml H_2O, 115 ml Glacial HOAc; autoclave. pH should be $\leq$ 5.6; actual = 5 M OAc^-, 3 M K^+.

10 M NaOH <u>solvation of NaOH is highly exothermic so this must be done in a pan of ice water.</u> For 500 ml place a 1 liter glass beaker containing 250 ml 18 megaOhm purified water and a spin bar in a pan of ice water. With the spin bar spinning for rapid mixing, slowly add the NaOH pellets so they continue to move (may need to increase speed). DO NOT ADD THE PELLETS ALL AT ONCE. When the pellets have dissolved and the solution is cool enough, then transfer to a measuring cylinder and bring to volume. Transfer to a polypropylene bottle for storage. YOU CAN NOT STORE CONCENTRATED ($\geq$ 1M) NaOH in glass because it will etch the glass. DO NOT AUTOCLAVE NaOH solutions. 10 M NaOH is sterile and destroys organic molecules so just make dilutions with sterile H_2O.

150 mM NaOH 1% SDS 98 ml H_2O, 1.5 ml 10 M NaOH, 1 g SDS (**Make Fresh on day of use**). Do not add concentrated NaOH to SDS as this forms an insoluble precipitate

NIB (Nuclear Isolation Buffer) 30 mM TrisCl pH 7.5, 100 mM NaCl, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.5% Nonidet P40. Made by dilution of sterile stocks and stored at 4ºC.

2 M NaPB pH 7.0 mix 500 ml 2 M Na_2HPO_4 and app. 149 ml 2 M NaH_2PO_4 (pH adjusted to 7.0 by amount of NaH_2PO_4)

PBS 150 mM NaCl, 10 mM $KHPO_4$ pH 7.2. The $KHPO_4$ is a mixture of KH_2PO_4 and K_2HPO_4 to make pH 7.2.

Phenol(See Organic Extractions in Introduction) Molecular biology grade crystallized phenol- add about 1/10th volume of 0.5 M TrisCl pH7.5 and thaw with occasional mixing at 37ºC until liquified, mix the bottle well, allow to stratify into two layers, remove the top layer and discard. Add 1/10th to 1/5th volume of TE, mix well, aliquot at about 100 ml in bottles and store frozen at -20ºC. To use, thaw at room temp or 37ºC and pipet from the lower level only.

PMSF Caution Toxic if ingested, or breathed. (M.W.=174) 87 mg/ml in Isopropanol is 500 mM. Dispense powder into tared tube and then weigh. Add appropriate volume of isopropanol. Store at -20ºC. To use, place at 37ºC and swirl occasionally until crystals dissolve.

Pronase 10 mg/ml Pronase (Boehringer Manheim #165921; Sigma P5147) dissolved in

TNE; incubate 1 hour at 37ºC, aliquot to microfuge tubes, store frozen at -20ºC. May be frozen and thawed several times. Thaw at room temp. (Not 37ºC) and hold on ice.

ProteinaseK Dissolve at 5 mg/ml in TNE. Store frozen in microfuge tubes at -20ºC. May be frozen and thawed several times.

RIB and 2x RIB (RNA Isolation Buffer) 20 mM TrisCl pH 7.5, 700 mM NaCl, 20 mM Na_2EDTA. Made by dilution of sterile stocks and stored at 4ºC. 2xRIB is diluted and urea added for the day of use by dissolving 8.4 g Urea in 10 ml 2x RIB + 2 ml 10% SDS and bring volume to 20 ml with sterile H_2O.

RNA, Yeast Competitor Yeast RNA (extracted with phenol, then $CHCl_3$ and EtOH pptd) in TE at 100 µg/µl.

RNAseA 10 mg/ml RNAseA type IIA (Sigma R5000) dissolved in TNE; 10' in boiling water bath; bring to slow boil over flame, aliquot to microfuge tubes, store frozen at -20ºC. May be frozen and thawed many times times.

10% Sarkosyl Add 10 g sarkosyl (sodium lauroyl sarcosinate) to 80 ml dH_2O with mixing. Drop wise add 1 M NaOH until the solution clears. Bring volume to 100 ml and autoclave.

10% SDS BioRad, BRL or Fisher SDS (NOT Sigma)- 10 g per 100 ml H_2O then filter sterilize through 0.45 µM filter. If you autoclave it will often precipitate when stored at room temp (reason unknown). Note: Sigma SDS has given us problems in the past for DNA studies.

20x SSC 175.3 g NaCl, 88.2 g NaCitrate to 1 L pH7 (autoclaved). Final con. for 1x SSC = 150 mM NaCl, 15 mM NaCitrate. **NOTE**: the pH of dilute (>= 1x SSC) may change based on H_2O pH; often to maintain pH of app. 7.0-7.2 for 0.5x SSC you may need to make a 20x SSC stock at pH 6.4 (pH of dilution to 0.5x determined empirically).

20x SSPE 3.6 M NaCl, 0.2 M NaH_2PO_4, 20 mM EDTA pH 7.5

1x SM 10 mM TrisCl 7.5, 10 mM $MgSO_4$, 100 mM NaCl, autoclaved

10x SM 12.1 g TrisCl, 12 g Mg_2SO_4, 58.4 g NaCl, to 1 L, pH 7.5

Te 10 mM TrisCl pH 7.5, 0.1 mM EDTA

TE 10 mM TrisCl, 1 mM Na_2EDTA pH 7.5

TNE 10 mM TrisCl, 1 mM Na_2EDTA, 100 mM NaCl pH 7.5

Media for E.coli

L-broth 10 g Tryptone, 5 g yeast extract, 5 g NaCl, 1 L H_2O (Agar- 15 g/L)

L-agar plates L-broth + 15 g/L agar (need 10-15 ml /plate)

Top Agar L-broth + 0.7% Noble or Purified Agar

Top Agarose L-broth + 0.5% Agarose

10x Minimal Supplement 10.5 g K_2HPO_4, 4.5 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 0.5 g NaCitrate, 99.5 ml H_2O, autoclave. Add sterile: 1 ml (0.2 g) $MgSO_4$-$7H_2O$

Minimal Medium for 10 ml: mix sterile components (at 50ºC if for agar plates or room temp for broth) 1 ml 10x Supplement, 80 µl 50% Glucose, 4 µl 50 mg/ml Thiamine and 9 ml of either 1.67% Agar for plates or sterile H_2O for broth.

XGal-IPTG top agar Melt sterile top agar, cool to 45 to 50ºC. Pipet 3 ml into 13 x 100 mm slip cap tube. Add 10 µl 100 mM IPTG and 20 µl 5% XGal. Keep at 42-45ºC until use, use within 15-30 minutes.

Drugs and Supplements: Autoclave the L-broth/agar 15' on slow exhaust, cool to 50ºC in H_2O bath, add sterile supplements, swirl gently but thoroughly so complete mixing but NO bubbles, pour plates.

L-broth supplements (depending on culture):

0.1% glucose from autoclaved 50% glucose stock

0.1 mM thymidine

50 µg/ml thiamineHCl from 1000x stock = 50 mg/ml ThiamineHCl in H_2O filter sterilized and stored at 4ºC

15 µg/ml Tetracycline from 1000x stock = 15 mg/ml in 50% EtOH, filter sterilize. Note: when tetracycline solution starts showing signs of a precipitate you should THROW IT OUT. This breakdown product of Tet is toxic to Ca-shocked cells. Additionally, do not use Tet plates that are more than 4-7 days old.

100 µg/ml Ampicillin from 100x stock = 10 mg/ml in H_2O, filter sterilize. Amp may be frozen and thawed many times and Amp plates will last for several weeks at 4º

Chloramphenicol: 40 mg/ml in 95% EtOH Filter sterilize

100 mM IPTG: 23.8 mg Isopropyl-B-D-thiogalacto-pyranoside, 1 ml H_2O; (filter through sweeny adaptor or other 0.2 micron sterile filter) store -20ºC

5% X-Gal: 50 mg 5-bromo-4-chloro-3-indolyl-B-D-galactoside, 1 ml N,N-dimethyl-formamide; store in polypropylene or glass -20ºC

X-Gal plates: per 100 ml of sterile 50ºC L-agar add 80 µl 5% X-Gal and 40 to 80 µl 100 mM IPTG, gently mix (avoid bubbles) and pour immediately. Alternatively, individual plates may be spread with 20 µl 5% X-Gal and 40 µl 100 mM IPTG then inverted at 37ºC for 30 minutes prior to use (if the plates are several days old so they are fairly dry then first spot 100 µl L-broth then add the X-Gal and IPTG to the L-broth before spreading).

Media for Yeast

references: YPD- Busbee and Sarachek (1969) Arch. Mikrobiol. 64:289-314
CM & MM- Haught and Sarachek (1985) Mutation Research 152:15-23.
YES- Moreno, Klar and Nurse (1991) Methods in Enzymology 194:795-??

YPD 20 g Glucose, 10 g Yeast Extract, 20 g Peptone, 1 L H_2O

CM (Complete Defined Medium) 20 g Glucose, 1.7 g Yeast Nitrogen Base (w/o amino acids and $(NH_4)_2SO_4$) , 3 g Casamino Acids, 1 L H_2O, adjust to pH 6.5 with KOH

MM (Minimal Defined Medium) 20 g Glucose, 1.7 g Yeast Nitrogen Base (w/o amino acids and $(NH_4)_2SO_4$), 3 g $(NH_4)_2SO_4$, 1 L H_2O, adjust to pH 6.5 with KOH

YNB with Ammonium Sulfate may be substituted in MM at 6.7 g/L. Difco recommends that YNB be dissolved with glucose at 10x strength and filter sterilized (store at 4ºC until use). For most purposes we have found that all reagents can be dissolved, pHed and then autoclaved. The YNB will form a precipitate that will not interfere with growth.

5FOA Selective medium for Uracil requiring mutants. Supplement added to CM medium. Do not autoclave 5-FluoroOrotic Acid in this medium as the pH is altered during autoclaving. For 5FOA media dissolve the 5FOA, YNB, Uracil and glucose at 2x strength (heat to 50ºC to dissolve), adjust pH, filter sterilize. Autoclave agar and Casamino Acids (plus other supplements such as adenine if needed) at 2x strength. Cool (or heat) all to 50ºC. Mix and pour.

YES 5 g Glucose, 30 g Yeast Extract, 1L H_2O. **Use for *Schizzosaccharomyces pombe***

FFF For growing *C. albicans* as hyphae. 3 g Casamino Acids, 1.7 g Yeast Nitrogen Base (w/o amino acids and Ammonium Sulfate), 1 L H_2O, adjust pH to 6.8 to 6.85 with KOH (takes about 1 KOH chip for every liter of medium). Autoclave 20' at 118ºC on liquid cycle (will form a precipitate). To use: prewarm to 37ºC or 25ºC. Grow unbudded cells (see section on Yeasts), harvest cells and wash several times with sterile H_2O. Inoculate at 10^6 to 10^7 cells/ml. Incubate with shaking at 37ºC for germ tubes or 25ºC for budding.

FFFF Prepare FFF as above but include 5 g/L $(NH_4)_2SO_4$

Yeast medium supplements:

16 g/L agar

25 µg/ml Adenine Sulfate: from autoclaved pH adjusted stock stored at 4ºC

25 µg/ml Uracil: from autoclaved pH adjusted stock stored at 4ºC

GENERAL PROCEDURES

These procedures are of general utility in a variety of different procedures and will be used throughout the following chapters.

Quantification of nucleic acids

Most experiments depend on accurate quantitation of the particular nucleic acid, RNA or DNA, which you are manipulating or analyzing. Several methods exist with different levels of accuracy and sensitivity. In the past nucleic acid concentrations have been primarily determined by measuring the absorbance of the sample for UV light at 260 nm. Advantages are that most labs have a UV spectrophotometer and the approximate purity (contamination by phenol or protein) can be assessed by absorbance ratios such as 260/280 or 260/230. Disadvantages are that it is relatively insensitive and generally requires significant volumes (based on the quartz cuvettes used) and nucleic acid quantities (as much as several 100 mg). Alternatively, RNA and DNA can be quantitated by fluorescence using either ethidium bromide with UV light or Hoechst dye in 600 nm (blue) light. The advantage of fluorescence quantitation is sensitivity and thus the small quantities of nucleic acid sample required (ng vs. µg). Disadvantages are that both are more sensitive for double stranded than single stranded. Further, ethidium staining requires a calibration curve (with limited sensitivity range), while Hoechst fluorescence requires a fluorometer.

UV Spectrophotometry- best for quantifying oligos, single stranded RNA and DNA.

1) Allow the spectrophotometer and UV source lamp to warm up (>30 min.).
2) Determine the minimum volume which can be measured in your cuvettes (dependent on the height of the beam and cross section of the cuvette. Add that volume of 10 mM NaOH to the cuvette (include the reference cuvette if double beam spec). Wipe the faces of the cuvette(s) with a Kimwipe® to remove smudges or fingerprints and place in respective holders. Zero the machine. **Note**: some people use H_2O for these measurements but these determinations may be more sensitive to base composition and `double stranded-ness' of the sample nucleic acid. Therefore, NaOH is recommended.
3) Add some of your sample to the sample cuvette (usually 1/10th to 1/100th of the volume in the cuvette) and mix. This should be an amount which will give a significant reading (see below) and that you don't mind not getting back.
4) Read the absorbance value. Significant readings are those in the range between 0.05 and 0.5, determinations based on readings outside of this range are less precise.
5) Calculate the nucleic acid concentration in the original sample according to the formula.

DNA Concentration from A_{260}

$$\frac{\mu g}{ml} nucleic\ acid = \frac{50\frac{\mu g}{ml} * A_{260}\ reading}{dilution\ factor}$$

$$where: dilution\ factor = \frac{sample\ added}{total\ volume\ in\ cuvette}$$

Ethidium fluorescence- best if you have small quantities and no fluorometer

Two alternatives are suggested either by drops or by in gel staining.

Drops: a) Prepare a range of mixtures encompassing the probable concentration of your sample and an aliquot of your sample in buffer (0.5x TBE or TE work fine). All samples should be adjusted

to 0.5 µg/ml ethidium bromide.

b) Spot 5 or 10 µl aliquots on a piece of Parafilm® on a UV transilluminator.

c) Photograph at the appropriate exposure for your system.

d) Compare the calibration curve to your sample and estimate the approximate concentration of your original sample.

In Gel: a) Run an aliquot of your sample vs. a known amount of an appropriate size standard DNA in a gel containing 0.5 µg/ml ethidium bromide.

b) Photograph under UV light according to the requirements of your system.

c) Compare your sample intensity to that of the bands in the size standard to estimate the band with a similar fluorescence.

d) Calculate the percentage of the total amount of size standard DNA present as the size band of similar intensity. For example, if your sample is approximately equivalent to the signal from a 2 k.b.p band in a 250 ng aliquot of a Lambda DNA digest. Then your sample is 250 ng * (2 kbp/50 kbp)=10 ng.

Hoechst fluorescence- best for small quantities of DNA.

The method described is for a Hoefer™ minifluorometer TKO100 but should be adaptable to other instruments. This method is best for double stranded nucleic acids and has a linear response over a wide range. It is relatively insensitive to RNA and single stranded DNA where the sensitivity is about 1/10th that of double stranded. Thus, you can accurately determine the DNA concentration of a sample despite a significant contamination with RNA. However, if your sample is primarily RNA (such as in crude genomic DNA preps) then you will overestimate the amount of DNA. Hoechst 33258 binds to DNA and fluoresces (Excitation = 365 nm, Emission = 460) when intercalated into the hydrophobic, base stacking region of double stranded nucleic acids.

Requires: Hoechst dye solution, DNA standard (salmon DNA at 100 µg/ml), Hoefer mini-fluorometer and micropipettors

1) Concentrates of Hoechst dye #33258 (1 mg/ml in H_2O) are stable if stored at 4°C wrapped with aluminum foil (to protect from light). Prepare a fresh dilution of Hoechst concentrate at 100 ng/ml (1 µl of 1 mg/ml per 10ml of diluent) in 0.5x TBE or 10 mM TrisCl pH 7.5 1 mM EDTA 100 mM NaCl. Dilute solutions of Hoechst dye are only good for about 1-3 days if stored in an amber bottle (much less if exposed to more light).

Measuring using the Promega GloMaxJr

2) Make certain the UV module is in the machine, and then turn it on (switch on back).
3) There is no warm up time.
4) Select the UV module on the display.
5) Choose calibrate, and choose Run new calibration.
6) Select ng/ul for the units
7) The machine will prompt for the blank, so insert a clean 1cm square cuvette (mark the front top for maintaining the orientation), add 2 ml of the dilute Hoechst from step 1. Follow the onscreen prompts to zero the machine.
8) When prompted for the first standard enter the concentration of the DNA standard (e.g., 60) and then press OK.
9) Follow the onscreen prompts for the first standard. Then choose Proceed with Current Calibration.
10) You are now ready to add additional samples subtracting the prior reading or replace the buffer and start from zero.

GloMaxJr Module	Excitation (nm)	Emission (nm)
UV	365	410-450
Blue	460	515-570
Green	525	610-660
Red	625	660-720
GFPUV	365	515-570

Measuring using a TKO fluorometer

2) Allow the fluorometer to warm up for >30 min.
3) To Calibrate the fluorometer
   A) Dispense 2 ml dilute Hoechst from step 1 above into the cuvette and wipe off the outside with a Kimwipe to remove fingerprints and smudges.
   B) Place the cuvette in the sample chamber **with the orientation mark on the cuvette to the front**, close the lid and adjust the zero setting to zero.
   C) Remove the cuvette and add 2 µl of a control DNA solution of known concentration in the range between 100 and 500 ng/µl. Mix well by either: i) covering the top with Parafilm and inverting several times, or ii) agitating the solution by rapid side to side movement. Be careful to not touch the sides of the cuvette; you can touch the bottom of the cuvette.
   D) Return the cuvette to the chamber and adjust the **SCALE** knob so the display reads as the initial concentration of the control DNA sample.
   For example: if your control was 2 µl of a 100 ng/µl DNA solution then the sample in the cuvette would be 100 ng/ml. Adjust the display to read 100 (in ng/ml in the cuvette)
   E) Remove the cuvette, dump out the contents and rinse the inside of the cuvette with H_2O.
4) Measuring a sample:
   A) Add a fresh aliquot (2 ml) dilute Hoechst. Return cuvette to the chamber and adjust the zero reading if necessary (adjust ONLY the zero knob or you have to recalibrate the machine).
   B) Add 2 µl of your unknown DNA sample to the cuvette and mix as above.
   C) Return the cuvette to the sample chamber and read the concentration on the display which is the concentration in ng/ml in the cuvette and also the concentration in ng/µl in your original sample. No conversion is necessary.
   D) An alternative approach when you are reading multiple samples takes advantage of the linearity of the flourometer. Samples can be added successively to the same aliquot of Hoechst solution and reading recorded. Just remember to subtract the previous reading. For example if the machine is calibrated using a 100 ng/µl solution then an additional unknown sample is added to that same Hoechst solution and the machine now reads 150 then the unknown solution is 50 ng/µl (150-100). If a second unkown is added and the machine now reads 225 then the second unknown is 75 ng/µl (225-150). This approach minimizes the work effort but is not going to be as "exact" as the method where the Hoechst solution is replaced for every reading.

Notes on Fluorometric measurements

1) Readings from 5 to 1500 are generally very accurate. Less than 5 and the errors involved are very large. If your sample is dilute and you need to add more to get a reading then remember to divide the final reading by the number of µl of sample added to the cuvette

and then multiply by two.
For example: if you add 6 µl to the cuvette and obtain a reading of 24 then your sample is 8 ng/µl; (24 / 6) x 2.

2) Caution should be exercised that small bubbles or debris should not be floating in the diluted samples in the cuvette as these will give anomalous readings. **Check to make sure nothing is floating around in the mixed cuvette when you add your sample**.
3) All readings or adjustments should be made quickly as Hoechst fluorescence rapidly quenches. Particularly dirty samples may quench rapidly displaying a progressive decrease in readings beginning almost immediately. In such cases the most probable concentration is the initial reading.
4) To prepare a good standard you should use highly pure double stranded DNA. Prepare a dilute sample (app. 100-200 ng/µl) and quantify using the UV spectrophotometer. The standard should be stored at 4ºC and good for several months. Every so often you may wish to verify the quality and quantity of your DNA. Eventually it will go bad and you will get anomalous readings or will be unable to adust the scale setting.
5) When you are unable to adjust the machine for the standard you should perform the following steps in this order:
   A) Make sure that the lamp is on in the machine. You should be able to see the lamp through the air holes in the back.
   B) If you can't zero the machine or set to scale then wiggle the knob back and forth pressing in slightly, then rotate the knob rapidly back and forth. Watch the readings and see if they stabilize. This may indicate you need to have the knobs cleaned with tuner cleaner.
   C) Are you sure the cuvette is clean and there isn't `lint' floating in the Hoechst dye solution.
   D) Check another standard solution or known solution to see whether the machine can be set to scale with that solution
   E) Prepare a new batch of Hoechst dye stock and fresh dilution.
   F) Call in an expert.

Organic Extraction

Extraction with organic solvents is used to purify nucleic acid solutions through removal of contaminating proteins and detergents (SDS) or at least inactivate enzymes through protein denaturation. These solvents work by interacting with hydrophobic amino acid side groups and disrupting the tertiary structure (denaturing) of the protein. Denatured proteins and amphipathic detergents attempt to partition equally between the organic and aqueous solutions. Centrifugation then separates the non-miscible solutions into their respective phases and the denatured proteins and detergents move to the region between the solutions.

1) The two most popular organic solvents are phenol and chloroform-isoamyl alcohol ($CHCl_3$-IAA). Phenol is more effective at denaturing proteins for two reasons: proteins are partially soluble in phenol and phenol is partly miscible with water solutions. $CHCl_3$-IAA is far less miscible with water and primarily denatures proteins without truly extracting them from the solution.
2) Because phenol is miscible with water (and vice versa) aqueous solutions exposed to phenol will contain sizable quantities of phenol and the phenol can remove some of the aqueous volume. Phenol also intercalates into nucleic acids and can be precipitated along with the nucleic acid. The contaminating, carry-over phenol will inactivate enzymes and absorb UV light at 270 nm. Thus the phenol will inhibit subsequent restriction digestion,

polymerase treatment, or ligation, and obviate spectrophotometric quantitation.

3) $CHCl_3$ has an extremely low solubility in water but phenol is much more soluble in $CHCl_3$ than in water. Residual phenol is effectively removed by extraction with $CHCl_3$-IAA.

4) Therefore, all extractions incorporating phenol must be followed by extraction with $CHCl_3$-IAA to remove the phenol. The only exception is when there are no planned enzymatic treatments; in some plasmid preparation protocols the $CHCl_3$-IAA extraction is delayed until later.

5) Phenol can decompose with exposure to oxygen or light and is usually purchased as molecular biology grade crystallized phenol stored under nitrogen. It is stored away from light at -20ºC. To prepare for normal use (exceptions are for acidified phenol for RNA isolation):

A) Add about 1/10th volume of 0.5 M TrisCl pH7.5 and thaw with occasional mixing at 37ºC. The TrisCl will buffer the phenol and saturate the phenol with H_2O rendering it liquid at room temp. Pure phenol is a crystal at room temp and is clear. Any brown, pink or yellow phenol should be disposed of and not used for nucleic acids.

B) After the phenol is liquified, mix the bottle well, allow to stratify into two layers, remove the top layer and discard (see **Waste organic solvents**, below).

C) Add 1/10th to 1/5th volume of TE, mix well, aliquot at about 100 ml in bottles and store frozen at -20ºC.

D) To use, thaw at room temp or 37ºC and pipet from the lower level only, leave the upper aqueous layer in the bottle.

6) $CHCl_3$-IAA is prepared by mixing 24 volumes of $CHCl_3$ with 1 volume of isoamyl alcohol (**Caution: isoamyl alcohol fumes can be harmful to your lung epithelium and vapors will settle in your lungs leading to shortness of breath, suffocation or internal bleeding; work with pure isoamyl alcohol in a well-ventilated area or in a fume hood)**, mix and store at room temp in a ground glass stoppered bottle or screw cap bottle with teflon lined cap.

7) **Performing an organic extraction**

A) add an equal volume of organic solvent to the aqueous solution. Mix well by shaking, vortexing, or rapid inversions. Open top tubes can be covered with SaranWrap held in place by your thumb during the mixing. Do not use parafilm which is soluble in the organic solvent. It is usually a good idea to wear latex gloves as most snap cap tubes leak a little.

B) **Phenol causes severe skin burns and $CHCl_3$ is a mild carcinogen. Handle with extreme caution, keep away from your eyes, clean up spills, and rinse any skin spills with lots of COLD water. Do NOT use ethanol as this will only speed absorption into your skin and make the burn worse.**

C) If the extraction involves **radioactivity you must use a screw cap tube with an O-ring seal** to minimize tube leakage or you will contaminate the centrifuge during the spin.

D) After thorough mixing to form a slurry of the aqueous and organic solutions, spin at room temperature at high speed (>2000 rpm, 8000 rpm is better) for 5-10 minutes to separate the solutions. Large amounts of detergents or denatured proteins will form a white or yellow interface between the lower organic and upper aqueous solutions.

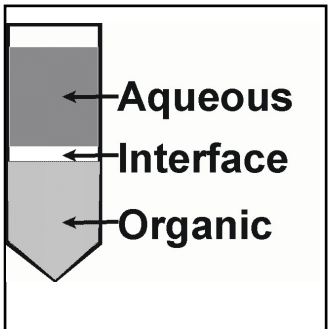


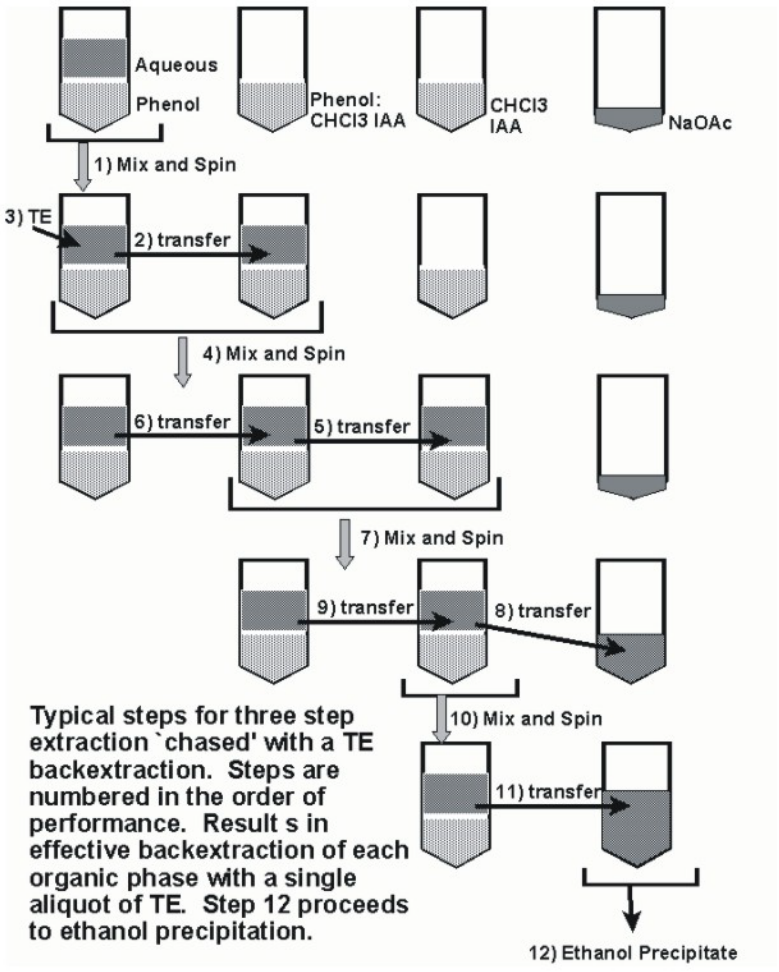
Figure 2. Extraction Phases

E) Be careful to not shake or jostle the tube to disturb the interface.

F) Carefully pipet the upper aqueous solution to a new clean tube. Start removing the aqueous layer at the upper surface and gradually lower the pipet tip as the solution level drops. Pipet slowly as the tip approaches the interface avoiding sucking up the interface as best as possible. If significant amounts of the aqueous solution remains in the original tube or if the interface is sizable, you may wish to `back-extract' the organic layer.

G) Back extraction uses 0.5 to 1 volume of TE added to the organic solvent, then repeat the mixing and spin. The back extraction is then pooled with the original aqueous solution (see figure). If the organic extraction employed phenol then remember that you need to do a $CHCl_3$-IAA extraction to extract residual phenol from the aqueous solution.

Figure 3. Back extraction flow chart



8) **Strategies for organic extraction** depending on the extent to which the nucleic acid is contaminated.

The most effective extraction is two to three successive phenol extractions followed by one or two extractions with $CHCl_3$-IAA. But this is time consuming. A less extensive extraction is successive extractions with phenol, then a 50:50 mixture of phenol:$CHCl_3$-IAA, and then a final $CHCl_3$-IAA extraction.

Minimal protein contaminants can be removed by extraction with 50:50 phenol:$CHCl_3$-IAA and then a $CHCl_3$-IAA extraction. If all you need to do is inactivate a restriction enzyme then you can probably get by with a single extraction with $CHCl_3$-IAA.

A) Protein removal can be accentuated by first adding small amounts of detergents such as SDS (add SDS to 0.1%, mix, and then perform extractions).

B) Any organic extractions should be followed by an alcohol precipitation. Extraction with $CHCl_3$-IAA leaves a small quantity of IAA in the aqueous solution that is removed by an alcohol precipitation.

9) **Extractions of high salt solutions** can present a special problem. If the aqueous phase is above app. 1 M salts then the density of the aqueous layer may be higher than the density of phenol (app. 1.2 g/ml) resulting in an inversion of the phases or failure to separate the two phases during the centrifugation. During extraction of high salt solutions be careful to make sure you are saving the correct phase before discarding the tube. If you are saving the wrong layer you will find that the solution you think is the aqueous phase doesn't form two phases when added to the next organic extraction solution. $CHCl_3$ (1.8 g/ml) is much denser than phenol, so addition of some $CHCl_3$-IAA will help to separate the phases.

Disposal of waste organic solvents

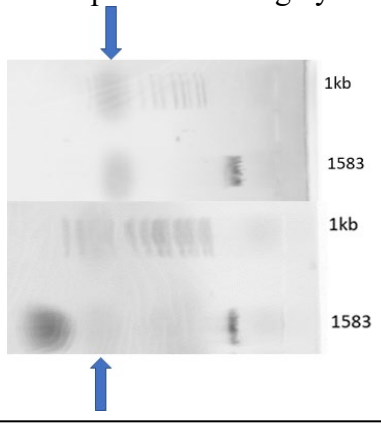
Used phenol and $CHCl_3$ should be collected in glass bottles. Mark the bottle **USED PHENOL/$CHCl_3$** (do not use the term WASTE until you are ready for immediate disposal as the word WASTE has an explicit meaning to hazardous waste personnel). When the bottle is full contact your hazardous waste office for pickup. If you are generating much used phenol then you may wish to keep used phenol in its own bottle as disposal of used phenol by burning is less expensive than disposal of phenol/$CHCl_3$ mixtures.

Cleanup of Genomic DNA using Nanosep Filtration

Nanosep spin filters (1.5ml can accept about 0.4ml) are available in different molecular weight cutoff (MWCO). The 100k MWCO, https://shop.pall.com/us/en/products/zidOD100C34, work well for final cleanup of high molecular weight genomic DNA to eliminate small contaminants. The filters have a maximum of 14k x g. For cleanup of bacterial genomic DNA before submission for library preparation for next-generation sequencing, we load 0.3 ml of extracted DNA and spun for 5 min. at 4 ºC at 8000 x g, in a swinging bucket rotor. Then discard the flow-through, then load 0.3 ml of Te to the top, and spin again. Then repeat the discard and add new Te. After the spin discard the flow-through and add Te to the top cup and put the tube vertically on a mixer at 500 rpm at room temp for 10 minutes to resuspend the DNA from 'trapped' on the filter membrane. An example of a contaminated DNA cleanup is in Figure 4. Pall makes a 50 ml version called the Macrosep which has a maximum of 5000 x g. Pall also has the 100k MWCO in 96 well format for cleanup of 96 DNAs at a time.

Figure 4. Cleanup of DNA by Nanosep spin filtration

Bottom is the gel of the dirty DNA and the top is a gel of the DNA post-Nanosep filtration. Blue arrows indicate the bromophenol tracking dye.



Purification using a G-50 Centrifuge Column

This simple and rapid procedure separates nucleic acids or proteins from small solutes such as free nucleotides and salts. It is a standard means for partial purification of labeled probes from unincorporated radioactivity.

1) mix approx. 300 mg dry Sephadex G50 and 5 ml TE in a glass tube and place in a boiling water bath for 10 min.

2) collect the sterile G50 in a centrifuge column. Equilibrate the column with TE by rinsing the column 1-2x with app. 4 ml aliquots of TE allowing rinses to drip through the column. Centrifuge the column in a 17x150 mm glass tube for 5' at 600 x g in a swinging bucket centrifuge to remove excess liquid.

3) trim off the lid from a 1.5 ml microfuge tube and place on a wadded Kimwipe pad in the bottom of a 17x150 tube. The column should just rest on top of the microfuge tube. Add the 200 µl sample to the column and spin as in step **2**.

4) rinse the column with an additional 200 µl of TE. 95 to 99% of the TCA precipitable counts will be in the void and first rinse which should be pooled.

Bradford method for protein quantification

Concentration of protein in samples is based on the Bradford method (Coomassie Blue binding) using a standard curve of BSA. This technique was developed by Dr. M. Bradford's observation that when Coomassie Brilliant Blue G250 binds to proteins in an acid solution its absorbance maximum shifts from 465 nm to 595 nm. The usefulness of this approach over the classic Lowry or Biuret methods is that the Bradford method works over a far greater protein concentration ranges and is not as sensitive to contaminating detergents or ions.

Requires: deionized H_2O, BSA standard solution (>140 µg/ml), clean test tubes (8 per group), dye reagent BioRad (2 ml per group), spectrophotometer (595 nm)

1) prior to use dilute 1 volume of the Dye reagent with 4 volumes of deionized water and filter through Whatman #1 filter paper. (diluted dye reagent is good for about 1-2 weeks)

2) Prepare a dilution series of the BSA (bovine serum albumin) standard in H_2O at 0, 20, 40, 80, and 140 µg/ml. Also dilute aliquots of the unknown at 1:10, 1:5 and 1:2

3) In a series of clean dry test tubes place 50 µl of either a standard or unknown. Add 2.5 ml of the diluted dye reagent, vortex (no foaming) and incubate for 5 min. to one hour.

4) Measure OD-595 for each sample. Plot the standards and use this standard curve to determine the protein concentration in your unknown. Remember to take into account your sample dilutions.

Microassay Bradford Protein Quantification.

Reference: Anal.Biochem. 72:248-254 (1976)

Reagent: Bio-Rad Protein Assay. (BIORAD cat. 500-0006)

1) Use a flatbottom 96-well plate. (Costar cat. 3596)

2) Make a standard curve of BSA from 0.1-3.0 µg of protein in a total of 20 µl sample buffer.

3) Make appropriate dilutions of samples in a total of 20 µl of sample buffer.

4) Make dilutions in different wells of the plate.

5) Add 200 µl of 1:5 diluted Bio-Rad Reagent in H_2O, diluted just before use.

6) Incubate 5' at RT, read out at 590 nm (Plate reader or EIA apparatus).

7) Draw standard curve and calculate protein concentration.

Advantages: Low reactivity with other chemicals, easy to use, low amount of sample usage.

Disadvantages: Variation with different proteins is larger than with Lowry, BSA is known to give a higher read out than actual amount, but can be used since most people are using this as a standard.

Quantification of protein by the Biorad DC assay

Using BioRad 500-0116 reagents

1) Mix 500 µl Reagent A with 10 µl Reagent S
2) Place 10 µl of each samples to measure and BSA standards (from 0 to 5 mg/ml) in clean 2.2 ml tubes
3) Add 50 µl of Reagent A+S mixture and vortex
4) Add 400 µl Reagent B and vortex
5) Incubate 15 minutes but less than one hour at room temp
6) Read A_{750}
7) Plot A_{750} of standards vs. Concentration. Use linear line to determine concentration of unknowns.
8) Not affected by SDS or detergent concentrations less than 1%. Best when standards are also in same buffer as unknowns.

Lowry Protein Quantification

Reference:: JBC 193: 265 (1951).

Reagent I : 50 ml 2% Na_2CO_3 in 0.1 M NaOH
0.5 ml 1% $CuSO_4 \cdot 5H_2O$
0.5 ml 2% NaK·Tartrate

Reagent II: 2.5 ml Folin reagent
2.5 ml H_2O

1) Prepare fresh mixtures for Reagent I and II just before use, use immediately and dispose of unused portions.
2) Samples and standard are diluted with H_2O to a total volume of 0.3 ml.
3) Add 1 ml reagent I and incubate 20' at 37ºC.
4) Add 0.1 ml of reagent II, mix well, and let stand 30' at RT to allow color to develop.
5) Spin if a precipitate forms, and read A_{750}. Plot protein standard concentrations versus absorbance and use this curve to determine the concentration in your unknown.

Suggestions:

- Make standard curve of BSA from 1-50 µg of total protein.
-High values can be read at 500 nm if standard values are available to be read at that value.
-Better accuracy is obtained by using a non-linear regression for the standard curve
-If your unknown is outside the range of readings for your standard curve you can NOT use the standards and must repeat the unknown using a more dilute or concentrated sample to bring the unknown reading into the range of the standards.

Disadvantages are: -reactivity with lots of chemical reagents such as: EDTA, Tris, Mg^{+2}, K^+ and others;
-time consuming and laborious as opposed to a Bradford quantitation.

Quantification of radioactivity by TCA precipitation

1) mix sample and 10 µl (100 µg) Yeast carrier RNA in 10x75 glass tube, add app. 0.5 ml cold

10% TCA, mix and incubate on ice 10 min. Some people add NaPyrophosphate (NaPPi) to their 10% TCA to bring to 10 mM to reduce background. This addition to the solution is not necessary unless you need exquisitely precise numbers.

2) fill half the tube with cold 5% TCA and filter through glass fiber filter (Whatman 934-AH)

3) repeat rinses with half full tubes 3x with cold 5% TCA and 1x with cold 10 mM HCl. Clamp off the vacuum release to suck most of the dampness from the filter

4) dry the filter completely 5' in an 80^{o} oven. Count filter in scintillation fluid. Use these counts to determine the cpm/µg, percentage incorporation, and total number of counts incorporated

Percent Incorporation

$$\%\ Inc. = \frac{100 * counts\ inc.}{\mu Ci\ added * 22x10^{6}\frac{cpm}{\mu Ci}}$$

ISOLATING EUKARYOTIC DNA

Preparation of Tissues or Cells

Blood

Most birds, fish, amphibians and reptiles have nucleated red blood cells so all cells should be processed. Mammals have non-nucleated blood so that only 1-10% of the cells (white blood cells) contain nuclei. Blood clots quickly upon isolation so collection is usually in the presence of anticoagulants. Vacutainers can be obtained for collection that contain dried anticoagulant. Alternatively the a collection syringe may be pre-loaded with anticoagulant. Anticoagulants include heparin, EDTA, and citrate:

Heparin should be added (usually in PBS) to a final concentration 10U/ml (about 0.1 mg/ml) and included in all cell washes.

Citrate solution is prepared as ACD: 0.48 g Citric Acid, 1.32 g NaCitrate, and 1.47 g Glucose in 100 ml H_2O. Add 1 ml ACD per 6 ml fresh blood. This blood can be stored at 4ºC for several days or frozen at -70ºC indefinitely before isolating DNA.

To process fresh blood several options exist: isolate nuclei, lysis of a cell pellet, direct lysis, or isolate buffy coat.

Options for frozen blood include lysis of a cell pellet or direct lysis. First try to pellet the cells, if no cells pellet then you only can use direct lysis.

Options for blood clots are only to do direct lysis.

Isolation of buffy coat cells-

This method usually works only on fresh blood treated with ACD and should only be used for blood from organisms with non-nucleated red blood cells. This method enriches the sample for the nucleated white blood cells. Spin at 1300 x g for 15 minutes at room temperature. Pipet off the yellow plasma supernate, do not disturb the pellet surface. The buffy coat should appear as a whitish layer on top of the cell pellet. If no buffy coat is evident then go to **Lysis of a blood cell pellet**. Use a pasteur pipet or 1 ml micropipettor to gently pipet off the buffy layer and transfer to a fresh tube. Pellet at 1300 to 2000 x g for 15 minutes. Resuspend the cell pellet in TE using 10 ml for every gram of cell pellet. Go to **DNA Purification step 1**.

Isolation of blood cell nuclei- pellet the cells and lyse with NIB as for suspension cells (see below). The nuclear pellet should be pelleted twice out of NIB to reduce clotting factors- resuspend nuclear pellet in fresh NIB solution (for this second pelleting use NIB made 120 mM NaCl; otherwise the nuclei will lyse), centrifuge again and resuspend in 10 ml TE per gram (1ml of cells is app. 1 gram of cells).

Lysis of a blood cell pellet- Spin the blood at 2000 x g 5'. Resuspend in cold PBS+ACD (6:1 v/v) and pellet again. Resuspend the pellet in TE using 10 ml of TE for every g of cell pellet. Go to **DNA Purification step 1**.

Direct lysis of blood cells- Add EDTA to 5 mM and SDS to 0.5%. Incubate at 65ºC for 15 minutes. Add pronase to 200 µg/ml and incubate at 37ºC for 4 hours. If there are signs of clotting add more pronase and continue the incubation for an additional 4 hours to overnight. Go to **DNA Purification step 5**. This DNA will be contaminated by RNA and

should be RNAse treated after extraction and precipitation.

Processing of clotted blood- If the blood clots do not despair, DNA can be recovered by successive digestion with pronase until the clot dissipates. This has worked with clotted cell pellets from blood, add TE+0.5%SDS+5 mM EDTA then add pronase to 200 µg/ml, incubate at 37ºC for 3-8 hrs then if necessary add more pronase and repeat the digestion, then go to **DNA Purification step 5**. This DNA will be contaminated by RNA and should be RNAse treated after extraction and precipitation.

Simplified preparation of DNA from blood

This DNA is suitable for RAPD, PCR or Southern analyses.

1) Blood is mixed with a hypotonic solution containing non-ionic detergent to lyse cell membranes but not nuclear membranes. The lysis solution is STM-Low (64 mM Sucrose, 20 mM TrisCl pH 7.5, 10 mM $MgCl_2$, 0.5% Triton X-100). For non-nucleated mammalian blood use equal volumes of blood and lysis solution. For nucleated blood such as avian blood use 1ml of lysis solution for every 0.1 ml of blood .
2) Gently but thoroughly mix the blood and lysis solution. Pellet nuclei at 6000 rpm (2000 x g) for 5 minutes at 4ºC. Decant and discard supernate.
3) For every ml of lysis solution used in step 1, resuspend the nuclear pellet by IMMMEDIATE trituration in the same volume (as used for STM) of 10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Triton X-100. To resuspend the pellet triturate with the same pipet or tip used to deliver the solution. If you do not triturate immediately the nuclei will start to lyse and form a clump that will never disperse. THE PELLET MUST BE COMPLETELY RESUSPENDED BEFORE GOING TO THE NEXT STEP. Failure to resuspend before adding the SDS will turn the pellet into a virtually insoluble lump.
4) Add 1/10th volume of a 50:50 mixture of 250 mM EDTA and 10% SDS. Mix well by inversion. Add Pronase to 200 µg/ml (Proteinase K or Pronase E can be used by Pronase is cheaper) (1/50th volume of 10 mg/ml stock). Incubate at 37ºC for 4 hours or overnight. If clots were formed during the lysis then additional pronase digestion may be necessary (after 4 hours if clots remain add more pronase and continue incubation at 37ºC).
5) Extract the aqueous solution twice with an equal volume of phenol:$CHCl_3$-IAA and then one time with $CHCl_3$-IAA
6) Add 1/10th volume of 3 M NaOAc, mix and add 2.5 volumes of cold 95% Ethanol. Mix by multiple inversions; mixing may take a while to occur because the DNA solution may be viscous.
7) DNA should precipitate as a "snot ball" that should float to the top and can be hooked out with a pipet tip. For extremely dilute samples you may have to pellet the DNA at 8-10000 rpm for 15 minutes at 4ºC. The precipitated DNA should be rinsed by immersion in cold 70% EtOH, then drained/decanted (remove as much liquid as possible and then dry *in vacuo*.
8) Redissolve the DNA in Te. Use 200 µl for every ml of nonnucleated blood or 6-800 µl of Te for every 100 µl of nucleated blood. Dispersal and solvation of the DNA pellet can be speeded by incubation at 37 to 50ºC for 30 minutes to an hour with occasional "thipping." Store concentrated solutions at 4ºC. Dilute solutions (<100 ng/µl) for PCR can be frozen.

Fast Isolation of DNA from blood for PCR

For rapid preparation of DNA samples suitable for PCR

1) Mix 1 - 10 µl avian blood with 50 volumes (50 µl/µl blood) of STM-Low (64 mM Sucrose, 20 mM TrisCl pH 7.5, 10 mM $MgCl_2$, 0.5% Triton X-100) on ice.
2) Pellet nuclei at 4-6000 xg for 5 minutes in the cold or room temp.
3) Resuspend the nuclear pellet in 50 volumes (50 µl/µl blood) of TEN (10 mM Tris-Cl pH 8.0, 1 mM EDTA, 10 mM NaCl. NOTE: pH is higher and salt lower for compatibility with Proteinase K and PCR.
4) Add proteinase K to 200 µg/ml and incubated at 65 ºC for 1 hour.
5) Heat in boiling water for 10 min.
6) Spin at 11,000 rpm for 5 min at room temp.
7) Collect supernate for use in PCR. One µl of this supernate can be used as template in every 10 µl PCR.
8) DNA samples prepared in this manner should be stored at -20ºC.

Storage of chicken blood for DNA isolation later

This protocol is based on the standard Anticoagulant Citrate Phosphate Dextrose Solution, USP (CPD) BLOOD-PACK™ Unit, with glycerol added so that the solution can be frozen and thawed several times. For the actual details on the STM-Low and TEN+pronase see **New and Improved method for fast isolations from fresh avian blood in 96-well format.**

1) Prepare SCDP20% (10% KH_2PO_4, 150 mM KCl, 11 mM NaCitrate, 2 mM Citric Acid, 2 mM NaH_2PO_4, 16 mM dextrose, 20% glycerol). Weigh out the dried ingredients and dissolve in 60% of the volume required of ultrapure H_2O, add the glycerol (be certain to back-rinse the graduated cylinder to get all the glycerol. Adjust pH to 7.2 with KOH, then bring to volume. Sterilize by filtration.
2) Aliquot 100 µl per well of a 0.5 ml assay block (assay blocks can be made up well in advance and stored with a silicone mat lid at 4 ºC. NOTE: actual volumes can be adjusted but always have at least 10x volume of SDCP20% for the amount of blood to be added.
3) Assay blocks should remain on ice during collection. The mat lid can be placed within a single fold towel to stay relatively clean.
4) Using a sterile lancet puncture a wing vein and use a micropipettor to collect 10 µl of blood. Triturate the blood (several pipettings) into the SCDP in a well. Record the wing band for that well in the collection sheet.
5) Assay blocks are stored frozen at -80 ºC. They are thawed in room temperature water. Then 50 µl is mixed by trituration several times into 200 µl of STM-Low on ice. Then incubated on ice for 10 minutes.
6) Centrifuge at 2-3000xg at 4 ºC for 10 minutes. Gently decant right after the centrifuge stops. It is not necessary to get rid of ALL of the supernate.
7) Resuspend by trituration (at least three times) into 200 µl TEN+pronase.
8) Incubate on an angle with shaking at 37 ºC for at least 2 hours or overnight
9) Quantification can be difficult because the solution will be viscous (requires mixing well with the Hoechst solution for fluorimetry.
10) General yields are 40-80 ng/µl.

New and Improved method for fast isolation <u>from frozen avian blood</u> in 96 well format

For rapid preparation of DNA samples suitable for PCR in 96 well format. For this method we use polypropylene assay blocks with 0.5 ml wells. Citation: Bailes, S., J. Devers, J. D. Kirby, and D. Rhoads. 2007. An inexpensive, simple protocol for DNA isolation from blood for high-throughput genotyping by polymerase chain reaction or restriction endonuclease digestion. Poult.

Sci. 86: 102-106.

1) Pipet 0.2 ml cold STM-Low (64 mM Sucrose, 20 mM TrisCl pH 7.5, 10 mM $MgCl_2$, 0.5% Triton X-100) into each well. The assay block should remain on ice while blood is added to all the wells
2) Add 2-3 µl avian blood.
   A) For frozen blood we have found it convenient to use sterile toothpicks.
   B) Allow the blood to partially thaw.
   C) Stick the toothpick about 3-5 mm into the blood and then stick the toothpick into the assay block well into the STM.
   D) After all wells have toothpicks added the toothpicks can be removed and thrown away. on ice.
3) Pellet nuclei at 1000 xg for 5 minutes in a centrifuge equipped to spin microplates.
4) Invert the microplate and dump off the supernate.
5) Use a multichannel pipettor to resuspend nuclear pellet by trituration into 200 µl TEN +pronase (10 mM Tris-Cl pH 8.0, 1 mM EDTA, 10 mM NaCl, 100 µg/ml Pronase)
6) Incubate the plate covered for 1 hour at 37ºC in a bacteriological incubator.
7) Transfer the plate to a 65ºC water bath or incubator 10 to 30 minutes to inactivate the pronase..
8) Samples are now ready for PCR screening and can be quantified by fluorometry.
9) For PCRs use 1 µl of this solution for every 10 µl PCR volume.
10) DNA samples prepared in this manner should be stored at -20ºC sealed with silicon mat lid.

New and Improved method for fast isolation <u>from fresh avian blood</u> in 96 well format

For rapid preparation of DNA samples suitable for PCR in 96 well format. For this method we use polypropylene assay blocks with 0.5 ml wells. Citation: Bailes, S., J. Devers, J. D. Kirby, and D. Rhoads. 2007. An inexpensive, simple protocol for DNA isolation from blood for high-throughput genotyping by polymerase chain reaction or restriction endonuclease digestion. Poult. Sci. 86: 102-106.

Note: this system has been very reliable except for one big experiment where we got NO significant DNA. We suspect that the blood set in STM-Low for too long before centrifugation. Therefore, we have developed an alternative whereby the blood is collected, and stored frozen in a citrate-glycerol solution (see Storage of chicken blood for DNA isolation later). Aliquots can then be processed for nuclear isolation by this method.

1) Pipet 0.4 ml cold STM-Low (64 mM Sucrose, 20 mM TrisCl pH 7.5, 10 mM $MgCl_2$, 0.5% Triton X-100) into each well of a 0.5 ml conical well assay block.
2) Assay blocks can be prepared days in advance, stored at 4 ºC, sealed with a silicone mat lid. The assay block should remain on ice while blood is added to all the wells
3) Record the bird wingband number on the collection sheet
4) Hold the bird on its back and remove downy feathers over a wing vein.
5) Use a 28-gauge lancet to puncture the wing vein
6) Collect 8-10 µl blood with a micropipettor.
7) Triturate the blood into the STM in the assay block well to distribute the blood and rinse out the pipet tip
8) After all wells have been loaded replace the mat lid. Assay blocks with blood added are good for several hours if kept on ice.
9) Prior to centrifugation invert the block several times to make certain the blood is well distributed in the wells

10) Pellet nuclei at 1-2000 xg for 5 minutes in a centrifuge equipped to spin microplates.
11) Remove the mat lid. Invert the microplate and gently "dump off" the supernate so that little liquid is carried over, but do not shake which will dislodge the white nuclear pellets
12) Use a multichannel pipettor to resuspend nuclear pellet by trituration into 200 µl TEN +pronase (10 mM Tris-Cl pH 8.0, 1 mM EDTA, 10 mM NaCl, 100 µg/ml Pronase) using 200 µl wide bore pipet tips.
13) After all wells have been resuspended add 200 µl more TEN pronase to each well.
14) Seal the block with the silicone mat lid
15) Incubate for at least 1 hour at 37ºC in a shaking bacteriological incubator with the assay block on a ~45 degree angle so that the solution mixes well in each well. If you don't angle the block then the debris can collect in the conical bottom and not get adequately digested by the pronase.
16) Transfer the plate to a 65ºC water bath for 10 minutes, or incubator for 30 minutes, to inactivate the pronase.
17) Samples are now ready for PCR screening and can be quantified by fluorimetry.
18) For PCRs use 1 µl of this solution for 1 to 3 replicate 20 µl PCRs.
19) DNA samples prepared in this manner should be stored at -20 ºC, sealed with a silicon mat lid.

Cleanup of DNAs from fast isolation in 96 well format for NGS library synthesis

This method has been validated for purification of DNAs from the New and Improved method for fast isolation from fresh avian blood. The DNAs then work well for NGS barcoded library synthesis using the iGenomX RipTide kit for Illumina sequencing.

1) Mix 100 µl of the crude DNA lysate with 100 µl TEN+Pronase (10 mM Tris-Cl pH 8.0, 1 mM EDTA, 10 mM NaCl, 100 µg/ml Pronase)
2) Incubate for at least 1 hour at 37ºC in a shaking bacteriological incubator with the assay block on a ~45 degree angle so that the solution mixes well in each well.
3) Transfer the plate to a 65ºC water bath for 10, or incubator for 30 minutes, to inactivate the pronase.
4) Setup a stack of filter plates on a 0.5ml assay waste collection block. The top plate is a 1.2 µm filter plate (Pall #8040), on top of a 100 MWCO filter plate (Pall #8036), on the assay block on the bottom. **NOTE:** when handling the filter plates do NOT touch the bottom surface. This will contaminate your samples.
5) Load the pronase treated DNA solutions into the corresponding wells.
6) Centrifuge the stack (use a Used stack as counter balance) at 1500 x g 10 min. at 5 ºC.
7) Dispose of the flow through in the assay block.
8) Add 200 µl of Te to the wells in the top plate, and repeat steps 6 and 7
9) Repeat the 200 µl Te rinse steps (steps 8 then 6 then 7)
10) Discard the top 1.2 µm filter plate
11) Add 100 µl of Te to the wells of the 100 MWCO filter plate, and cover
12) Place on a platform orbital shaker and "shake" for 10 min. at 500 rpm, to resuspend the DNA from the MWCO filter
13) Transfer the resuspended DNA to a labeled 96 well raised lip PCR plate,
14) Quantify the samples by Hoechst fluorescence.
15) Seal with a 96 well silicone mat lid and store frozen at -20 ºC.

Simple Isolation of DNA from Feathers for PCR analysis

Bailes, Devers, Kirby and Rhoads. Poultry Science (In Press)

1) Use freshly pulled quills with shafts of 1-2 mm that have visible pulp. Clip off about 3-5 mm of the quill point into a microfuge tube. You can use 1-2 quills.
2) Add 200 µl TEN+Pronase (10 mM Tris-Cl pH 8.0, 1 mM EDTA, 10 mM NaCl, 100 µg/ml Pronase) and incubate at 37ºC with shaking or mixing for 2-4 hours.
3) Inactivate proteases at 65ºC for 5 minutes. Pipet the solution to a fresh tube and store at -20ºC. Should yield 5-0-150ng/µl DNA.

Simple Isolation of DNA from Semen for PCR analysis

Bailes, Devers, Kirby and Rhoads. Poultry Science (In Press)

1) Triturate 10-20 µl fresh or frozen-thawed semen into 400 µl TEN+Pronase (10 mM Tris-Cl pH 8.0, 1 mM EDTA, 10 mM NaCl, 100 µg/ml Pronase) and incubate at 37ºC with shaking or mixing for 2-4 hours.
2) Inactivate proteases at 65ºC for 5 minutes.
3) This solution should be 150-400 ng/µl DNA and suitable for most PCR screenings.
4) To further clean up the DNA for other uses, add 400 µl CHCl3-IAA, mix well, spin.
5) Transfer the upper aqueous to a new tube containing 40 µl 3 M NaOAc, mix and then add 1 ml cold 95% EtOH. Invert to mix several times
6) Spin 5 minutes 10 krpm at either room temp or 4 ºC. Decant, add 0.5 ml cold 70% EtOH. Spin 1 minute, decant, and dry
7) Redissolve in 200 µl Te. Should yield 150-500 ng/µl DNA.
8) Store DNAs frozen at -20ºC.

Suspension cells or cultured cells

Adherent cultured cells may be collected by trypsinization or scraping. Cells should be pelleted at 2000 x g 5'; resuspended in cold PBS and pelleted, then either:

a) resuspended at 1 x 10^7 cells/ml (10 ml/g cells) in cold TE (10 mM TrisCl 1 mM EDTA pH 7.5)

or b) lysed with 10 ml cold NIB/g of cells (10 ml NIB/ml cells) and spun in cold 5000 x g 5', Decant and resuspend in TE 10 ml/g of starting cells.

go to DNA Purification step 1

Animal Tissues: Frozen cells or tissues can be used but the quality (size) of the DNA isolated will be lower (20-60 kbp vs. >100 kbp). Chill on ice, mince with sterile single-edge razor blade rinse with cold PBS. Then either:

a) Freeze in liquid N_2 and pulverize with lN_2 chilled mortar and pestle. Transfer powder to glass Dounce homogenizer and warm in ice water. Add 10 ml NIB per g starting tissue and homogenize with 5-10 strokes of a B-pestle. Transfer to cold centrifuge tube and pellet nuclei 5' 3000 x g 4ºC. Resuspend pellet in same volume of TE and proceed with SDS lysis (step 1 below), or

b) Add 5 ml TE per gram tissue and process with a `tissuemizer' homogenizer, go to step 1 of DNA purification. The homogenizer generator should be cleaned in between uses by successive immersion with motor running in beakers containing 300 ml of: i) soapy water, ii) running tap water, iii) 100 mM HCl, iv) 100 mM NaOH, v) running deionized H_2O, vi) rinse with 95% EtOH from squirt bottle; and then run motor briefly in air to dry.

Bacterial Cells: Should be washed with TE and then protoplasted with 1 mg/ml Lysozyme

in GTE then proceed to step 1.

Yeast Cells: For high molecular weight genomic DNA prepare protoplasts as described in chapter on **Yeast** then proceed to step 1. If crude, partially fragmented DNA is OK then use either **Smash and Grab** or **Microwave Isolation** (see below).

DNA Purification

1) Add 10% SDS to final concentration of 0.5%. Incubate with occasional inversions for 10' at 50°C.
2) Bring to 37°C and add boiled RNAse A to final concentration of 100 μg/ml. Incubate at 37°C for 30'.
3) Make 10 mM with EDTA, invert to mix and incubate 10' at 50°C.
4) Bring to 37°C, add pronase to final concentration of 100 μg/ml, incubate at 37°C for >8 hours.
5) Extract 3 times with 50:50 phenol:$CHCl_3$.

NOTE: Extractions should be gentle mixing of the two solutions for several minutes. Spin 1-2000 x g 5-10' room temp. Carefully pipet out and discard the lower organic layer.

6) Extract once with $CHCl_3$:IAA and transfer upper, aqueous layer to fresh tube.
7) Add 1/10th vol 3 M NaOAc and mix well. If the cation concentration is not high enough then the yield will be low as DNA needs sufficient cations to precipitate.
8) Add 2-3 vol cold 95% EtOH. Gently invert to slowly mix solutions and precipitate the DNA as a mass. Hook out the white string-like, mass of DNA wit-h a bent pasteur pipette. Rinse in cold 70% EtOH and then place the mass of DNA in a new tube and briefly dry under vacuum. Redissolve the DNA in TE. (re-solvation may require hours to days and can be a 4°C.
9) Determine the optical quality of your DNA (260/280 should be around 1.8 to 1.85) and determine the A_{260} concentration. If the DNA is not sufficiently concentrated repeat the EtOH precipitation or N-butanol can be used to reduce the volume followed by dialysis to remove the butanol.
10) Test the quality of your DNA by testing restrictability with an enzyme such as Hind III. Also run an uncut sample (100 ng) to see if there is significant RNA contamination.

Yields: Mammalian cells yield approximately 200 to 400 μg of DNA per 10^8 cells or 5 mg DNA/g of cells.

Plant Leaf Tissue

Taken from Bernetzky and Tanksley (1986). Theoretical and Applied Genetics 72:314-321. DNA prepared in this manner is generally suitable for Southern analyses, or PCR or RAPD reactions.

Solutions

Extraction Buffer: 100 mM Tris pH 8.0, 350 mM Sorbitol, 5 mM EDTA, 1% 2 mercaptoethanol. The solution without mercaptoethanol can made up, autoclaved, and stored at 4°C. Prior to use remove required amount and add 2 mercaptoethanol to 1%.

10% CTAB: hexadecyl trimethyl ammonium bromide (Sigma) is dissolved in H_2O and autoclaved. Store at room temp.

1) Use fresh leaf material. Young leaves are better than older leaves. Dice to fit into tube and determine weight of leaf material.
2) For every gram of leaf tissue add 6 ml of extraction buffer and homogenize on ice with hand-held homogenizer for several minutes. The homogenizer generator should be cleaned in between uses by successive immersion with motor running in beakers containing 300 ml of: i) soapy water, ii) running tap water, iii) 100 mM HCl, iv) 100 mM NaOH, v)

running deionized H_2O, vi) rinse with 95% EtOH from squirt bottle; and then run motor briefly in air to dry.

3) Pour the homogenate through a double layer of cheesecloth and collect in a centrifuge tube or bottle. Spin for 15' at 700 x g at 4ºC to pellet cells and nuclei, discard the supernate. Resuspend the pellet in Extraction Buffer using 1 ml for every gram of starting leaf material.
4) For every 10 ml of Extraction Buffer add 1 ml 10% CTAB, 2 ml 5 M NaCl, 1 ml 250 mM EDTA, and 1 ml 10% Sarkosyl. Final concentrations are approximately, 1% CTAB, 1 M NaCl, 25 mM EDTA and 1% Sarkosyl.
5) Heat at 60-65ºC for 20 minutes.
6) Extract 1 time with an equal volume of $CHCl_3$-IAA
7) Collect the aqueous phase is mixed with 2/3rd volume of isopropanol. Allowed to stand at room temp for 10 minutes and then the nucleic acids are collected by centrifugation for 15-20 minutes at >8000 x g at room temp. The pellet is rinsed with cold 70% ethanol and then vacuum dried.
8) Redissolve the pellet in a suitable volume (usually 500 µl) TE + 50 µg/ml boiled RNAse A. Incubate for 30-60 minutes at 37ºC.
9) Extract with 50:50 phenol:$CHCl_3$-IAA then with $CHCl_3$-IAA
10) Add 1/10th volume 3 M NaOAc and 2 volumes of 95% ethanol. Incubate in ice water for 15-30 minutes or an hour at -20ºC. Collect the DNA by centrifugation at high speed and rinse the pellet with cold 70% ethanol, dry and redissolve in 100 to 1000 µl Te depending on the quantity of starting material. Quantify.

Differential precipitation of Polysaccharides from DNA

DNA isolated from some tissues and organisms (plants, liver, some bacteria) may contain significant amounts of polysaccharides as these molecules have generally the same properties as the nucleic acid backbone. If your pellet is mostly a clear gelatinous pellet that redissolves very poorly then you may wish to try differential precipitation. According to S.D. Michaels, M.C. John, and R.M Amasino (1994), Removal of polysaccharides from plant DNA by ethanol precipitation, Biotechniques 17(2):274-276; the following protocol can reduce the contamination significantly.

1) Bring the solution to 0.3 M with NaCl.
2) Slowly, with mixing, add 0.35 volumes of cold ethanol. Good mixing is necessary to keep the local concentrations of ethanol from becoming high.
3) Incubate on ice for 15 minutes.
4) Spin 5 minutes at 10k x g in microfuge. Collect supernate. The clear pellet should be polysaccharides.
5) Add an equal volume of isopropanol to the supernate. Invert to mix and incubate 5 minutes at room temp.
6) Spin to pellet DNA or hook out the DNA.
7) Rinse with 70% EtOH. Dry and redissolve.

Determination of cellular DNA and Protein concentrations

For whole cells process as above through step 3. Skip any any pronase or protease treatment if you need to determine total protein concentration. The sample may need to be sheared to allow accurate sampling. To shear pass through a syringe several times. Measure the relative

concentration of DNA and protein in the sample by measuring the relative absorbance at 260 nm and 280 nm or use fluorometry to determine the DNA content and the Bradford method to measure protein content.

Microwave Isolation of Total Plant or Fungal DNA for PCR/RAPD Analysis

This procedure was developed by D. Goodwin and S. Lee (slee@goldng8.univnorthco.edu) at U of North Colorado. Reference: Biotechniques (1993) 15:438-444. Purportedly works with everything: bacteria, plants, fungi and protists.

Solutions:

Lysis Buffer- 50 mM TrisCl pH 7.2, 50 mM Na_2EDTA, 3% SDS, 1% β-Mercaptoethanol

Phenol-$CHCl_3$- 50% Phenol, 48% $CHCl_3$, 2% Isoamyl alcohol

1. Place 0.5 cm^2 of mycelium (fungi: 0.1-2 g wet weight or equivalent weight of other sample) in 1.5 ml microfuge tube containing 50-100 µl of lysis buffer.
2. With tube open and loosely covered with plastic wrap, microwave on high (400 W **check your microwave output**) for 15", then 10", and then 5". Total of 30" but NO BOILING.
3. Add 300-350 µl lysis buffer (vol=400 µl) and incubate 10' at 80ºC
4. Add 400 µl Phenol-$CHCl_3$, vortex and centrifuge 10' room temp 10k x g.
5. Remove aqueous to new tube, add NaOAc to 0.3 M and isopropanol to 54% (about 1.1 volumes), spin 2' at 15 krpm or 4' at 10k x g at room temp., decant and rinse pellet with cold 80% EtOH.
6. Resuspend in 50 to 150 µl Te (depending on experience)

YIELD: 0.1 to 10 µg/µl depending on source and starting quantity of tissue

DNA Extraction from Feathers

Adapted by Lowell Overton from M. Sorenson (Boston University) and Wenrui Duan (Ohio State)

1) Use feather pulp or feather tip (4-5 mm in length). Sterilize by quick rinse with 70% ethanol
2) Chop or grind feather root into very small pieces (can slice the feather in half lengthwise and the dice)
3) Add 30 µl of 100 or 200 µM DTT (to break disulfide linkages in the keratins and other proteins)
4) Add 10 µl 10% SDS, 4 µl 10 mg/ml Pronase (or Proteinase K), 4 µl 250 mM EDTA, and 180 µl TE
5) Mix well and incubate overnight at 37ºC for Pronase or 55ºC for Proteinase K
6) Extract twice with Phenol:$CHCl_3$-IAA and then twice with $CHCl_3$-IAA. (generally one transfers the upper aqueous layer but if there is a large interface then you can maximize yield but increase contaminants by removing and discarding the organic layer)
7) Precipitate with 1/10th volume of 3 M NaOAc and 3 volumes of 95% EtOH, overnight -20ºC
8) Pellet DNA, rinse with cold 70% EtOH, dry, and resuspend in 200 µl Te, expect 10-50 ng/µl

Fungal DNA Extraction

A tried and true method communicated from Dr. James Correll, Dept. of Plant Pathology,

University of Arkansas, as modified from: Lee, S. B., Milgroom, M. G., and Taylor, J. W. 1988. A rapid, high yield mini-prep method for the isolation of total genomic DNA from fungi. Fungal Genetics Newsletter 35:23-24. This procedure starts with lyophilized fungal mycelium. The lyophilized mycelium is then ground to a fine powder in liquid nitrogen in a frozen mortar with a pestle.

9) Fill 2-ml tubes ¼ to 1/3 full with finely ground (lyophilized) mycelium (the finer the better). Do not fill more than 1/3 full. Tubes may be numbered (1-18, etc.) if a corresponding list of isolate designations is prepared. This will simplify tube labeling during extraction.
10) Add 1300 µl extraction buffer (0.2 M Tris, pH 8.0; 0.25 M NaCl; 25 mM EDTA; 0.5% SDS). This is best done in two 650 µl aliquots. Mix well (use multimixer or vortex until mixed).
11) Incubate at RT for 30 minutes.
12) Spin at 14k rpm for 5 minutes. Pour supernatant into new 2-ml tube. This should yield about 1 ml. Add extraction buffer to any tubes with significantly less volume.
13) Add 500 µl (1/2 volume) phenol (TE saturated). Add 500 µl (1/2 volume) $CHCl_3$:IAA. Mix well by repeated inversion for several minutes to form a milky emulsion. (For this and subsequent mixing, place an empty rack over tubes and invert the entire rack at once).
14) Spin for 5 minutes at 12k rpm.
15) Remove aqueous (top) phase to new 2-ml tube. Use large mouth tips to avoid shearing DNA and pipette slowly to avoid drawing up the debris at the interface. Bring volume to approximately 1 ml with extraction buffer, if necessary.
16) Add 10 µl RNase A (stock concentration of 10mg/ml). RNase can be placed in cap of open microcentrifuge tubes to use a single pipette tip. Mix. Incubate 30-60 minutes at 37°C.
17) Add 1 ml (1 volume) $CHCl_3$:IAA. Mix well by repeated inversion for several minutes to form an emulsion.
18) Spin for 5 minutes at 12k rpm.
19) Remove aqueous (top) phase to new 2-ml tube. Use large mouth tips and pipette slowly.
20) Add 100 µl (1/10 volume) 3M NaOAC (pH 5.2). Mix by version.
21) Add 1 ml isopropanol (1 volume). Allow DNA to precipitate for a few minutes then mix very slowly and gently by inversion. Tubes may be held overnight at -20 °C, if necessary.
22) Spin 30 seconds at 14k rpm to pellet.
23) Pour off supernatant, drain inverted tubes on paper towels.
24) Re-suspend pellet in 360 ml TE. (Allow pellets to re-hydrate for a few minutes before gently flicking tubes). DNA must be re-suspended before proceeding to the next step.
25) Add 180 ml 7.5 M NH_4OAc. Mix well by inversion. Chill tubes on ice for 5-10 minutes.
26) Spin 20 minutes at 14k rpm, 4°C. While these are spinning, label final 1.5 ml tubes with isolate designations for storage of the DNA.
27) Pour supernatant into final tubes. Verify correct isolate designations as you proceed. Discard pellet.
28) Add 1 ml (2 volumes) cold 100% ethanol (EtOH). Allow DNA to precipitate for a few minutes while mixing very slowly by gentle inversion.
29) Tubes may be held overnight or longer at -20 °C, if necessary.
30) Spin 5 minutes at 14 k rpm, 4°C to pellet. Pour off supernatant, invert to drain.
31) Wash pellets by filling tubes with cold 70% ethanol. Spin 5 minutes at 14k rpm, 4°C , pour off supernatant, invert to drain.
32) Repeat wash, if desired.
33) Dry pellets under vacuum centrifugation for 30 minutes or in oven at 55°C for 5 minutes

34) Re-suspend pellets in 50-100 µl TE or Te. Start with 50 µl if unsure of yield. Te is best if yields are low and DNA is for restriction digests. (Allow pellets to rehydrate for 10-20 minutes before gently flicking tubes to resuspend).

35) Quantify concentration and dilute DNA with storage buffer (TE or Te), if necessary. Yields should be in the range of 10-50 µg per DNA prep, and may be diluted to a convenient concentration for pipetting, generally 200 to 500 ng/µl.

GELS: STANDARD

Ethidium Bromide (EthBr)

- WARNING THIS IS A CARCINOGEN AVOID SKIN CONTACT. To prepare the stock solution weigh out the powder and then dissolve with mixing to 10 mg/ml in H_2O. Filter through whatman #1 filter paper and store at room temperature in an amber plastic bottle (amber to block light and plastic to avoid breakage). For DNA visualization by UV illumination the EthBr can be applied either by inclusion in the gel and buffer at 0.5 µg/ml, staining the gel after electrophoresis in 1 µg/ml, or inclusion of EthBr at 10-20 µg/ml in the 5x sample loading buffer. One simple method that we prefer is to prepare the gel without EthBr and then add EthBr at approximately 0.5 µg/ml to the running buffer. This may lead to some band smearing due to differences in binding of EthBr but is generally avoided by slow electrophoresis or allowing equilibration of gel and buffer prior to sample loading.

Agarose Gels

Concentrations for DNA Separation

% Agarose	Resolving range (bp)
0.7	500-20,000
1.0	200- 5,000
1.5	100- 1,500

Casting the gel

1) Set up your gel tray so it is completely sealed on all sides, normally this is either a self sealing gel tray or gel tray which needs to have the ends sealed with labeling tape. Orient the comb so it is suspended in place very near one end of the tray and just barely above (0.5-1 mm) the lower surface of the gel tray.

2) Weigh out the appropriate amount of agarose into a screw cap bottle or flask, add gel buffer to volume and bring to boil, either a good boil on a stirring hot plate or just boiling several times in a microwave with occasional swirling; **you must be SURE that the agarose is completely melted and dispersed evenly in solution**.

3) Cool the mixture to about 50°C in a water bath (may cool at room temp. with occasional swirling for uniform cooling) and then pour into gel frame to a depth of 5 to 8 mm depending on the needed well volume for your sample. Melted agarose may be prepared in advance and kept at 50°C but the agarose tends to coalesce after several hours; the solution may be boiled and cooled anew.

4) Allow plenty of time for the agarose to cool and solidify before you **gently** remove the comb or you will tear the bottoms of the wells; this will result in your sample leaking out the bottom of the well.

5) Place the gel in the gel chamber and add gel buffer (0.5xTBE) so that the gel is just barely submerged in the buffer (about 1mm above gel surface). If you add too much buffer then more current will be passed (remember Ohm's law: Watts=Amps x Volts). The more current (mAmps) the more heat.

6) If you are adding EthBr only to the running buffer add it now (1 µl of 10 mg/ml stock per 10 ml of buffer), gently rock the chamber to distribute the EthBr, and allow to equilibrate for 10-15 minutes before loading.

Sample Size and Resolution:
Typically the minimum amount of DNA which can be visualized as a band with EthBr staining is: using photography and a transilluminator, 5-10 ng by photography; or by eye and a hand-held illuminator, 20-25 ng. Of course this is based on the dimensions of the well which will determine the width of the band, the detection levels above assume a 4-5 mm wide well. The height of the well as determined by thickness of the gel is less important for visualization because the band is viewed from above; however, gel depth should be kept at a minimum to conserve expensive agarose, minimize transfer times in blotting, and retain flexibility of the gel when handling (thick gels don't bend well). The thickness of the comb used normally varies from 0.5 to 2 mm and will affect the thickness of the DNA band (whether it is a fine line or a broad band. Thick combs are less important for big DNA than small DNA molecules. This is based on the relative migration rates in aqueous solution (the well) and in the agarose gel. On 0.7% gels the big DNAs (>2 kbp) will tend to 'stack' when entering the gel, while smaller DNAs readily move on in to the gel. Bands for 500 bp fragments on 0.7% gels generally have the same dimensions as the well.

The amount of DNA which can be loaded into a single well depends on the degree of resolution required and the Voltage gradient applied (V/cm). Especially in the case of genomic DNA blots or fragment purification where large amounts of DNA are applied: **the slower the gel is electrophoresed the better**- 20 Volts (<1 V/cm) will give much better separation and reduce 'rocketing'. Rocketing is the smearing of the band either as a V shape or as a bunch of ugly streaks. Rocketing results from either: Over-loading (to much DNA in one spot at the same time), Over-voltage (racing the sample through the gel) or excess Salts in the sample. Larger DNA >8 kbp, especially >20 kbp, is much more susceptible to rocketing and over-loading.

Densitometric Analysis of a Gel Using ImageJ

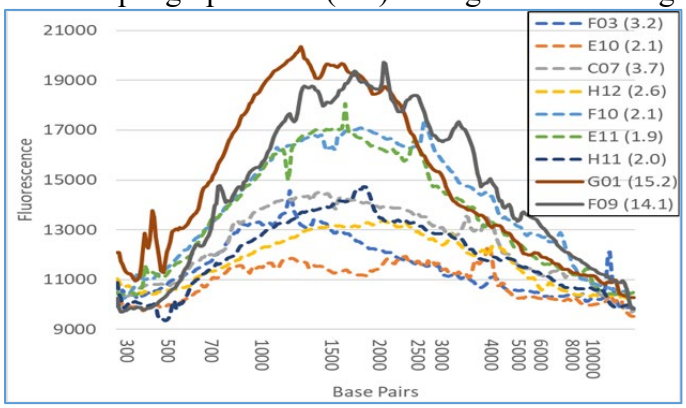
ImageJ is a java application that can be downloaded from the NIH website https://imagej.nih.gov/ij/download.html. It can be finicky to get started because it needs to find the java.exe, but once you get it installed it is fairly straightforward.

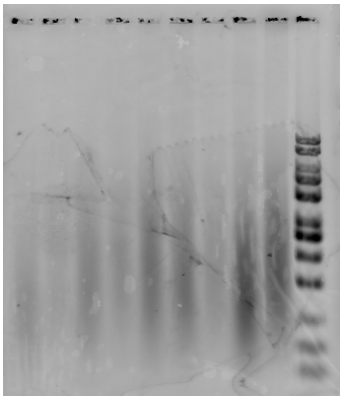
1) Agarose gels can be stained with Et Br (see above) and then scanned on the Typhoon (ex 532/em 580 or 600) at 200nm resolution. Set the PMT to 500V or 600V depending on the amount of DNA in each well.
2) Save the results to a .gel file which will load directly into ImageJ because a .gel is really a tif format.
3) Select Image>Adjust and then use the sliders to adjust the image to make the DNA and markers as visible as possible
4) Select Edit>Options to set Line Width to 10 (you can adjust the width based on width of the wells in your gel.
5) Draw a line down one of the lanes that covers all of the bands and ends at a point which can be used when you move the line to other lanes to read them all from the same start or end point (like the bottom of the gel).
6) Select Analyze>Plot Profile to get a scan of the first lane
7) Save the scan data for that lane to a .csv.
8) Set the Plot Profile to Live so that when you move the line to successive lanes you can Save the profile for that lane.
9) Then when you have all the lanes (including marker) saved as .csv you can load all the .csv into Excel and then create a single worksheet containing the intensities for all of the lanes as a table with the distances in column 1.
10) In column 2 enter the marker sizes based on the “peaks” in the fluorescence signal for the

marker lane

11) In Excel make a line graph of the entire table using the marker lane as the X-axis, and fluorescence of the experimental lanes on the Y-axis.

12) An example graph result (left) for a genomic DNA gel (right) might look like this:





Acrylamide DNA Gels

Nondenaturing gel

		4%	**6%**	**10%**
39:1 acrylamide:MBA		4 ml	6 ml	10 ml
10x TBE		2	2	2
H_2O		34	32	28
		**********DEGAS**********		
10% NH_4persulfate	7.5 µl/ml	300 µl	300 µl	300 µl
TEMED	0.75 µl/ml	30	30	30

Run in 0.5x TBE (no Ethidium Bromide) at up to 200 V with cooling at 20°C.

Denaturing TEB Acrylamide gels

28x40x0.04 cm= 60 ml, 22x40x0.04 cm= 40 ml

acrylamide conc:	**4%**		**6%**		**8%**		**10%**		**20%**	
gel volume-	**40**	**60**	**40**	**60**	**40**	**60**	**40**	**60**	**40**	**60**
acrylamide:MBA (38:2)	4 ml	6	6	9	8	12	10	15	20	30
Urea	20 g	30	20	30	20	30	20	30	20	30
20x TEB	2 ml	3	2	3	2	3	2	3	2	3
H_2O	17 ml	25	15	22	13	19	11	16	1	1
TEMED (µl)	60 µl	90	60	90	60	90	60	90	60	90

	dissolve and equilibrate to room temp									
10% NH_4persulfate (µl)	80 µl	120	80	120	80	120	80	120	80	120

Pour into gel mold, rap out bubbles with screwdriver handle, lay gel flat, insert comb, clamp plates over comb, and leave level during polymerization. Allow gel to polymerize about 1 hour. Remove comb and rinse top of gel. Place in gel apparatus under gel buffer: 1x TEB.
Pre-electrophorese for 5 minutes at 40-50W.
Flush wells with buffer to remove excess urea just prior to loading samples.
Loading buffer: 90% formamide, 1x TEB, 0.02% Bromophenol Blue.
Samples mixed with minimum of 1-2 volume of Loading buffer, heated to 90-95ºC for 5', quick chilled to 0ºC and loaded immediately.
Electrophoresis is constant wattage. There is no need to monitor temperature for TEB gels. For a 6% 30x40cm gel usually 40-50W for around 120 Watt-hours will keep 80bases on the gel (about xylene cyanol). Typically run the BPB to the bottom. These gels can be run overnight but reduce the number of Watt-hours to 60-65. At low Watts the DNA runs faster per Watt-hour.

Denaturing TEB Minigels

For 6 x 6 cm mini gels such as the MiniProtean II® (BioRad). 10 ml of gel solution is enough for two 0.75 mm thick gels. Great for screening PCR labeling products or other radiolabeled DNAs between 50 and 1000 bases

1.5 ml Acrylamide:MBA (39:1), 5 g Urea, 0.5 ml 20x TEB, 4 ml H_2O.
Mix/dissolve components then add 20 µl 10% APS and 15 µl TEMED. Pour into gel mold and insert comb. Allow to polymerize for 30-45 minutes. Assemble into chamber using 1x TEB buffer. Pre-electrophorese for 5 minutes at 150 Volts (5-6 mAmps). Loading buffer: 95% Formamide (deionized), 1x TEB, 0.02% Bromophenol Blue∘Xylene Cyanol. Samples are mixed with at least an equal volume of Loading buffer, heated to 95ºC for 5 minutes, snap cooled in ice water, and loaded. Don't forget to flush the wells with buffer before loading samples. Electrophorese at 150 Volts for 30-40 minutes (xylene cyanol near or off of gel bottom).

Denaturing TBE Acrylamide gels

28x40x0.04 cm= 70 ml, 22x40x0.04 cm= 50 ml

acrylamide conc:	**4%**		**6%**		**8%**		**10%**		**20%**	
gel volume-	**50**	**70**	**50**	**70**	**50**	**70**	**50**	**70**	**50**	**70**
acrylamide:MBA (39:1)	5 ml	7	7.5	10.5	10	14	12.5	17.5	25	35
Urea	25 g	35	25	35	25	35	25	35	25	35
10x TBE	2.5	3.5	2.5	3.5	2.5	3.5	2.5	3.5	2.5	3.5
H_2O	20.5	28.5	18	25	15.5	21.5	13	18	0	0
	dissolve all ingredients and equilibrate to room temp									

10% NH_4persulfate 1.8 µl/ml	90 µl	130	90	130	90	130	90	130	90	130
TEMED 1.5 µl/ml	75 µl	105	75	105	75	105	75	105	75	105

Pour into gel mold, rap out bubbles with screwdriver handle, lay gel flat, insert comb, clamp plates over comb, and leave level during polymerization. Allow gel to polymerize 0.5-1 hour then place in gel apparatus under gel buffer, 0.5x TBE.
Flush wells with buffer to remove excess urea.
Pre-electrophorese to heat gel to 45 to 55ºC
approximate values: 28x40 cm- 100 W 22x40 cm- 90 W
Samples in 60% formamide, 0.3x TBE, 0.02% dye are heated @ 90ºC for 5', quick chilled to 0ºC and loaded immediately.
Electrophoresis is constant wattage to maintain a gel temp of 50ºC.

Gradient Sequencing Gels

TEB gels are prefered as there is no need to monitor gel temperature. The TBE formulations are presented for those that just don't like newer concepts and techniques.

0.4 mm thick gels	TBE GELS						TEB GELS				
% - Size (cm)	8%-20x60			6%-20x80			6%-20x80			6%-30x40cm	
Buffer conc:	4x	1x	0.5x	4x	2x	0.5x	9x	6x	1x	9x	1x
final volume (ml):	20	20	60	10	10	80	10	10	80	20	40
acrylamide:MBA											
39:1	4	4	12								
38:2				1.5	1.5	12	1.5	1.5	12	3	12
Urea (g):	9.2	9.2	27.6	4.6	4.6	36.8	5	5	40	10	20
10xTBE (ml)	8	2	3	4	2	4					
20xTEB (ml)							4.5	3	4	9	2
H_2O (ml)	0	6	22.8	0.5	2.5	34.4	0	1.5	32	0	16
Sucrose (g)	2			1			1				
BPB dye	trace			trace			trace				
10% APS (µl)	40	40	110	20	20	160	20	20	160	20	80
TEMED (µl)	30	30	90	15	15	120	15	15	120	15	60
Use (ml):			rest			rest			rest		rest[1]

notes:

[1]- to make the gradient gel add persulfate and TEMED then mix 5ml each of 1x and 9x to make 10 ml of 5x. Proceed with pouring the gel using the three different buffer concentration solutions.

Pouring the gel. Add persulfate and TEMED (do not degas). Pull indicated amount of middle gel concentration (1x or 2x TBE, 6x TEB) into 25 ml pipette followed by the amount for the high concentration underneath. Pull two air bubbles into pipette which will slightly mix interface between solutions. Pipet gel solution into angled (30 degree) assembled gel plates trying to maintain the gradients integrity. Complete the gel by addition of low concentration. Rap out bubbles with plastic screwdriver handle (may need to orient gel near vertical to get all bubbles out. Lay the gel down horizontally and insert blank comb (flat side of sharks-tooth comb), cover top with saran wrap and used Bulldog clamps to secure plates directly over the comb (clamp the plates down against the comb to make a tight seal). Allow to polymerize in horizontal position app. 1 hour. Remove comb, rinse gel top and insert Shark's Tooth comb. Mount in electrophoresis apparatus. Gel buffer is 0.5x TBE for TBE gels; 1x TEB for TEB gels.

- **DNA samples** are in 65-90% formamide, [0.5x TBE or 1x TEB], 0.02% dye. Immediately prior to loading, samples are heated @ 90ºC for 5', quick chilled to 0ºC, and then loaded.
- **TBE gels <u>MUST</u> be pre-electrophoresed to heat gel to 45 to 50ºc before loading. approximate Wattage: 20x60- 100 W 20x80- 90-95 W.** Gel temperature varies during the run so occasionally check the temp. and adjust the Wattage accordingly.
- **TEB gel are pre-electrophoresed for <u>ONLY</u> 5-10 minutes at 70 Watts (don't over do it). Gels of 20x80 cm usually run fine at 70 W or less; they can be run Overnight at low wattage. Typical run times: for 20x80 cm TEB gels run at 10 to 50 W and run for 380 to 400 W hours; for 30x40cm gels run at 5-50 W for 100 to 120 Whours.**

¬ **Use tracking dyes to determine when to stop. Suggestions:** for sequence 5-10 bp from primer's 3'-end run Bromphenol blue to gel bottom; for sequence 20-30 bp from primer's 3'-end xylene cyanol to 13 cm from gel bottom.

Recovering Samples from Gels

DNA Electroelution from Gels

This technique works for acrylamide or agarose gels with an IBI style electroelutor
Trapping solution: 4 M NaCl, 20% Glycerol, 0.02% BPB
Trapping solution is constituted from sterile solutions of: 5 M NaCl; 80% Glycerol; and 2% BPB. Mix 8 ml 5 M NaCl, 2 ml 80% Glycerol and enough BPB solution to turn the solution a deep blue. Store at R.T.

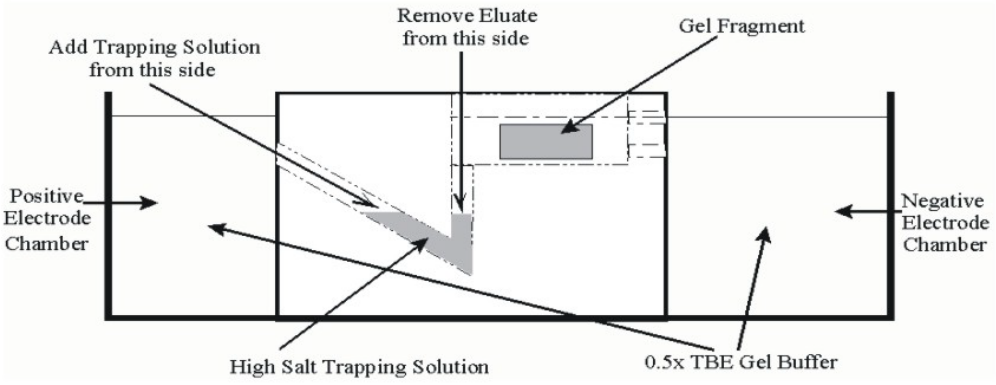
Gel preparation

1) DNA samples should be run in a 0.5x TBE 0.5 µg/ml EthBr buffer. The best separation and quality of recovered DNA is with samples run at low voltage (≤20 volts on minigels). Using scalpel pre-rinsed with water then EtOH, excise the band(s) of interest from the gel. Illumination should be with a handheld UV lamp not on a trans-illuminator so as to minimize UV exposure to the DNA-EthBr complex. Over exposure will lead to nicking and cross-linking and reduced biologic activity.

Electroelution

2) Fill the electroelution chamber with 0.5x TBE (no EthBr). Fill the channels high enough that the buffer will just cover the gel fragments but not so high as to bridge the top of the center bar. Remove any air bubbles trapped in the channels with a pasteur pipette.

Figure 5. Diagram of Electroelution Chamber.



3) Place the gel fragments in the chamber depression (well) in the bar and hookup the power supply leads. **Once the trapping solution is added you should not move or vibrate the chamber.**

4) Using a 200 µl pipettor SLOWLY introduce 100-150 µl of trapping solution into the V-tube. Trapping solution should be added from the angled portion (back side) of the tube so that no trapping solution is introduced into the well where the gel fragment(s) are. Use a

separate pipette tip for each aliquot.

5) Electrophorese towards the positive (red) terminal at 75-100 V. Small fragments from agarose gels will be done in 30-40 min. Larger fragments or acrylamide gel samples may take longer (up to 1.5-2 hrs). Most elutions may be monitored occasionally using a hand-held UV light. Do not over-irradiate the samples as this will damage the DNA.
6) After the sample has eluted from the gel fragment allow a few minutes for it to pass down the channel and trap in the salt solution. Turn the power down to 20 V. To recover the high-salt trapping solution use a new, sterile 100 µl glass micropipet. Insert the micropipet into the vertical tube (the well side) down to just above the blue trapping solution. Start withdrawing the blue solution and lowering the micropipet so as to continually draw out the trapping solution from the interface. Your DNA should be primarily trapped at the interface as this is where the DNA enters the high salt environment. It generally takes two pipet fulls to remove all of the blue using the same micropipet. Total volume recovered should be about 300 µl.
7) If you are isolating large quantities of DNA(>1-5 µg) you may wish to extract the solution one time with isoamyl alcohol (H_2O saturated) to remove some of the dye and EthBr Note: Isoamyl alcohol is less dense than water so discard the top organic layer and keep the aqueous phase on the bottom.
8) Extract with $CHCl_3$-IAA (discard the bottom layer).
9) Add 1 ml 95% EtOH and incubate >1 hr at -20ºC.
10) Pellet the DNA by spinning 20 min at 12000 rpm at 4ºC. Decant, add 1 ml cold 70% EtOH and spin an additional 2 min. Decant and dry <u>in vacuo</u>.

Comments: If the DNA must be ultrapure you may wish to electroelute it again to insure purity. If salt contamination would be a problem for future manipulations (and if lots of DNA was recovered) you may wish to do another EtOH precipitation. If the first EtOH precipitation is overnight the excess NaCl and TBE salts may precipitate in which case a visible salt pellet may form.

Electrophoresis onto Whatman Paper

Provided by Ron Okimoto

1) Electrophorese the sample in an appropriate gel for good separation.
2) Cut a piece of Whatmann 3MM chromatography paper and dialysis membrane (small MW cutoff such as 3500 MW). The paper and membrane should be about the same size and have dimensions wider than the band of interest and the height of the gel. Soak the paper and membrane in a puddle of gel buffer on plastic wrap.
3) Turn off the gel power
4) Gently cut a slit in the gel in front of the band of interest
5) Using forceps or gloved hands, insert the paper and membrane into the slit so that the paper is on the band side and the membrane backing the paper. The object is that the band will enter the paper but not be able to leave the paper because the membrane blocks the bands progress.
6) Electrophorese the band into the paper under moderate voltage (40-6 Volts). Monitor the progress occasionally with a handheld UV lamp.
7) Turn off the power , use forceps to pull out the paper and membrane. Transfer the paper to a microfuge tube with 400 µl TE. Place on a mixer or vortex for 5-10 minutes.
8) Transfer as much of the TE to a new tube. Extract once with $CHCl_3$-IAA. Transfer the aqueous to a new tube.
9) Add 40 µl 3 M NaOAc and 1 ml 95% EtOH. Incubate at -20 ºC for 1-2 hours as appropriate

for DNA band size and DNA quantity.

10) Collect the DNA with appropriate centrifugation time and speed, do a 70% EtOH rinse, dry, and redissolve in Te (volume dependent on recovery of about 20-30% of the starting band).

DNA Fragment Recovery by Crush-Soak

1) Place the gel fragment in a microfuge tube and mash the gel slice to a paste with a siliclad, sterile glass rod, add 500 µl 500 mM NH_4OAc 2 mM EDTA, vortex and incubate at 37ºC for 3-4 hr (<750 bp) or 5-6 hr (750-3000 bp).

2) Spin out the gel debris, collect the supernate, rinse the debris with 300 µl of the same solution (vortex and spin). For ^{32}P fragments, elution may be monitored with a hand-held Geiger counter.

3) Reduce the vol to app. 200 µl with 2-butanol, $CHCl_3$:IAA extract, 50 µl backextraction (same buffer), add 5 µg carrier RNA (if necessary). Precipitate with EtOH.

Recovery by Agarase Digestion of Low Melting Agarose

Based on procedures recommended by FMC for use with their product β-agarase, a bacterial enzyme that digests the agarose backbone to oligosaccharides rendering the agarose non-geling and alcohol soluble.

11) Separate the DNA sample in a low melting point (LMP) agarose gel and identify the band(s) of interest. Alternatively, separate in standard agarose and then cut out a well in front of the band of interest, form a block of LMP agarose in the well and then electrophorese the band into a block of LMP agarose.
12) Excise the band of interest and place in a microfuge tube. Add 2 to 5 gel-fragment-volumes of 1x Agarase buffer and incubate at room temperature for 15 to 60 minutes to exchange gel buffer for agarase buffer.
13) Remove buffer and then melt agarose at 65ºC (2-5 minutes).
14) Equilibrate molten agarose at 40ºC.
15) Add 1 µl β-Agarase per 200 µl molten agarose, gently mix, and incubate at 40ºC for one hour. The digested solution may then be used for enzyme digestion, PCR, or further purified by alcohol precipitation. Note: Fluorometric quantitation is not possible in the digested agarose solution.
16) Alcohol precipitations are performed at room temperature so as to not precipitate the agarose oligosaccharides. For small quantities of DNA carrier glycogen can be added (generally 2-10 µg) and mixed in prior to adding the ethanol. Add ammonium acetate to bring to 2.5 M or NaOAc to bring to 0.3 M, mix and then add 2.5 to 3 volumes of room temperature 95% ethanol. Mix and let stand at room temperature for 1 hour to overnight. Pellet the DNA at 10-11 krpm for 20-30 minutes at room temperature, wash the pellet with 70% EtOH, dry, redissolve and quantify.

Purification and removal of digested agarose oligosaccharides can be facilitated by use of Microcon™-50 spin filters:

A) Suspend the sample in 200-500 µl of Te (<500 µl per unit) in the filter units upper chamber.
B) Spin at 10,000 rpm in angle microfuge rotor for 8 min. Discard solution in lower collection tube.
C) Add additional Te to the upper chamber and spin at same speed for 5 min.
D) Invert filter chamber into new collection tube and spin at 1000 x g for 3 minutes. Recover liquid from collection tube and add 5 µg Glycogen.

E) Extract once with $CHCl_3$:IAA, add NaOAc and Ethanol precipitate

Note: remaining agarose fragments apparently quench fluorometric quantification based on Hoechst dye binding. However, agarose contamination will not adversely affect ligations and transformations. Quantification can be either by estimation based on samples run on mini gels or by UV spectrophotometry.

Formaldehyde Gels for RNA separation

Reference: Focus 8(3):5 or Maniatis Cloning Manual

Size markers for 1.2% Formaldehyde gels

BPB dye	about 200 bases
28S human rRNA	6333 bases
18S human rRNA	2366 bases
23S E.coli rRNA	3566 bases
16S E.coli rRNA	1776 bases

10x MOPS-EDTA: 0.5 M 3-(4-Morpholino)-Propanesulfonic acid, 10 mM Na_2EDTA, pH 7.0 with NaOH

Sample Buffer: 50% Formamide 2.2 M Formaldehyde, 1x MOPS-EDTA, (20-40 µg/ml Ethidium Bromide), 0.03% Bromophenol Blue and Xylene Cyanol.

Formaldehyde 37% Stock Solution = 12.3 M

Electrophoresis buffer: 1x MOPS-EDTA

Gel Preparation and Electrophoresis

1) For a 1.2% gel weigh out 1.2 g agarose and melt by boiling in 10 ml 10x MOPS-EDTA, 72 ml H_2O. Cool to 60ºC, add 18 ml 37% Formaldehyde (final conc. =2.2 M), mix well and pour immediately.

2) After the gel has cooled (about 1 hour), submerge in electrophoresis buffer and pre-electrophorese for 30 min. at 60 V then load samples.

3) To prepare the samples dry the RNA and resuspend in Sample Buffer. Heat to 80º-90º (hot block will work) for 5 min. then quick-cool on ice. To visualize the samples others recommend staining the gel in Ethidium bromide (5 µg/ml) in H_2O 60 min., followed by destaining in H_2O for several hours. Alternatively, it is easier to add Ethidium bromide to the Stop Buffer (final conc. when added to the sample should be around 20-40 µg/ml) this can be done for only the marker samples. However, no adverse effects have been observed for blotting of RNA samples loaded in Ethidium Bromide containing sample buffer.

4) Initial electrophoresis is 60 V for 60 min. and then turn up to 90 V for the remainder of the run. Typical mA for a 28 cm electrode path are the same as the Voltage reading, i.e., 90 V 90-100 mA. Be sure to recirculate the buffer after the dyes have entered the gel.

Gel Treatment and Blotting

6) Treat the gel 2x 15 min. with 50 mM NaOH to partially hydrolyze the larger RNAs for subsequent transfer.

7) Treat 2x 15 min. with 0.5 M Tris pH 7.5, 1.5 M NaCl to neutralize.

8) Transfer to GeneScreenPlus as per any other gel.

Glyoxalation and Glyoxal Gels for RNA Samples

Solutions

15% glyoxal: 150 µl deionized glyoxal (deionized with Dowex-1 (OH-) and Dowex 50 (H+)), 500 µl DMSO, 20 µl 0.5 M KPB pH 6.5, 330 µl H_2O

0.5 M KPB pH 6.5: 685 ml 0.5 M KH_2PO_4, 315 ml 0.5 M K_2HPO_4

Preparation

1) Dissolve the dry nucleic acid in 25 to 30 µl of the 15% glyoxal solution and incubate for 45' 50°C
2) Add 1/2 vol 30% glycerol with BPB to color
3) Load the samples on a 1.5% agarose gel made in 10 mM KPB pH 6.5
4) Electrophorese in 10 mM KPB pH 6.5 buffer 250 V (60 mA) for 2.5 hr dye should migrate app. 13 cm
5) You can now stain with acridine orange or blot according to procedure for formaldehyde gels (see above)

Loening Gels for Resolution of rRNA

Reference: JMB 38:355-365

10x Loening Buffer (1 L): 36.3 g Tris, 49.7 g NaH_2PO_4-H_2O, 2.92 g Na_2EDTA, pH 6.8

	Bottom	Resolving	Running Buffer	Sample Buffer
H_2O	16.4 ml	47.5 ml	to 2 L	17 µl
30:0.8 Acrylamide:MBA	10 ml			
10x Loening buffer	3 ml	6 ml	200 ml	25 µl
10% SDS	600 µl	1.2 ml	4 g(solid)	8 µl
Agarose		0.36 g		
24:1.2 Acrylamide:MBA		5.28 ml		
Glycerol				50 µl
TEMED	60 µl	60 µl		
10% NH_4persulfate	180 µl	300 µl		
Bromophenol blue				to color

Gel: Best in vertical gel with one frosted glass plate using 0.3-0.5 cm spacers. Pour the bottom and allow to polymerize, melt the agarose in H_2O cool to 45-50°C, add buffer and acrylamide, degas, add SDS, TEMED and persulfate, swirl to mix and pour immediately, insert comb and allow to polymerize. Dissolve RNA 1 A_{260} in 15 µl H_20 then add 5 µl Sample buffer and load on gel

Staining gel: stain several hours in H_2O+0.001% methylene blue may need to destain in H_2O

Photograph through red filter $^1/_{60}$th sec f22

Laemmli SDS Protein Gels

Reference: Nature 227:684 (1970)

Resolving gel for 24 ml	**10%**	**12.5%**	**15%**	**20%**
H_2O (ml)	9.72	7.72	5.72	1.72
1.5 M TrisCl, pH 8.8 (ml)	6	6	6	6
30:0.8, Acrylamide:MBA (ml)	8	10	12	16
	**********DEGAS**********			
10% SDS (µl)	240	240	240	240
TEMED (µl)	20	20	20	20
10% NH_4Persulfate (µl)	45	45	45	45

Tape bottom of gel or place in frame. Remove 1 to 5 ml of gel solution for plug (depending on gel size) and add TEMED and APS at 2x. Pipet solution down sides to seal both spacers and form 2-3mm high plug in bottom, allow to solidify for 15 minutes and then drain and blot extra solution from top of plug so resolving gel sticks to plug. Then pour rest of gel sets in 15-30 min.
Stack and teeth not required for mini-gels.

Stacking gel and teeth (optional) 8 ml	**3.0%**	**4.0%**	**5.0%**
H_2O	5.1 ml	4.8	4.6
0.5 M TrisCl pH 6.8	2.0 ml	2.0	2.0
30:0.8 Acrylamide:MBA	0.8 ml	1.1	1.3
	**********DEGAS**********		
10% SDS (w/v)	80 µl	80	80
TEMED	10 µl	10	10
10% NH_4Persulfate (w/v)	30.µl	30	30

Buffers:
Electrode buffer: 25 mM Tris, 192 mM Glycine, 0.1% SDS (w/v), pH 8.3
for 2 L: 6 g Tris, 28.8 g Glycine, 2 g SDS, pH should=8.3
Sample buffer: 62 mM TrisCl pH 6.8, 1% SDS (w/v), 10% Glycerol (v/v), 5% 2-Mercaptoethanol (v/v), 0.001% Bromphenol blue (to color)
RUN - TO + FOR 1000 to 2000 V·Hr for 10cm gel

Gel Staining Procedures

Coomassie blue for proteins

1) Soak the gel for >6 hr in 0.1% coomassie blue 50% Methanol 7.5% HOAc
2) Destain by passages in 50% Methanol 7.5% HOAc; the gel may shrink in this destaining and can be swelled in 30% Methanol 7.5% HOAc before returning to 50% MeOH 7.5% HOAc. The gel can therefore be cycled between the two solutions as the 50% solution destains better.
3)Soak in 30% Methanol 7.5% HOAc for 1 hr
4)The gel may now be impregnated with Salicylate for ARG or stored in a sealed bag with 30% Methanol 7.5% HOAc
For Coomassie stained gels photograph through an orange filter

DMSO/PPO for fluorography

1) Soak the gel for 30' in two passages of DMSO making sure that the second passage is fresh

DMSO

2) Soak the gel for 3 hr in 16% (w/w) PPO in DMSO

3) Rinse the gel with H_2O three times and then soak the gel in H_2O for another 30'

4)Dry the gel under vacuum for >4 hr <70°C

1 M Salicylate for fluorography

1) Soak the gel in H_2O for 30'

2) Soak the gel for 30' in >10-gel-volumes of a freshly made 1 M NaSalicylate solution (16 g/100 ml)

3) Rinse with H_2O and dry under vacuum for > 2 hr <70°C

NOTES:

Gels may be DMSO/PPO or Salicylate treated after Coomassie blue staining but not vice versa.

For fluorography DMSO/PPO is app. 2x more sensitive than Salicylate

Ponceau S stain of western blot membranes

1)Stain the membrane in 0.2% Ponceau S (diluted in H_2O) app. 10'

2)Destain in H_2O to best color difference

3)Photograph with ruler(s). For polaroid 107C/667 film with back lighting use dark blue filter at f/32 for $^1/_{125}$th sec

Silver Stain for Nucleic Acids

from M. Balas, Volcani Centre, Rehovot, Israel via E. Rybicki Univ. of Cape Town

Short Procedure

1) Soak in 30% EtOH 10% HOAc 10' room temp. no shaking (can go overnight)

2) Soak 2x 10' in 10% EtOH

3) Wash 3x 5' in H_2O

4) Soak 30' in 6 mM $AgNO_3$ (50 ml/gel??) the quick rinse with H_2O

5) Rinse rapidly in fresh 2.5% Na_2CO_3 0.02% HCHO then replace with fresh for 2' more. Replace with fresh solution and allow bands to develop.

6) Stop with 1% HOAc and store in same. Can destain with 2% NH_4peroxydisulphate. Gels can be shrunk with 100% EtOH, then acetone and then air dried.

Long Procedure

1) Soak gel in 50% EtOH 10% HOAc 1-3 hrs or overnight

2) Soak in 10% EtOH 1% HOAc 1 hr. then rinse 3x with H_2O

3) Soak in 12 mM $AgNO_3$ 1 hr. rinse 3x 5' in H_2O

4) Rinse rapidly in fresh 0.75 M KOH 0.28% HCHO, then replace with fresh and allow bands to develop

5)Stop with 0.07 M Na_2CO_3 or 5% HOAc. Store in 1% HOAc.

Coomassie Quantitation of Proteins in Polyacrylamide Gels

Reference: E.H.Ball (1986) Anal. Biochem. **155**:23-27.

Note: This procedure is non-destructive. The polypeptide remains after dye removal and may be

analyzed further. Protein amounts in the range of 0.5 to 10 μg can be assayed. Coomassie dye binding varies for different proteins; therefore, only relative amounts (not absolute) of the same protein may be determined. However, dye binding appears to be linear for a wide range of protein amounts.

Stock solutions:
Staining solution: 50% Methanol, 7.5% Acetic Acid, 0.1% Brilliant Blue Coomassie R250.
Destaining solution: 30% Methanol, 7.5%Acetic Acid.
Extracting solution: 3% SDS in 50% isopropanol.

1) Staining: soak the gel in 10 gel volumes of the staining solution: 2 hr for 6 to 10% polyacrylamide gels; overnight for 12.5% to 15% polyacrylamide gels.
2) Destaining: soak the gel several times in 10 gel volumes of destaining solution until gel areas without proteins are almost totally destained.
3) Extraction of dye: Gel pieces containing stained protein are excised from the gel with a cork borer (the same size of gel fragments must be excised for each sample). In addition, for each gel, a similar sized gel fragment without protein is also excised to give a baseline value. Place each gel piece in a glass 12x75 mm screw-cap tube. Add 1 ml of extracting solution to each tube and incubate in a 37ºC bath for 24 hr without agitation.
4) Quantitation at 595 nm: cool the samples to room temp and collect the extracting solution. Determine the optical density (absorbance) at 595 nm using the extracting solution as the reference control. Determine the baseline values from the gel piece(s) containing no protein. Subtract the baseline value from experimental values.

BLOTS & HYBRIDIZATIONS

Note on using this chapter: There are many ways of affixing nucleic acids onto membranes and then probing those nucleic acids with either proteins or other nucleic acids to detect sequences. This chapter describes use of several different membranes based on your preference. The nucleic acids may be applied to the membrane by dot blotting, Southern blotting, colony lifts, plaque lifts, etc. Choose the method that works best for your application (plaque lifts are described in the chapter on Lambda). For Southern and northern blots there are several transfer methods depending on the nucleic acid to transfer and the membrane onto which to blot. Once the nucleic acid is on the membrane then you proceed to the section on hybridization. Again, there are different hybridization conditions and solutions depending on the sensitivity desired and the nature of the probe (labeled oligonucleotides vs. longer probes, etc.).

Southern blotting is a commonly used technique. There are many variations on these procedures affected by the type (DNA/RNA) and amount (genomic vs. plasmid level) of nucleic acid to blot and detect, and the membrane used for the blot. There are probably as many hybridization procedures as there are laboratories that do Southern blotting. DNA is usually resolved in standard agarose gels and while RNA can be partially resolved in standard gels they are better separated in Formaldehyde gels as described in the chapter on **Gels: Standard**. Blotting of RNA is slightly different than DNA in the method for partial fragmentation. For DNA the DNA is partially depurinated in a specifically timed treatment with HCl followed by NaOH denaturation. This NaOH treatment also breaks the phosphate backbone at sites of previous depurination. RNA is fragmented by a timed exposure to NaOH. Fragmentation greatly facilitates the quantitative transfer of larger (>2kbp) nucleic acids from the gel. If you don't need quantitative transfer then skip the fragmentation (skip the HCl treatment for DNA DON'T SKIP THE NaOH TREATMENT). Nucleic acids are transferred as denatured single strands onto the membrane so they are accessible to subsequent hybridization to the probe.

For hybridization we recommend you try Church buffer for simplicity and low cost. If you have problems with background then experiment with the other hybridization buffers. We have tried to reduce the complexity and generally found that with the newer membrane types most of the accessory reagents (Denhardt's solution, Formamide, etc.) can be avoided with little or no loss in sensitivity or increase in background.

First we will describe the recommended methods for standard blots. Variations or special applications follow after the recommended methods, later in the chapter.

Preparation of the Gel

Plan ahead- have all solutions made up and ready to go

For mammalian genomic DNA blots: use 5-10 µg of DNA per lane. The thicker the gel and the wider the comb the better your bands will be. Recommendation: use 7-8 mm wide comb and pour the gel at least 5-7 mm thick. The best band patterns are obtained when the gel is electrophoresed at <50 volts. Typically, for a 18 cm long gel and 30-40 cm electrode path (distance between electrodes) electrophoresis is for 600 volt-hours.

Notes on gels to be blotted:

For blotting to nitrocellulose: run the gel in 1x TAE or 0.5x TBE.

For blotting to Nylon membranes: GEL SHOULD BE RUN IN 0.5x TBE (Can use 1x TAE but do not run over 2 V/cm) **For all genomic level or any gel being run at >2 V/cm for > 200 V-hours THE BUFFER SHOULD BE RECIRCULATED (+ TO -) to inhibit over-heating, acidification of the gel and high backgrounds for the blot. Acidification is also prevented by running the gel at LOW voltage (1 V/cm); but for long run times use recirculation.**

Treating the Gel for Blotting

All Gel treatments are performed at Room Temperature

Never handle the gel or membrane with bare hands, wear vinyl or latex gloves; wash them with soap and water then rinse well before handling the gel.

1) Trim away all but app. 1 cm from either edge of the gel and any gel more than 0.5 cm above the wells.
   To indicate marker band positions either:
   A) punch holes through the gel where the marker bands are with a 100 µl glass micropipet. After transfer the positions of the holes are marked through the gel onto the membrane with a pencil.
   B) Photograph the gel next to a ruler so that distances on the gel/blot can be associated with a particular marker band size and migration distances.
2) To monitor pH changes Bromophenol blue becomes yellow in acid, blue in neutral and basic. For thick gels check to be sure the tracking dye has changed color **completely through the gel** before proceeding to the next treatment.

Choose step 3 for RNA gels or step 4 for DNA gels

3) For RNA gels
   A) Treat the gel 2x 15 min. with 50 mM NaOH to partially hydrolyze the larger RNAs for subsequent transfer.
   B) Treat 2x 15 min. with 0.5 M Tris pH 7.5, 1.5 M NaCl to neutralize.
   C) Transfer to modified Nylon membrane with 10xSSC as per any other gel.

4) For DNA gels- preferred method is Alkaline Denaturation and Neutral Transfer Method but some have had success with Alkaline Denaturation and Alkaline Transfer

Alkaline Denaturation and Neutral Transfer.

Preferred method, works best for many types of nylon membranes

A) Acid Depurination: This step specifically removes about one of every 400 purine bases. Subsequent alkaline treatments break the DNA backbone at the site of depurination, thus fragmenting the DNA for more rapid and complete transfer. **HCl treatment may be omitted with significant reduction of transfer of large DNA (>10-20 kb). HCl treatment should NOT be used on RNA gels or when the largest DNA fragments to transfer are <1000 bp**. Treat the gel 1x 15' with 0.25 M HCl or 2x 10' with 50 mM HCl then rinse 2x briefly with sterile H_2O.

B) Alkaline Denaturation: for DNA transfer to most nylon or nitrocellulose membranes. Treat the gel 2x 15' with 0.6 M NaCl 0.4 M NaOH, rinse 2x with sterile H_2O.

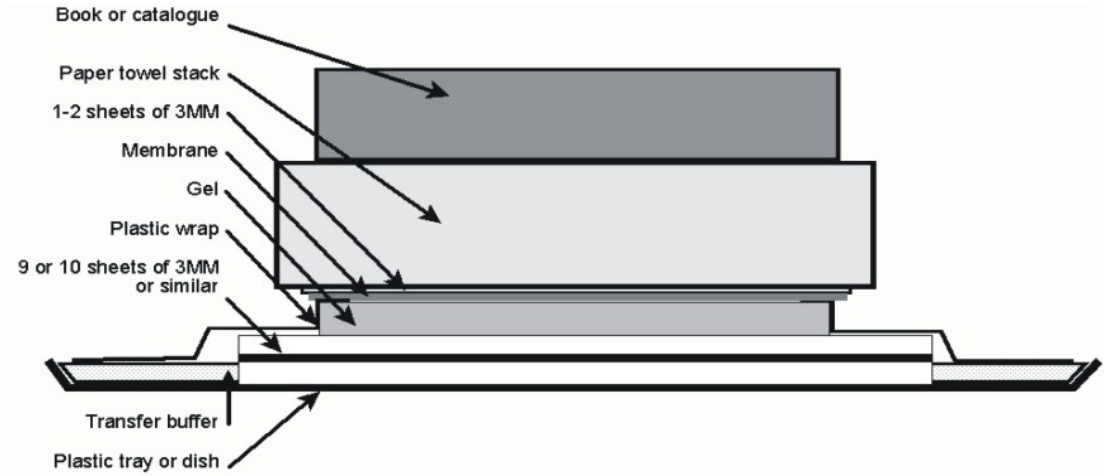
C) Neutralization: Treat 2x 15' with 0.5 M Tris 1.5 M NaCl pH 7.5, proceed with transfer using 10xSSC.

5) **The Membrane:** cut a sheet just larger than the trimmed gel size and treat according to the membrane type:
Nitrocellulose: wet with H_2O (if the membrane does not wet completely then discard and cut new membrane) and immerse in 2x SSC for 15' use 20x SSC for the transfer.
Charged or Neutral modified Nylon: [for Gene-Screen$^+$ indicate the concave side as side B with a pencil] Float the sheet on H_2O (if the membrane does not wet completely then discard and cut new membrane) and immerse in 10x SSC for 15', use 10x SSC for transfer (unless alkaline transfer).

6) **The Transfer:** there are two alternative transfer setups, the traditional **Wicked Reservoir** or the **Saturated Stack**. The most economical and convenient is the Saturated Stack.
Wicked Reservoir: Place a glass bridge over a pyrex dish filled with Transfer Solution. Saturate a 5-layer 'wick' of Whatman 3MM paper with Transfer Solution. Place it over the glass plate hanging down into the solution.
Saturated Stack: Use 6-8 layers of Whatman 3MM cut about 2 inches bigger than the gel. Place them in a flat bottomed plastic pan (microwave cookie sheets or plastic dinner plates work well) or in any flat bottom pan with low walls) and saturate them thoroughly with Transfer Solution.

7) Invert the gel onto the wick (bottom side up) and place Saran wrap so it just covers the edges of the gel and sticks out over the dish. Next place the membrane on the gel and then 2 sheets of Whatman 3MM prewet with Transfer Solution. With light pressure roll out all air bubbles between the wick, gel, membrane and cover sheets. Place a 3" stack of absorbent paper toweling (cut 2" larger than the gel) on top and weight it down with a baking dish of water or a big thick book like an old Sigma catalog.

Figure 6. Typical Southern Blot Transfer Setup.



8) Allow to transfer over night. Changing paper toweling can facilitate transfer and may shorten transfer time to 2-4 hours.

9) **Disassembling the Transfer Stack**. Remove the weight, paper toweling and Saran wrap without moving the gel, blot or filter paper. Invert the gel on the blot and using a Soft-lead (HB) pencil (stuck through the blot) mark the well and marker positions onto the blot.
A) For Nitrocellulose:
a) Rinse for 30 seconds in 1 M NH_4OAc then blot dry on 3MM paper Place between 3MM sheets and bake in vacuo for 2 hr 80°C.

B) For Nylon Membranes:

a) Wash the blot 2 time for 5' with 2xSSC at room temp. with slow shaking. Make sure no agarose is stuck to the membrane.

b) Blot on 3MM paper, place between 3MM sheets and affix the DNA to the membrane by either:

I) dry for 1 hr at 50ºC to 80ºC (vacuum not required)

II) dry for several hours to overnight at 37ºC.

III) UV crosslink with the membrane damp using optimal UV settings for your machine

IV) Most charge modified nylon membranes may be taken directly to the pre-hyb step with a ca. 50% reduction in sensitivity.

Hybridization of Blots

The Probe

For genomic blots the probe should be $>10^8$ cpm/µg DNA, use 1 x 10^6 cpm/ml. **The probe must be as free as possible from contamination by proteins and unincorporated radioactivity. G-50 purified and EtOH precipitated seem to do the trick**.

For plasmid or viral DNA blots less probe (2 x 10^5 cpm/ml) may be added; however the intensity of positives decreases relative to the negatives.

Church Buffer vs. Traditional Hybridization cocktails. Church buffer is a simple mixture of BSA, SDS and salts. Although not as complex as other hybridization solutions it has performed well in Southern blots and plaque lifts. The advantages are lower cost and ease of use. Sensitivity is probably equivalent. To use: heat the buffer to 65º to get the SDS back in solution and then add the appropriate amount (0.2 ml/cm^2) to the filter. Pre-hybridize for 1-2 hr then boil your probe (no competitor DNA is needed) and add to pre-hyb solution. **DO NOT ADD THE PROBE DIRECTLY ONTO THE MEMBRANE, ADD THE BOILED PROBE TO THE SOLUTION AND MIX RAPIDLY TO DISPERSE OR YOU WILL GET A BIG BLOTCH ON YOUR BLOT.** Hybridize for the appropriate length of time then proceed to the washes.

Prehybridization

1) Rinse the blot for 5-10' @ 65ºC in 0.1x SSC 0.1% SDS (this step is not required but may reduce background on colony lifts
2) Place in a Kapak heat-sealable bag, seal-a-meal bag, or hybridization oven bottle and add 0.2 ml Church buffer solution per sq.cm. of blot.
3) Expel air by pressing the bag between two glass plates or angle the bag and expel air bubbles. Seal the bag, submerse in a 65ºC bath and incubate shaking for at least 30 min. For sealed bags we find that the bag works best if "floating" unimpeded in a plastic sealed tub of water in a shaking water bath.

Hybridization

4) Denature the probe in a boiling water bath or 90ºC hot block for 5', add immediately to the Church buffer in the hybridization bag. DO NOT add onto the membrane but add to the solution away from the membrane. Seal the bag and rock to distribute the probe evenly, return to 65ºC water bath. Incubate with shaking for 18 to 24 hr.
5) Carefully slice the bag around the blot, transfer the blot to a plastic wash tray containing Wash buffer (0.5x SSC 0.05% SDS 3mM PB) prewarmed to 65ºC, rock gently, drain, add new wash buffer, rock and drain. Add new wash buffer and return to 65ºC for 5 min. Drain

and repeat 65ºC wash for 5 min.

For RNA probes: after the wash place the membrane in a Seal-a-Meal bag and add 0.2 ml/sq.cm. of blot: 2x SSC 10 µg/ml RNAseA. Incubate 30' at 37ºC. Then wash the 3x 5' each at 37ºC in 1x SSC 0.05% SDS 3mM PB. RNAse removal of probe is best only when working with a homologous probe, heterologous probes with multiple mismatches may be removed by this treatment.

6) Blank areas of the blot should have no radioactivity detectable with a hand held monitor
7) Place damp membrane in a Seal-a-Meal bag or wrap in Saran Wrap, squeeze out excess moisture and seal.
11) Mount for ARG at -90ºC.

Probe Elution for Reprobing (for Nylon Membranes only)

1) Wash blot 2x 30' in 0.01x SSC 0.1% SDS 65ºC
2) The blot should now be ready for prehybridization.

Alternative Procedures for Blotting

Alkaline Denaturation and Alkaline Transfer.

Do NOT use for RNA gels. works for Zetaprobe membranes not recommended for GeneScreen or Magnagraph. NOTE: experience has shown that with some nylon membranes (Magnagraph) there is poor binding of DNA under alkaline transfer conditions. Although signal detection is still possible at plasmid (cloned DNA) levels, genomic blots may not work unless the probe is VERY hot.

A) Immerse the gel briefly in 0.4 M NaOH then proceed with transfer using **0.4 M NaOH as the transfer buffer**.

In Gel Hybridization for oligonucleotide probes

According to Harrington and Descenzo, communicated through J. Correll.

1) After the gel has been photographed, assemble on a gel drier and dry under vacuum pressure at room temp for 45 minutes and then apply heat (50ºC) for 45 minutes. Gels can be dried onto either cellophane or whatman paper but the paper or backing must be removed during the soaking steps.
2) Treat the gel in denaturing solution 0.5 M NaOH 1.5 M NaCl for one hour (probably 30 minutes is plenty) using 150 ml per 600 cm^2 gel.
3) Neutralize the gel in 1 M TrisCl pH8.0 1.5 M NaCl for one hour use same volume as step 2.
4) Rinse in sterile water for 30 minutes
5) Pre-hybridize the gel for one hour in 250 mM NaPB pH 7.0 7% SDS at hybridization temperature (see section below on oligonucleotide probes for hybridization temperatures based on oligonucleotide probe length
6) Add the labeled oligonucleotide and hybridize overnight at hybridization temperature
7) Wash the gel three or four times in 6xSSC 3 mM NaPB 0.5% SDS at hybridization temperature for 5 to 10 minutes per wash.
8) Wrap in Saran Wrap and mount for autoradiography

Dot Blotting Nucleic Acids

1) Preparation of dsDNA

For dot blotting to Nylon membranes substitute 10x SSC for all references to 20x SSC

A) DNA should be free of proteins and contaminating RNA
B) DNA in 10 µl 10 mM Tris 1 mM EDTA
C) Add 10 µl 0.6 M NaOH and denature by heating for 5' in boiling water bath or 20' in °C water bath
D) Place in ice and add 80 µl of cold 20x SSC made 75 mM with HCl (7.5 µl conc. HCl/1 ml 20x SSC)
E) Keep on ice and run through filter as soon as possible

2) Preparation of RNA or m13 ssDNA

NOTE: RNA should only be blotted to Magnagraph, GeneScreenPlus, or other charged nylon membrane

A) RNA should be free of proteins and contaminating DNA
B) RNA in 100 µl 10 mM Tris 1 mM EDTA
C) Denature by heating for 10' in 90° hot block
D) Place in ice and add 100 µl of cold 20x SSC (to make 10x SSC)
E) Keep on ice and run through filter as soon as possible

3) Preparation of m13 phage lysates

NOTE: for phage lysates dotblot only 10 µl as 20 µl may clog the filter. For purified phage ssDNA treat as for RNA.

A) 10 µl of m13 phage lysate is mixed with 1 µl 1%SDS and heated to 65° for 10'.
B) Remove from heat and add 90 µl 20x SSC (10x for GS^+). **DO NOT CHILL BELOW ROOM TEMP.**
C) Run through filter as soon as possible

4) Preparation of filter

A) Wet filter with H_2O, then soak for 10' in 20x SSC (10x for GS^+)
B) Place membrane in manifold and block unused portion with double layer of Saran Wrap
C) Apply vacuum to manifold. It is critical that the vacuum suction is low pressure so the samples pass through the membrane slowly, 100 µl should take app. 15-30 sec.
D) Apply samples by pipetting onto side of well so that no air bubble is trapped at the bottom and with sufficient force to spread the sample across the entire sample well bottom. Rinse each sample well with 200 µl 20x SSC.
E) Trapped air bubbles may be dislodged by applying light air blasts with a pasteur pipet
F) Disassemble manifold and mark well locations. Rinse and dry blot as for any Southern blot depending on membrane used.

Colony Lifts for Hybridization Screening

Filter membranes can be sterilized by microwaving for 30".

Low density colony lifts < 5000 cfu/100 mm plate

1) Spread cells on plate and grow until 1-2 mm diameter (app 15 hr). Mark a NC for orientation and aseptically place on the colonies. Do not slide or trap air bubbles under the filter.

Mark the bottom of the plate to correspond with the orientation marks.
2) When the NC is completely wetted peel it off and place it colony side up on growth plate (amp or tet) @ 37ºC for 2 hr (to allow the colonies to reform- do not let them get to big (>1mm) or there will be problems later). Lift the NC filter and place on L agar + 500 µg/ml chloramphenicol @ 37ºC (for amplification).
3) The master plate is incubated until colonies are visible again (app. 2 hr), sealed and inverted @ 4ºC. (Go to step 4)

High Density Screens
1) Place a sterile NC on an agar plate. When it is wetted invert the NC on the plate and spread on it the bacteria (up to 5 x 10^4/100 mm plate) in 100 µl leaving a 3-5 mm border on the NC. Incubate O/N (app. 15 hr) until the colonies are 0.5-1 mm.
2) Transfer the master NC (colonies up) to a sterile paper towel. Prewet a NC (both sides) on an agar plate and place it with no sliding or bubbles on the master NC. Cover with a sterile paper towel and press together with a roller. Mark the filters for orientation, peel apart placing the master in a petri dish and the replica on the plate used to wet it. The original is incubated 2 hr @ 37ºC to reform the colonies and then sealed and stored @ 4ºC.
3) The replica is incubated until the colonies have reformed (app. 2 hr) and then transferred to L-agar + 500 µg/ml chloramphenicol for 12-24 hr.

Lysis and Fixing
4) The NC is peeled from its plate and treated successively by placing colony side up on a 3.5 inch square of 3MM saturated with 7 ml of the indicated solutions for the indicated times:

7 minutes with 0.5 M NaOH
2 minutes with 1 M Tris 7.5
4 minutes with 0.5 M Tris 7.5 1.5 M NaCl

5) Transfer the NC to a dry piece of 'coarse' filter paper (Whatman #1) in a Buchner funnel, apply suction and pull through the membrane first 100 ml 0.5 M Tris 7.5 1.5 M NaCl and then 100 ml $CHCl_3$
6) Air dry the filter then bake 80ºC <u>in vacuo</u>. Bake, colony side up on a single sheet of 3MM or the colonies will stick to filter paper.
7) Hybridize by your favorite protocol.

Alternative Procedures for Hybridizations

Oligonucleotide Probes:
method 1: (for ref:Current Protocols in Molec. Biol. sec 6.4.1) Use the same prewash, pre-hybridize at 42º for 1-2 hours (competitor= denatured Herring DNA). Hybridization contains only Yeast RNA competitor (no DNA) and is at an appropriate temp. (14mer=25º, 17mer=37º, 21mer=42º, 23mer=48º) Wash buffer=6x SSC, 3 mM PB, 0.05% SDS wash 3x 5' at hybridization temp then 3x 5' at 14mer=37º, 17mer=48º, 21mer=55º, 23mer=60º
method 2: Hybridization is in 7% SDS, 250 mM NaPB pH 7.2, 1 mM EDTA. Pre hyb for 30' at hybridization temp then add probe to liquid, mix well and continue hybridization for 8-20 hours. Wash the membrane with 6x SSC, 3 mM NaPB 3x 5' each at hybridization temp. 15mer=42-45ºC, 17mer=45-48ºC.

For library screens (multiple filters) use at least 2.5 ml hyb' solution per 85 mm filter circle. Prepare complete (including probe/competitor) pre-hyb' and hyb' solution right before use (at temp.) and then transfer membranes (one at a time) into the solution. Individual transfer assures

even distribution of hyb' solution over each filter. For 85 mm filter circles High-form, glass, covered 100 mm petri dishes make good containers but should be sealed with Saran-Wrap under the lid to control evaporation. When washing the filters be sure to use ample quantities of wash buffer and occasionally separate the filters to assure complete and uniform washing.

Traditional Hybridization Solutions

Per 10 ml Solution:	**Nitrocellulose**		**Nylon Membrane**	
Stock Solution	**Pre-Hyb**	**Hyb**	**Pre-Hyb**	**Hyb**
20x SSC	2.5 ml	2.5 ml		
10% SDS			100 µl	100 µl
50x Denhardt's	2.0 ml	2.0 ml		
2 M PB	250 µl	100 µl	250 µl	250 µl
50% Dextran SO_4		2.0 ml		2.0 ml
H_2O	4.75 ml	2.2 ml	7.1 ml	5.1 ml
Reserve: (See **Notes** below)				
5 M NaCl			2.0 ml	2.0 ml
Salmon DNA	0.5 ml	1.2 ml	600 µl	600 µl

Use of Traditional Hybridization Solutions:

1) For RNA probes add 100 µg/ml yeast competitor RNA to the Salmon DNA.
2) For Nylon membrane heat the solution to 65º then add 65º NaCl maintain at 65º, until use.
3) Place in a Kapak heat-sealable bag, seal-a-meal bag, or hybridization oven bottle prepare 0.2 ml of Prehy per sq.cm. of blot.
4) Warm Pre-Hyb to 65ºC (see note on NaCl for GeneScreenPlus). Heat salmon DNA in a boiling water bath for 10', add immediately to the Pre-Hyb solution, mix and transfer to the bag. Expel air by pressing the bag between two glass plates. Seal the bag, submerse it in a 65ºC bath and incubate shaking for 4 hr
5) Cut one side of the bag and squeeze out the Pre-Hyb solution
6) Prepare Hyb solution use 0.2 ml/sq.cm. of blot.
7) Warm the Hyb solution without salmon DNA to 65ºC (see note on NaCl for GeneScreenPlus). Mix salmon DNA and appropriate amount of probe (see above); heat in a boiling water bath for 10', add immediately to the Hyb solution, mix and transfer to the bag being certain to distribute evenly, seal the bag and place in a 65ºC water bath. Incubate with shaking for 18 to 24 hr.
8) Wash the blot according to standard procedure

Preparation of Aminothiophenol Paper

Reference: B. Seed. Nuc. Acid Res. 10:1799-1810(1982)

1) Cut eight sheets of Whatman 540 paper to 14x22 cm and place into large heat-sealable freezer bag.

Perform steps 2-7 in a fume hood:

2) Add 70 ml 0.5 M NaOH containing 2 mg/ml $NaBH_4$ to bag.
3) Add 30 ml 1,4-butanediol diglycidyl ether (Aldrich 12, 419-2). **CAUTION:** DIGLYCIDYL ETHER IS CARCINOGENIC!!! WEAR GLOVES AND WORK IN FUME

HOOD.

4) Seal bag with 100-200 ml air space and mix by end-over-end rotation for 8-16 h (overnight).

5) Pour contents of bag into 100 ml 2 M NH_4OH and leave for 48 h in hood before disposal down sink. 58% NH_4OH = 16.55 M ; 2 M NH_4OH= 12.1 ml 16.5 M NH_4OH + 87.9 ml H_2O

6) Wash papers in glass tray in 500 ml 0.5 M NaOH: 95% ethanol (1:1) for 15 min on rotary table.

7) Add the papers individually to 300 ml of 0.5 M NaOH:2-ATP solution (1:1).
2-ATP solution = 2% (w/v) ATP in 95% ethanol made just before use (3.0 g ATP in 150 ml ethanol). 2-ATP = 2-Aminothiophenol Aldrich 12, 313-7. Immediately before adding paper, add 150 ml 0.5 M NaOH.
Agitate on rotary table for 4 hrs.

8) Drain ATP solution into bleach in hood before disposing down the sink. Remove papers and wash in clean glass tray with two cycles of 300 ml 95% ethanol then 300 ml of 0.1 M HCl. Each wash for 15 min. (60 mins. total). Final wash of 300 ml 95% ethanol.

9) Dry papers in dark in air, and store wrapped in saran wrap in dark at room temperature.

Blotting Gels to ATP Paper

Note: plan ahead have all solutions made up.

Preparation of gel for blotting

1) Trim away all but app. 1 cm from either edge of the gel and the gel above the wells. Marker bands are marked by cutting a plug through the gel with a 100 µl glass micropipet. It is advantageous to use bromcresol purple as a tracking dye to monitor pH changes (yellow=acid, purple=neutral or basic).

2) For RNA gels or small fragment blots to ATP paper:
a) At room temp- treat twice 15' each with 50 mM NaOH then 4x15' each with 1 M NaOAc pH 4 <57.5 ml glacial HOAc pH 4.0 with NaOH for 1 L>. The final wash should be in 4ºC NaOAc and move to the cold room for transfer.
b) When you start the second NaOAc wash start the ATP activation
1) add 3.2 ml cold 10 mg/ml $NaNO_2$ to 120 ml cold 1.2 M HCl
2) add the sheet of ATP paper incubate at 4º for 20'
3) rinse the paper 3x with cold 1 M NaOAc pH 4
c) Use 1 M NaOAc pH 4 as a transfer solution

4) Place a glass bridge over a pyrex dish filled with transfer solution. Saturate a 5-layer 'wick' of Whatman 3MM paper with transfer solution and lay it over the glass plate hanging down into the solution.

5) Place the gel on the wick and place Saran wrap so it just covers the edges of the gel and sticks out over the dish. Next place the ATP paper on the gel and then 4 sheets of Whatman 3MM. With light pressure roll out all air bubbles between the wick, gel, membrane and cover sheets. Place a 3" stack of absorbent paper toweling (cut 2" larger than the gel) on top and weight it down with a baking dish of water. Allow to transfer over night. Changing paper toweling can facilitate transfer.

Next day

6) Remove the weight, paper toweling and Saran wrap without moving the gel, blot or filter paper. Invert the gel on the blot and using a soft-leaded pencil (HB) trace the outline of the gel and any markers onto the blot.

7) Place in a pyrex dish in a 50ºC water bath and cover with 1 M NH_4OAc 50 mM Glycine incubate 15' with agitation then decant and treat with the same solution again.

8) Rinse the paper 3x with sterile H_2O then hang to air dry

9) Store dry wrapped in Saran wrap.
10) Hybridize the same as for nitrocellulose (see 'blots')

Filters for Message Fishing

Activation of ATP papers

1) Circles of app. 2.4 cm diameter cut from ATP paper
2) Add 640 µl cold, fresh 10 mg/ml $NaNO_2$ to 24 ml cold 1.2 M HCl, add circles and incubate on ice with occasional gentle swirling for 30'
3) Wash 3x with ice cold H_2O for 1' each wash
4) Rinse 1x with 30 mM NaOAc pH 6.2
5) Place in individual siliclad vials and aspirate as dry as possible or blot to damp on clean 3MM paper

Preparation of DNAs

1) RNA free DNA dissolved in total of 36 µl H_2O
2) Add 4 µl 300 mM NaOH
3) Place in boiling water bath for 5' then chill rapidly in a ice-salt bath

Loading DNAs onto paper

1) Make up fresh 60 mM HOAc <400 µl Glacial HOAc in 100 ml H_2O>. Check for proper titration by diluting 300 µl 300 mM NaOH with 2.7 ml H_2O and then add 1.5 ml of 60 mM HOAc; pH should be between 5.5 and 6.0. Chill the 60 mM HOAc to ice cold.
2) In the cold room, taking care to not warm the tube with your hand, use a cold pipet tip to add 20 µl 60 mM HOAc, mix and transfer the solution to the damp ATP circle. (**Note**: care should be taken to insure that the ATP paper is not dry more than a few minutes and remains ice cold. Likewise the DNA sample should never get above 4ºC during or after the neutralization step.)
3) Incubate the filters O/N in cold room 4ºC
4) Next morning transfer filters in vials to 50ºC water bath and treat 3x for 10' each with 500 µl per filter of:

750 µl formamide; 187.5 µl 8x Pipes hybridization buffer; 30 µl 500 mM Glycine; 532.5 µl H_2O

5) Wash once with hybridization wash buffer prior to adding mRNA samples for hybridization <see 'fishtrans'>

LABELING DNA

Nick-Translation

DNA to be labeled should be relatively free of contaminating RNA and salts. DNAs purified by CsCl centrifugation, PEG precipitation, or extensive extractions and alcohol precipitations work best.

Nick translation is best for probes made from DNAs greater than about 6-800 bp in length. Nick translation of smaller DNAs does not yield good probes nor high incorporation levels owing to the DNAse `nicking' reducing the size of the DNA. DNA fragments of less than about 3-400 bp are very poor substrates for nick-translation, these small fragments make better probes if labeled by T4 kinase or terminal transferase.

Enzyme solution:

To Make	Use	of Solution
1000 µl	153.5 µl	H_2O
100 µg/ml	100 µl	1 mg/ml Acetylated BSA
50%	625 µl	80% sterile Glycerol enzyme grade
0.1 mM	2.5 µl	PMSF 7 mg/ml in isopropanol
1 mM	7 µl	1:100 of 2-mercaptoethanol (or make 5 mM DTT)
5 mM	6 µl	500 mM $Mg(OAc)_2$ (or make 5 mM $MgCl_2$)
50 mM	50 µl	1 M TrisCl pH 7.5
10 µM	1 µl	10 mM $CaCl_2$ (also works at 50 µM)
150 ng/ml	15 µl	10 mg/ml DNAseI either Worthington DPFF 1872 U/mg, BMB Grade II #104 159, or equivalent. Make fresh in 10 mM TrisCl pH 7.5, 1 mM $MgCl_2$, 100 mM NaCl, 100 ng/µl BSA
0.4 U/µl	40 µl	*E.coli* DNA Pol I BRL#8010 (10 U/µl) or Promega M205

Aliquot at 200 µl per tube and store at -80°C

Nucleotide solution:

To Make	Use	of Solution
500 µl	116.5 µl	H_2O
100 µg/ml	50 µl	1 mg/ml Acetylated BSA
2 mM	5 µl	200 mM DTT
50 mM	50 µl	0.5 M $MgCl_2$
500 mM	250 µl	1 M TrisCl pH 7.5
200 µM	10 µl	10 mM dCTP ┐ for labeled nucleotides
200 µM	10 µl	10 mM dGTP ┘ other than dATP substitute
200 µM	10 µl	10 mM dTTP dATP for that nucleotide

1) For each 0.5 µg of DNA to label use 10 µl α-^{32}P-dATP 3000 Ci/mmole (or other labeled dNTP). Isotope in Tricine buffer may be used directly. If the isotope is shipped in 50% Ethanol then it must be speed-vaced just to dryness, DO NOT ALLOW TO BE DRY FOR MORE THAN A FEW MINUTES to prevent radioautolysis. If a lower specific activity probe is required, 'cold' dATP can be supplemented in place of radioactive dATP.

2) On ice add: isotope from step 1
0.5 µg DNA to be labeled
2.5 µl nucleotide solution
H_2O to make 22.5 µl
mix by gently thipping the tube
add: 2.5 µl Enzyme solution
mix, spin lightly and place in 15ºC water bath.

3) Monitor ^{32}P reactions by Cherenkov counting:
a) spot a small aliquot (app. 0.2 µl) on a glass fiber filter
b) dry the filter and count dry
c) wash the filter 3x with 5% TCA and 1x with 10 mM HCl (or cold 95% EtOH)
d) dry the filter and count dry
e) the ratio of counts in the ^{14}C window is the approximate incorporation percentage.

4) The reaction may be monitored by sampling the reaction at different time points as described in '3'. Kinetics should be that incorporation increases through about 50-60', levels off and then begins to slowly decline after 75'.

5) Normally, after 60-70' incubation the reaction is terminated by adding 1 µl 250 mM EDTA per 10 µl of reaction volume and 2-10 µg carrier RNA.

6) For most probes dilute to 200 µl with TE. For extremely clean probes you should $CHCl_3$ extract: dilute to 100 µl with TE, $CHCl_3$:IAA extract and back extract the $CHCl_3$ with 100 µl TE.

7) Purify over G-50 centrifuge column or other spin column (MW cutoff filter). You can either quantitate and use directly (works pretty good for most hybridization probes) or EtOH precipitate for 1 hour, redissolve in 200 µl TE, then quantitate.

8) Determine the specific activity by direct TCA precipitation (see **General Techniques**) of an accurately measured volume of the sample (2 µl). Typically, you should expect 30-60% incorporation of radioactivity

9) Label your sample as to DNA source, date, and specific activity and store at -20ºC. These probes are good for about 2 days.

Note: Certain hybridization membrane/buffer systems may require G50 purification and EtOH precipitation of the probe in order to minimize non-specific backgrounds caused by trace contaminants or unincorporated nucleotides. For many uses G50 purification is sufficient but may not guarantee low background. You must weigh the gamble of your time required for additional purifications, to the time that could be wasted by lost experiments because of high backgrounds. We have had numerous genomic blots of mammalian or plant DNA that have worked fine with G50 purification alone. This isn't a guarantee for your own experience.

PCR Labeling

ref: D. Heiny and D. Rhoads modification of Schowalter and Sommer 1989, Analytical Biochemistry:177:90-94.

For the production of very hot probes. Probably only efficient for amplicons ≤1100 bp. Efficient PCR labeling requires low concentrations of unlabeled deoxynucleoside triphosphates (dNTPs) relative to standard PCR. For longer amplicons (>500 bp) the limiting amount of ^{32}P-dATP causes accumulation of short products. For longer inserts up to around 1100 bp, doubling the dATP concentration usually allows production of full length amplification products. Amplification is from ≤1 ng of a target PCR fragment or insert-containing-plasmid. The size and source of the DNA insert to amplify (amplicon), as well as the buffer formulation can affect the final percent incorporation. Since incomplete extension of the primers results in undesirable small products

(30-100 bp), total TCA precipitable counts are poor indicators of the quality of the probe synthesized. Therefore, for new amplicons or those of larger size you should determine an approximate size for the products by resolving a small aliquot (1/500th of final product) on a small TEB denaturing polyacrylamide gel. Incorporations generally range from 50-90% and produce up to 20 ng of probe at 4-6 x 10^9 cpm/µg. The major advantage of this method over nick-translation or random priming is that final probe is not contaminated with cold template (i.e., specific activity 6-10x higher). One disadvantage of this method is the size limit of the amplified region (amplicon).

The following protocol represents optimal conditions for samples sealed in glass capillary tubes and cycled in a Idaho Technology 1605 Air Thermo-Cycler.

Table 1. Reaction Mixtures for PCR Labelling

Final	For DNA amplicons <500 bp Use (µl)	For DNA amplicons >500 bp Use (µl)	of Solution
20 µl	8.2	3.2	H_2O
1x Buffer*	2	2	10x Idaho Buffer
2.5 µM	1	1	50 µM dCTP, dGTP, dTTP
500 nM	1	1	10 µM forward primer
500 nM	1	1	10 µM reverse primer
50-250 pg/µl	1	1	1-5 ng/µl plasmid containing amplicon
0.125 U/µl	0.8	0.8	3 Units/µl *Taq* DNA Polymerase
0.825 µM	5		α-^{32}P-dATP (3000 Ci/mmol)
1.65 µM		10§	α-^{32}P-dATP (3000 Ci/mmol)

*- Idaho Buffer 1x concentration = 50 mM Tris, pH 8.3; 250 ìg/ml BSA; 1 mM MgCl2; 0.5% ficoll; 1 mM tartrazine.

§ - the amount of total radioactivity may be reduced to 5 µl by substitution (supplementation) with cold dATP (i.e., 5 µl of 3.3 µM dATP).

PCR Parameters:

90°C	30 sec		
90°C	15 sec	┐	
45°C§	15 sec	├	25x
72°C	90 sec	┘	Ramp set to S1
72°C	3 min		

§Anneal temperature should be adjusted for the particular primers in use according to what normally works for the primers being used. Times for anneal and extension may also be adjusted to fit particular PCR methods and machines other than the Idaho Aircycler.

At end of cycling, add 180 µl TE and 5 µg carrier yeast RNA. Purify over G-50 spin column (see **General Techniques**). Quantitate by TCA precipitation and possibly test on denaturing mini gel (see **Acrylamide DNA Gels**). Should be used within one day as a hybridization probe.

Labeling a DNA fragment or oligonucleotide using Terminal deoxynucleotidyl Transferase

1) Mix:4 µl 5x TdT Buffer
   0.5-2 pmole of DNA fragment (200-300 ng of 300-500 bp; 1-2 pmoles of oligonucleotide)

3-10 µl α-^{32}P-dATP (depends on counts needed)
H_2O to 19 µl
0.5 to 1 µl (10 U) Terminal Transferase

2) Incubate 1-2 hours at 37ºC. The longer the better the incorporation and the `hotter' the probe will be.

3) Stop by addition of 180 µl TE and 1 µl 250 mM EDTA. Purify on G50 column (**General Techniques**). These probes can generally be used directly after G50 purification. Expect 5 x 10^7 cpm total from 5 µl of fresh isotope which will mean about 4-5 Adenine residues added per end.

Labeling by Gap Filling or by Oligonucleotide Priming

Gap filling may be used for generation of labeled markers or labeled fragments for S1-mapping or reverse translation sequencing. Oligo-priming is used to generate radioactive probes at high specific activity. Generally, random primers (random hexamer oligonucleotides) are used to prime Klenow fragment synthesis along a denatured target DNA and is usually effective for DNAs >100bp. DNA fragments smaller than about 600 bp should be labeled by oligonucleotide priming rather than nick-translation as the DNAse activity in nick-translation would reduce the average molecule size to less than 300 bp; thus reducing the quality of the probe. Specific primers may also be substituted for the random primers. For example, cDNAs generated by C-tailing into G-tailed plasmids may be labeled using oligo-$(dG)_{12\text{-}15}$ primers or PCR generated fragments may be labeled using the same primers used in the PCR.

Oligo or Random Priming for Labeling

1) In a final volume of 10 µl (use H_2O to bring to volume: combine 100-500 ng (0.5-2 pmoles) DNA with either 1 µg of random hexamer primers (for random priming) or 15 pmoles of oligonucleotides complementary to the DNA ends (oligo-dG primer for C-tailed cDNAs or specific PCR primers)

2) Boil for 5' then snap-cool in ice water. Proceed to step 3 (below) for Klenow labeling by gap filling.

For Labeling by Gap-filling start here:

use restricted DNA fragment with 5' overhang end(s) in 10 µl H_2O. Make sure the restriction digestion leaves a Thymine residue in the 5' overhang so that the ^{32}P-dATP will be incorporated.

3) Add 2 µl 10x Klenow Buffer (500 mM Tris 7.8, 50 mM $MgCl_2$, 10 mM DTT, 100 µg/ml BSA.

4) Add 2 µl 0.2 mM dCTP,dGTP,TTP in 10 mM Tris pH 7.5 1 mM DTT.

5) Add 1-6 µl based α-^{32}P-dATP (10-100 pmoles depending on the required specific activity- of course for gap-filling there is no need for using more than 10-20 pmoles) and add the mix from step 2 which should now bring the volume to 19 µl.

6) Add 2-4 Units of Klenow fragment and incubate 30-60 min. at room temp. (20-24º).

7) Stop the reaction as appropriate: for probles dilute to 200 µl with TE and run over a G50 column for further manipulations dilute to 100 µl with TE, add SDS to 0.05% and phenol:$CHCl_3$-IAA extract, the $CHCl_3$-IAA extract and ethanol precipitate.

Kinase Labeling of a Unique Restriction Fragment end.

This technique relies on removing 5' phosphate groups with alkaline phosphate, labeling with T4 polynucleotide kinase, restriction at an internal site and subsequent purification of the specific

fragment.

Solutions:
5x Kinase Buffer: 300 mM Tris pH 7.6, 75 mM DTT, 50 mM $MgCl_2$ (store @ -20°)

Primary Restriction:
Restrict the DNA with the appropriate enzyme
Dephosphorylation can be with Calf or Bacterial Alkaline Phosphatase CAP or BAP). CAP is more labile and therefore easier to get rid of. For CAP treatment see procedures for Plasmid cloning and transformation

BAP Treatment
1) The DNA should be in 10 mM Tris pH 8.0 100 mM NaCl (BRL React#2 is adequate). Add 20 U BAP/pmole of DNA ends. Incubate 37°C (5' overhang) or 60°C (blunt or recessed) for 1 hr.

pMoles of Fragment Ends

$$pmoles\ ends = \frac{pg\ DNA\ x\ number\ of\ ends}{size\ of\ plasmid\ (bp)\ x\ 660\frac{g}{bp}}$$

2) Dilute to 100 µl with TE extract with: phenol, 50:50 phenol:$CHCl_3$, and $CHCl_3$ twice, backextract with 100 µl TE
3) EtOH precipitate with 20 µl 3 M NaOAc and 1 ml EtOH -20°C >2 hr

Kinase Labeling: labeling is obviously best for dephosphorylated DNAs but can also work less efficiently as an exchange reaction.
1) Spin out the DNA, rinse the pellet with 70% EtOH, speedvac dry and redissolve the DNA in 10 µl H_2O
2) Blunt or recessed ends: heat the DNA @ 90°C for 3', quick chill to 0°C
3) Add 5 µl γ-^{32}P-ATP 1-3000 Ci/mmole.
4) Add 5 µl of 5x Forward Kinase Buffer, H_2O to 24.5 µl and 0.5 µl (12 U) T4 polynucleotide kinase (for 10 pmole 5' overhang, 5 pmole blunt or recessed)
5) Incubate 30' @ 37°C, dilute to 100 µl with TE, $CHCl_3$ extract, 50 µl TE backextraction, run over G-50 column, 150 µl rinse
6) Add 30 µl 3 M NaOAc (may add carrier RNA or DNA if quantity of labeled fragment is low) and fill tube with EtOH -20°C 2 hr

Secondary Restriction and Fragment Purification
1) Spin out the DNA, rinse with 70% EtOH, dry, and redissolve in restriction buffer (14 µl 1x enzyme buffer, 100 ng/µl BSA), add sufficient enzyme (remember the carrier) and incubate several hours at the recommended enzyme temperature.
2) Add stop buffer and load on an acrylamide gel in 1x TBE (no Ethidium) (>600 bp,3%; 300-600 bp,4%; <300 bp,6%) and electrophorese for an appropriate time.
3) Separate the plates, wrap the gel (on one plate) with Saran wrap, spot on the Saran Wrap with Glow-in-the-Dark glue (available at most hobby shops).
4) In the dark room, lay the gel face down on film on top an intensifying screen for 5-10'. Develop the film and cut out the band in the film.
5) Align the glue spots with their respective signals on the film and use the film as a template to excise the band of interest. Fragment may now be recovered from gel piece by electroelution or by crush soak. (see **Gels: Standard**)
6) Redissolve in 40 µl H_2O if you are process for Maxam Gilbert Sequencing

PCR: DNA Amplification

Reagents :

10x PCR buffer	500 mM TrisCl pH 8.3, 10 mM $MgCl_2$, 3 mg/ml BSA (or from enzyme supplier)
2 mM dNTPs	Deoxynucleotides in 10 mM TrisCl pH 7.5, 1 mM DTT
Primers	Oligonucleotides as pure as possible in Te
BSA	Fraction V crystalized, dissolved in sterile H_2O, cleaned by biphasic extraction and dialysis

Procedure Notes:

Typical Reaction

2 µl	10 x PCR Buffer + Mg
2 µl	2 mM dNTP
1 µl	Target DNA (0.1 to 50 ng depending on genome size)
2 µl	10 µM Primer 1
2 µl	10 µM Primer 2
x µl	H_2O to bring to 19 µl
1 µl	Taq Polymerase 1 to 5 U

anneal temperatures for typical primers

SP6, T7, M13 Forward, M13 reverse primers use 45ºC. Other primers are independently determined according to personal experience and can range from 40 to 70ºC.

The Force:

PCR is somewhat of a `magic' art. Many things that make sense don't necessarily work the way you think until you `feel the power of the force.' Especially when it comes to the mathematics and problems of amplification. PCR reactions may fail miserably when you `swear' you did everything the same way, but clearly something was wrong. Amplification will accumulate product much faster as the amplified product increases in concentration. Therefore, although unproven, amplification from minute amounts of template may require longer initial extension times; followed by shorter extension times. Attempts to over amplify may also lead quite rapidly to a degradation of the yield of specific fragments and lead to production of a heterogeneous smear or artifactual bands. Because PCR is driven by relative concentrations of target DNA, primers, dNTPs and enzyme changes in any of these between reactions may give different artifacts. Don't waste a lot of time chasing artifacts. PCRs from small amounts of complex genomic DNA will produce lesser quantities of larger or smaller bands. Adding more bacterial genomic DNA to a PCR will probably produce more artifactual bands and less of your specific band. Don’t just trust the results from PCR on one day unless you can repeat it on subsequent days. Unless you want to submit your findings to the International Journal of Meaningless and Irreproducible Results, don't waste your time chasing artifacts. That is a big reason to run proper controls and repeat the PCR on another day so that you know when you can and can't believe your results.

Another observation worthy of note, at least for Promega Taq polymerase, is that yields are much higher when you `prime' the reaction with an initial concentration of 1-10 ng vertebrate DNA in a 50 µl reaction; otherwise nothing happens. Therefore, when amplifying from small amounts

of input DNA you may need to supplement with a heterologous carrier DNA which is NOT a substrate for amplification (non-homologous to primers). Alternatively, adding BSA at 50-100 ng/µl may replace the carrier DNA. Don't use acetylated BSA, purified fraction V BSA is best.

Mg^{+2} Concentration:

Most of the parameters for a given set of oligonucleotide primers (annealing temperature and extension time) must be determined empirically. Usually, dNTPs are present at 100-200 µM and primers at 0.5 to 1 µM, but for different dNTP concentrations, initial DNA concentration, or degenerate primers where you add 2-3x the concentration, you may need to titrate the Mg^{+2} concentration. Start with Mg^{+2} at 1 mM and run reactions in parallel increasing the Mg^{+2} in steps of 0.25 mM up to a maximum of 2.5 mM.

Cocktail where possible:

Because PCR usually involves multiple reactions you should not set them up individually, but should prepare a cocktail containing all common reagents such as buffer, dNTPs, water, and Taq Polymerase. Usually, reactions vary by either the input target DNA or the primers being utilized. If the target DNA is what varies then add the primers to the cocktail, aliquot target DNA into separate reaction tubes, mix the cocktail containing all other reagents and then carefully add the cocktail to each reaction tube; use the same tip just add the cocktail up on the side of the tube. If the primers are what varies between reactions then the cocktail should include the target DNA and the primers are added to the reaction vessels prior to the cocktail. One big problem in PCR is the small volumes being measured (0.5 to 2 µl) which can vary considerably from sample to sample. Using cocktails can minimize these variations since volumes for the cocktail are much larger and can't vary from one reaction to the next. **VERY IMPORTANT**: When making a cocktail put in the water first, then add everything else except the Taq Pol, mix well, then add the Taq Pol and mix well again.

Primers:

Your choices of primers include those published, sold, or those you design. Primer design is usually best using software and there are many choices of design software. Recommended is Primer3 or Primer4 which are available for use on several webservers. It has many options, that you will need to understand if you are designing your own primers.

How much Taq to add:

Generally Taq polymerase is added to about 1-2 U per 20 µl reaction. Taq can polymerize at least 100 bases per second under optimal conditions. However, Taq is limited by its "processivity," how far it replicates before it falls off (about 500 bp). Therefore, for longer amplicons (>1000 bp) the PCR may fail because the Taq falls off and another enzyme fails to get "loaded" back on to finish the job before the next cycle starts. Adding more Taq can fix this problem or allowing longer extension times. We have seen 1.2 kbp amplicons fail using 1 minute extensions but doubling the Taq to 5U per 20 µl reaction gave great amplification still with 1 minute extensions. In a project amplifying cDNAs we had numerous failures using 5 U Taq in 50 µl PCRs and 1.5 minute extensions. Almost all those failures were large inserts. Doubling the Taq and increasing to 3 minutes solved most of the problems and amplified many inserts of 4 to 6 kbp. Sometimes it just takes trial and error to find the best conditions.

Proper Controls

Because PCR can fail for a number of reasons and can amplify from trace contaminants, **ALL**

PCR EXPERIMENTS SHOULD INCLUDE BOTH POSITIVE AND NEGATIVE CONTROLS. The best positive control is 1-2 ng of a plasmid or appropriate amount of a known positive genomic DNA known DNA. If the No DNA negative control gives a positive band then one or more reagents may be contaminated; throw out the cheap reagents like dNTP, dilute primer, and water out, and then try the experiment again. It is always a good idea to get in the practice of rinsing your pipettor barrel with a squirt bottle of dilute bleach and dry with a paper towel, to reduce DNA contamination on your pipettors.

Theory-

A DNA target and an excess of specific primers are mixed together with Taq polymerase and then taken through successive cycles of DNA denaturation, primer annealing, and primer extension against the template DNA. PCR occurs by multiple rounds of this cycle; whereby 2 copies exist after one cycle, 4 after 2 cycles, 8 after 4 cycles. Theoretically, after 30 cycles there should be 2^{30} (10^9) copies of the amplifiable sequence for each copy available at the start. Therefore, starting with 100 ng of human DNA, PCR should be able to generate 10^{16} copies of a 1 kbp fragment in 30 cycles [hints: 100 ng genomic DNA contains 0.03 pg of a specific 1 kbp fragment; this 0.03 pg=3 x 10^7 copies (see Equation box). 10^{16} copies of a 1 kbp fragment should equal 11 µg of DNA. In practice the yields are far lower (yields of about 100 ng).

Genomic DNA Copies for PCR

$$= \frac{3x10^{-14}g \; x \; 6x10^{23} \frac{molecules}{g}}{660 \frac{g}{mole \cdot bp}}$$

Annealing Temperature-

Approximate primer annealing temp can be determined by comparison of oligo length, GC content and sequence composition relative. These can be compared to other primers to establish the most likely conditions. These three primer attributes affect annealing temperature differently and are each important, however no one has yet established a reliable formula for accurately predicting the Tm for oligo stability. A number of computer programs claim to calculate Tm for oligos they are all just approximations. In our lab we use a Primer Tm Calculator in an Excel spreadsheet that adjusts the Tm calculation by an empirical adjustment for the salt concentration in our PCR buffer. But even that can be off by 5 or more ºC.

Primer Annealing Temperature

$$\boldsymbol{Annealing\ Temp}\ (℃) = \mathbf{2} * (\boldsymbol{A} + \boldsymbol{T}) + \mathbf{4} * (\boldsymbol{C} + \boldsymbol{G})$$

Therefore, until someone establishes a reliable integration of length, and stacking energies, try to use an online or local program to select your best guess for annealing temperature. A simple but flawed alternative is to calculate annealing temp as shown in the accompanying box. Thus, a 20mer of 8 A/T and 12 C/G would give (2x8)+(4x12)=66ºC. Usually, you calculate this simple maximum temperature and then use an anneal that is slightly lower (2 to 4ºC less). In the end you may need to try a couple of different annealing temperatures to find the best.

Extension Time-

When amplifying from plasmid sources (0.1 to 5 ng), extension times have been found to be much shorter than when the target is less prevalent. Taq pol should polymerize about 300 to 500 bp/min at 72ºC, However, when amplifying a 900 bp fragment from 100 ng genomic DNA, I have found that 3-6 min of extension may be required for reliable signal after 30 to 35 cycles. I have also detected 6 kbp amplification products from plasmid templates with extension times of 3 minutes. On the other hand, amplification of a 300 bp region has been demonstrated where the cycle was

92° 20s, 55° 30s and no extension time. This suggests that the enzyme was capable of fully extending 300 bp when only present at its optimal temp for a few seconds. In the end, if you know the primers are annealing at the annealing temp but get no product, then you either need to raise the amount of target DNA (don't go over about 10 ng bacterial DNA/rxn) or increase the extension time. For long sequences from purified target DNAs it may be advantageous to use short extension times when the number of suitable priming events are low and then use longer extension times during the later cycles when the number of polymerase molecules may become limiting due to the large number of potential priming events.

How Many Cycles and Other Issues

What to do when your positive controls work (or are weak) and you aren't getting bands in your experimental. Run more cycles, add more target DNA. You probably need to look at your PCR efficiency and consider how much DNA you need to produce to see the specific band. To see a DNA band in a minigel you really need to produce 5 to 10 ng of that specific band. The following table shows how efficiency can dramatically affect the number of cycles in order to see a 5ng band (yellow highlight) when you start from 2 ng of bacterial DNA or 100ng of human DNA. Adding twice as much template DNA will make little difference if the efficiency is the problem. Adding more template DNA may actually only drive production of artifact bands because if you aren't making the correct product efficiently the Taq polymerase is likely just making more of non-specific products. In qPCR especially it is pretty easy to find reactions that start to amplify something out around 30-35 cycles, but if you do a melt curve on that PCR or run it on a gel you find it is non-specific product.

Cycle	**duplications**			**2 ng DNA from 2.5 Mb Genome**			**100 ng DNA from 3 Gb Genome**		
	2e(cycles-1)			ng in 300bp band			ng in 300bp band		
	Efficiency			Efficiency			Efficiency		
	100%	75%	50%	100%	75%	50%	100%	75%	50%
1	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2	2	0.8	0.5	0.0	0.0	0.0	0.0	0.0	0.0
3	4	1.3	0.8	0.0	0.0	0.0	0.0	0.0	0.0
4	8	2.3	1.1	0.0	0.0	0.0	0.0	0.0	0.0
5	16	4.0	1.7	0.0	0.0	0.0	0.0	0.0	0.0
6	32	7.0	2.5	0.0	0.0	0.0	0.0	0.0	0.0
7	64	12.3	3.8	0.0	0.0	0.0	0.0	0.0	0.0
8	128	21.5	5.7	0.0	0.0	0.0	0.0	0.0	0.0
9	256	37.7	8.5	0.0	0.0	0.0	0.0	0.0	0.0
10	512	66.0	12.8	0.1	0.0	0.0	0.0	0.0	0.0
11	1024	115.5	19.2	0.1	0.0	0.0	0.0	0.0	0.0
12	2048	202.0	28.8	0.2	0.0	0.0	0.0	0.0	0.0
13	4096	353.6	43.2	0.4	0.0	0.0	0.0	0.0	0.0
14	8192	618.8	64.9	0.8	0.1	0.0	0.1	0.0	0.0
15	16384	1082.8	97.3	1.6	0.1	0.0	0.2	0.0	0.0
16	32768	1894.9	146.0	3.3	0.2	0.0	0.3	0.0	0.0
17	65536	3316.1	218.9	6.6	0.3	0.0	0.7	0.0	0.0
18	131072	5803.2	328.4	13.1	0.6	0.0	1.3	0.1	0.0
19	262144	10155.7	492.6	26.2	1.0	0.0	2.6	0.1	0.0
20	524288	17772.4	738.9	52.4	1.8	0.1	5.2	0.2	0.0

21	1048576	31101.7	1108.4	104.9	3.1	0.1	10.5	0.3	0.0
22	2097152	54428.0	1662.6	209.7	5.4	0.2	21.0	0.5	0.0
23	4194304	95249.0	2493.9	419.4	9.5	0.2	41.9	1.0	0.0
24	8388608	166685.7	3740.9	838.9	16.7	0.4	83.9	1.7	0.0
25	16777216	291700.0	5611.4	1677.7	29.2	0.6	167.8	2.9	0.1
26	33554432	510474.9	8417.1	3355.4	51.0	0.8	335.5	5.1	0.1
27	67108864	893331.2	12625.6	6710.9	89.3	1.3	671.1	8.9	0.1
28	134217728	1563329.5	18938.4	13421.8	156.3	1.9	1342.2	15.6	0.2
29	268435456	2735826.7	28407.6	26843.5	273.6	2.8	2684.4	27.4	0.3
30	536870912	4787696.7	42611.3	53687.1	478.8	4.3	5368.7	47.9	0.4

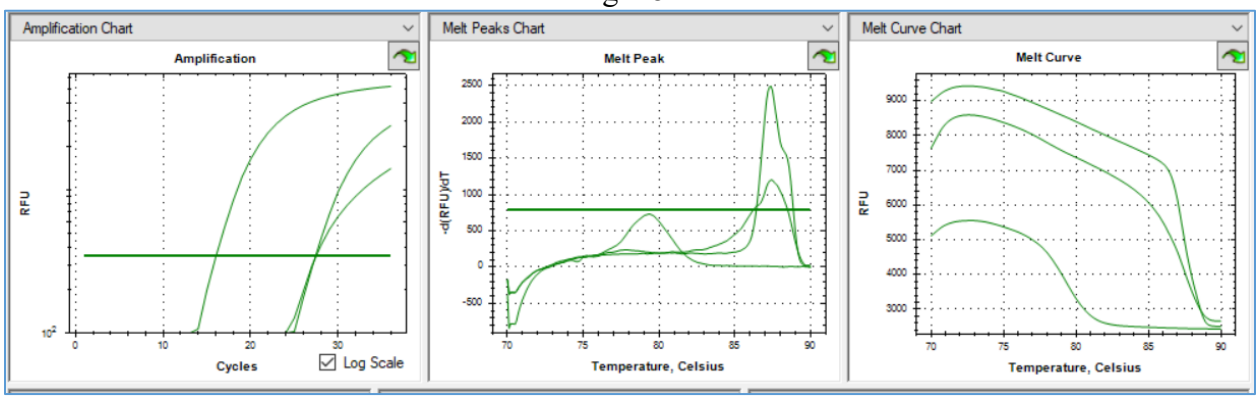
qPCR, Intercalating Dyes and High Resolution Melt, and delta Ct

Intercalating Dyes

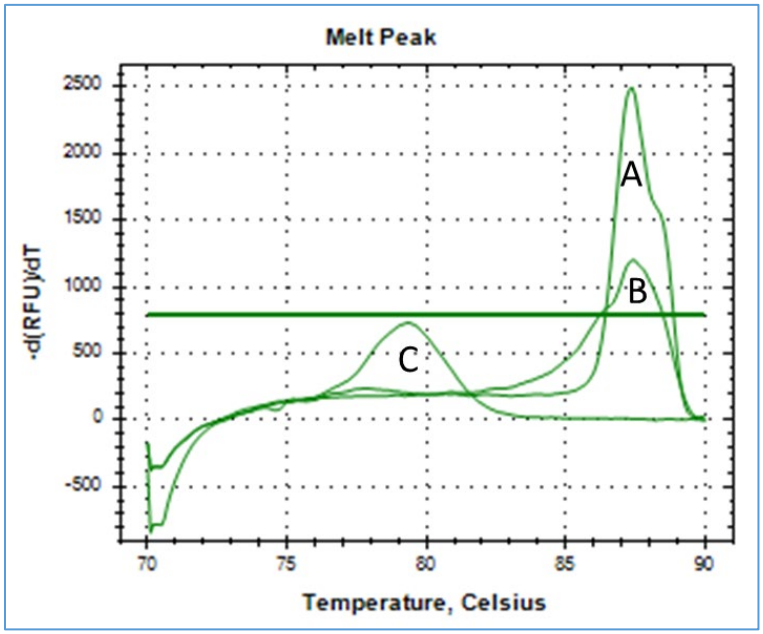
Many qPCR protocols rely on intercalating dyes that fluoresce when bound to double stranded (ds) DNA. This is much like ethidium bromide fluorescent staining of DNA in agarose gels. The dye fluoresces when bound to the somewhat hydrophobic electron rich environment of the stacked heterocyclic DNA bases. So, in the qPCR the more dsDNA product you make the more fluorescent signal you get. The first dye popular for this was SybrGreen, but newer versions have been developed that have slightly better properties primarily related to High Resolution Melt (HRM), which is discussed below. All of the dyes are detected in the same channel as FAM. We investigated SybrGreen, FastGreen and EvaGreen for the quality of their performance for HRM (which is the major difference between different dyes), and found EvaGreen to be the best for HRM discrimination 16S rDNA V2 regions of different Staphylococcus species (Sura Zaki, Dissertation, University of Arkansas).

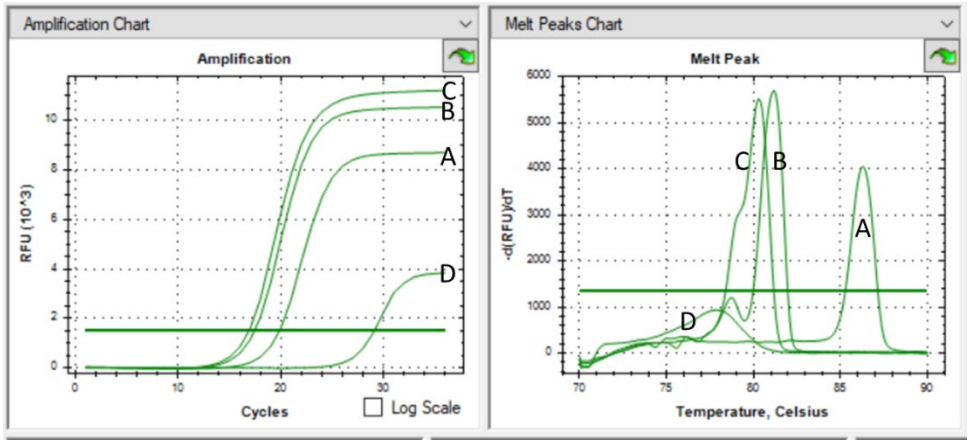
High Resolution Melt (HRM)

During PCR, depending on the conditions (salts, impurities, annealing temperature), the reaction can make specific products or non-specific products. Non-specific products could be so called off-target PCR products from other genomic locations, or primer-dimers. One way of evaluating the quality of the PCR products is to run an aliquot of the product on an agarose or acrylamide gel to determine whether the major product is the correct size, or even clean up the PCR product and send it for sequencing. Gels and/or sequencing can be labor-intensive, or expensive. HRM is an alternative analysis that can be used to assess the specificity of the PCR product when using intercalating dyes in qPCR. Post PCR the sample is denatured then allowed to reanneal and then subjected to a step wise series where the temperature is raised (generally in 0.1 ºC increments) and the fluorescence measured after each incremental step. This will generate a melt curve, which can then be plotted as the integral of fluorescent change with respect to temperature (dF/dT) so that the transition points (melt points) are viewed as peaks. This Figure shows examples of Left: amplification plot (log scale); Middle: melt peaks; and Right: melt curve.



These will be used to understand the need for HRM and then also for calculation of ΔCt. Inspection of the melt curves (larger image to the right with peaks labeled) shows that there is one sample (C) with a HRM peak just below 80 ºC while the other two samples (A & B) have a peak around 88 ºC. Sample A is the positive control DNA, and samples B and C were environmental samples. The qPCR was using the 8F and 936R universal primers targeting the bacterial 16S rDNA. So what we can see from the HRM is that sample B gave a product that melted at approximately the same temperature as the product from the positive control (purified bacterial DNA). Conversely, Sample C gave a HRM peak below 80 ºC which is most likely primer dimer although it could be a larger PCR product that is much lower in GC content, and thus melts at a much lower temperature. So what we can conclude is that sample B is most likely making the specific product (928 bp product of the 16S rDNA), while even though we got amplification in sample C, the product was not the expected one, so we can not, and should not, use the amplification plot (Ct value) for sample C. In a second example (to the right), we have four different samples subjected to qPCR-HRM using the same 16S primers but we see four different HRM patterns. Sample D is likely primer dimer or some other non-specific product. Samples A, B and C, were different bacterial species (A was *E. coli* and B and C were two different

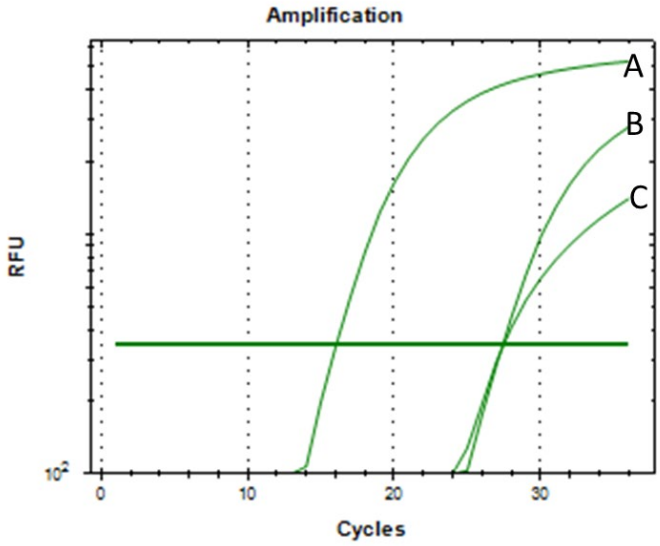




Staphylococcal species). WE can see that the melt point for B and C is significantly lower that A signifying that A has a higher GC content. Not that sample B has a small melt inflection (shoulder peak) around 79 ºC), and C also has a minor inflection (shoulder). These represent regions of the 928 bp product that melt first, followed by the rest of the molecule at a slightly higher temperature. The bigger the PCR product the more likely you are to see 2 or more peaks due to local variations in GC/AT basepairs. These can be quite important for diagnostics. Just be aware that minor differences in salt concentrations can shift the HRM peak by up to a degree.

Ct, ΔCt, and ΔΔCt

Once you have run your qPCR-HRM and flagged the samples with bad HRM (meaning you won't be using those samples), then you can begin analyzing the samples for quantity. The quantity of the input template for the PCR is proportional to the cycle at which the fluorescent signal reaches true logarithmic amplification (steepest increase). The investigator then can set a "threshold" and then determine during which cycle did each sample cross that threshold. So in this example from above (figure on right), we have three samples, A, B, and C), with the horizontal green line positioned so that all three samples cross in their logarithmic phase of amplification. The software then interpolates the Ct value for each sample with A=16.0, B=27.3, and C=27.4. We already learned above that C has a bad HRM so we can not compute any thing from the Ct value from C. But A and B gave "valid" specific HRM peaks so now we can compare the Ct values for A and B. The difference in their Ct values (ΔCt) reflects the difference in the amount of template in each sample put into the PCR. So, ΔCt=27.3-16.0=11.3. Under ideal PCR (see Amplification Efficiency below) the reaction would be doubling the amount of product every cycle, so the relative difference in the input DNA would be $2^{\Delta Ct}$, which in this example is $2^{11.3}$=2521. So if we put 2 ng of DNA into A, the positive control then we would compute the amount of template DNA (not total DNA) in B as 2 ng/2521 = 0.8 pg. Customarily ΔCt is experimental Ct value minus the reference Ct value. So, if the unknown sample (B in this case) has a higher Ct than the reference (A in this case) we get a positive number, but if the experimental gives a lower Ct then ΔCt becomes negative, and $2^{\Delta Ct}$ becomes less than 1. Note that one can also "invert" the math by using $0.5^{\Delta Ct}$, such that you multiply rather than divide, which in the example would be 2 ng x $0.5^{11.3}$ = 2 ng x 0.0004 = 0.0008 = 0.8 pg. The important thing is that you make certain you are applying the math correctly so that when the experimental has a lower Ct than the reference the resulting computations compute a higher amount of target in the experimental, and when the experimental Ct value is higher than the reference you compute a lower amount of target in the experimental. It is important that you run multiple PCRs (triplicates) for computing ΔCt for relative concentrations. Many things can happen to a single PCR (evaporation, poor pipetting, contamination), so if computing the relative concentration is important then run triplicates of the reference, and triplicates of the experimental. Then evaluate each PCR for HRM to discard false Ct values, take the average of the Ct values and compute the ΔCt from the averages. Generally



speaking, if the Ct values for your triplicates have a wider variance than 1 cycle, then you have a mixing and/or pipetting problem. Best to make up experimental and reference mixtures sufficient for 3 reactions and then aliquot to three wells of your qPCR plate (or some variation of that). You can put everything for three reactions into one well of the plate, use a pipettor to gently mix by trituration, and then distribute aliquots to two wells producing three wells containing that PCR mixture.

The next step in this progression is how to compare two different samples such as for two different mRNA samples for different tissues from the same organism, or the same tissue from two different individuals. For these comparisons we use an internal reference, which is assumed to have a relatively constant concentration in both samples (i.e., a mRNA that does not vary in expression levels) so that we can then use that reference as an internal standard to compute a ΔCt for each sample for our gene of interest relative to the reference. There are a number of references that have been proposed but many of those references used have been found to vary quite significantly from tissue to tissue (e.g., rRNA, actin, GAPDH). For further discussion of suitable references see: Radonic, *et al.,* 2004. BBRC 313:856-862, https://www.sciencedirect.com/science/article/pii/S0006291X03025646. For vertebrate samples we have found good references to include TBP, B2M, or RP2B, based on consistent expression across multiple tissues. Ideally the reference selected should have a Ct value close to that for the gene of interest, so do some test PCRs to find the best reference. To then calculate the relative amount of your experimental in each sample you compute the ΔCt for the reference and the gene of interest in each sample, and then the relative difference in expression of the gene of interest in two different samples is the difference in the ΔCt values in each sample, the ΔΔCt. In other words, ΔΔCt= [ΔCt sample 1] – [ΔCt sample 2]. If for sample 1 we get a ΔCt of 5.5 while in sample 2 we get a ΔCt of 3.5, then ΔΔCt = 5.5 - 3.5 = 2.0. So the expression in sample 1 is LOWER than in sample 2 because the ΔCt was MORE POSITIVE relative to the reference. Under ideal PCR conditions (see Amplification Efficiency below) that would mean that the relative expression in sample 1 was $2^{\Delta\Delta Ct} = 2^2 = 4$ times less than in sample 2, As mentioned above, you should never compute ΔCt or ΔΔCt from individual reactions, you need at least triplicates, and many recommend running 5 replicates for ΔΔCt. As mentioned above, if the Ct values for your replicates have a wider variance than 1-2 cycles in your RT-qPCR, then you have a mixing and/or pipetting problem. Best to make up gene of interest and reference gene mixtures sufficient for 3-5 reactions and then aliquot to the wells of your qPCR plate or you can put everything for the replicates into one well of the plate, use a pipettor to gently mix by trituration, and then distribute aliquots to other wells producing the replicate wells for each reference or gene of interest. The best reference for using ΔCt and ΔΔCt is Schmittgen and Livak. 2008. Nature Protocols 3:1101-1108, https://doi.org/10.1038/nprot.2008.73.

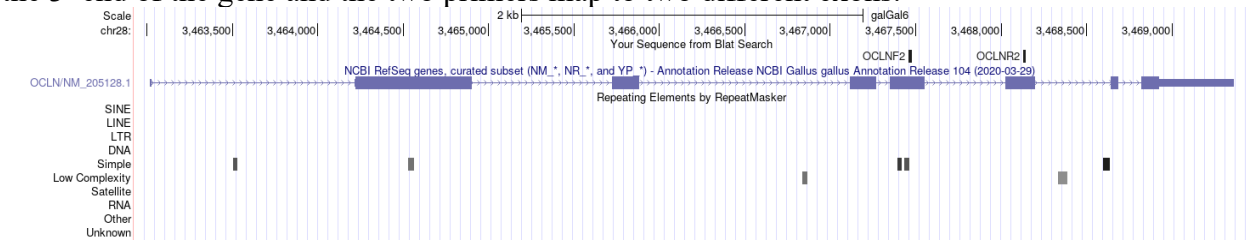
Amplification Efficiency: typically just having the relative amount based on ΔCt or ΔΔCt is enough and you can present the $2^{\Delta Ct}$ or $2^{\Delta\Delta Ct}$ computed relative amounts. However, PCR is never 100% efficient, meaning you never truly double the amount of product in any cycle. If you need to know what the amplification efficiency is then you need to do qPCR (in triplictes) on a dilution series of a known standard so that you can plot the change in Ct (ΔCt) with dilution to determine what the true efficiency of your PCR is which will vary with enzyme concentration, enzyme source, primers, and PCR target.

RT-qPCR Primer Design

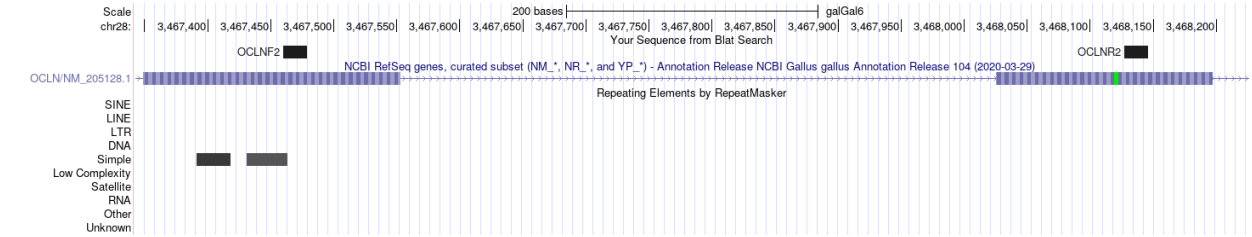
RT-qPCR (Reverse Transcriptase-quantitative PCR) employs intercalating dyes, HRM and ΔΔCt to measure relative gene expression. The technique requires high quality RNA that is converted to cDNA by reverse transcriptase which is then analyzed by PCR. The actual techniques are covered in the sections on RNA Isolation and Analyses for isolation of RNA, First Strand

Synthesis for qPCR, and Two-Step RP-qPCR. The primers for RT-qPCR should be designed to be specific for cDNA and not amplify from genomic DNA (gDNA). The simplest way to do that is to design the primers to exons that flank an intron that is big enough that any contaminating gDNA will not be big enough to allow PCR amplification. It is advisable to design the primers to exons near the 3' end of the gene because if you make cDNA by oligo-dT priming then there is an extreme bias for the 3' end of the mRNA. What follows is two examples for RT-qPCR primers for chicken genes that each present a different need regarding checking your data for whether the amplification is from cDNA or gDNA>

This is a BLAT for the OCLNF2-R2 primers as viewed at UCSC Genome Browser where we see the 3' end of the gene and the two primers map to two different exons.

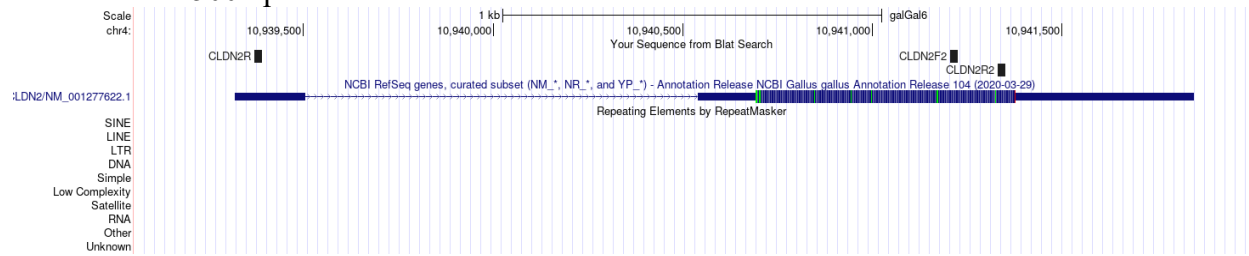


Zoom in on the BLAT hits and you can see that the primers bind in different exons with an intron of ~500 bases in between.



Therefore if you amplify from cDNA you get a product of around ~200 bases but with gDNA you get a product of ~750 bases. Those two products would have different HRM patterns and you could run the RT-qPCR on cDNA and gDNA to see the difference in HRM patterns. You could also verify the product by agarose gel electrophoresis. Once you have identified the HRM for the cDNA and gDNA products then you can monitor your RT-qPCR for different RNA preps to catch any that have gDNA contamination (can't trust those samples for this gene!).

The design for CLDN2 is better because the exon is slightly greater than 1000 bases which makes it more difficult to amplify from gDNA because it is harder to make a 1500 bp PCR product than it is a 700 base pair product and sheared gDNA that might be contaminating might not even be >1500 bp.



So as long as you do the correct testing and verifications then you will know that your RT-qPCR results are valid and represent mRNA levels and not contaminants. If you are lucky your gene will have two 3'-exons that flank an intron bigger than 3 kbp and then you will know that the RT-qPCR will only amplify from cDNA.

RAPD and the Dark Side-

In my experience RAPDs are one of the most frustrating PCR methods when you first start. Again,

owing to the mystical art of PCR, when you first start running RAPDs you will find that you can run a few reactions but that when you run many samples many of them fail. This probably derives from failures in complete mixing of the cocktail or taking too long getting the reactions into the cycler. Don't despair, start out with a few samples (4 or 6) and work your way up. If some of the reactions don't work then back off on the number. Work your way up. Eventually, you will become confident running 40 or 50 samples simultaneously. Your confidence will rub off on the reactions through good will and `the force' and you will `live long and prosper.' RAPD can be frustrating and misleading, but when you know what you can and can not expect RAPD can be quite informative.

Other observation on RAPD besides building up on the number of samples. There are variations between RAPDers, cyclers, and enzyme preps. Don't go switching around or you'll go crazy. First, determine the optimal amount of input DNA, enzyme units, and cycle parameters (time and cycles), then stick with them. Try and do everything the same, day to day. Even switching between cyclers can have drastic consequences. Never compare between separate runs on separate days, you'll go crazy. Only compare between samples run in parallel (and then you still may go crazy). Repeat unbelievable experiments and don't worry until the ubelievable repeats several times with newly isolated template DNA. Finally, many commercial Taq Pol preps are heavily contaminated with sheared *E. coli* DNA. Check your Taq on a fluorimeter for DNA contamination and if it reads over 5 or 10 ng then call and complain. Lots of sheared DNA will lead to meaningless smears in your reactions.

PCR-based Gel Purification

Simple method to purify a PCR amplifiable fragment from a heterogeneous mixture of fragments separated on a gel.

1) Electrophorese the amplification products in an agarose gel. Use a low voltage sample run (20-25V on a mini gel) to slowly separate the fragments for maximal resolution.
2) Excise the gel section containing the band of interest and place in a microfuge tube. The smaller the fragment the better the isolation and yield.
3) Add 1 ml TE to the gel fragment and incubate 10 minutes at room temp. Remove and discard the TE. Add anothher 1 ml of TE and incubate an additional 10 minutes at room temp. Remove and discard the TE.
4) Flame the tip of a 1 ml disposable micropipet tip to seal the tip. Use the sealed tip to crush the gel fragment into small pieces.
5) Add 100 to 150 µl of Te to the crushed gel, vortex, and incubate overnight or several hours at 4ºC.
6) Vortex the sample and quick spin to move the sample to the bottom of the tube.
7) Using a 200 µl pipettor with the tip inserted to the bottom of the tube, slowly collect most of the solution leaving the gel fragments behind. Transfer the solution to a new tube.
8) Use 1-2 µl of the solution in a 20-40 µl PCR using the original PCR conditions and primers for 20-35 cycles. Test 1-2 µl of this reaction on an agarose gel to verify the purity of the amplification products. If minimal other bands are present then the PCR product should be ready to extract and precipitate for use as a probe or for cloning.

PCR-based Plasmid Screening

Simple method for testing colonies for plasmid inserts. Sterile disposable pipet tips can be used in place of toothpicks but the expense is higher than for toothpicks.

1) Items needed are microfuge tubes containing 20 µl of sterile water (one for each colony to test) and a labeled master plate (see Plate Grids in Chapter on Plasmids) divided into labeled sections (one section for each colony to test). Up to 70 or 80 colonies can be streaked onto a single master plate.
2) Using a sterile toothpick (do not use round pointed toothpicks, use the cheap, flat toothpicks autoclaved to sterilize), touch the colony to screen. Keeping the same side that touched the colony to the bottom, drag the toothpick to make a streak of app. 0.5 cm on the labeled master plate. Remember to pick a negative control colony (blue colony in LacZ disruption cloning) and if possible a positive control (plasmid with known insert).
3) After streaking the toothpick onto the master plate, immerse the same end of the toothpick into the 20 µl of H_2O and rotate the toothpick between your thumb and forefinger to twirl the toothpick tip in the H_2O. Discard the toothpick and close the tube.

An alternative to simultaneous picking to the master plate and suspension in water is to toothpick onto the master plate, incubate until visible growth and then toothpick into 100 µl of H_2O. Use this larger volume because more cells will be present. You DO NOT need lots of cells, in fact, visible turbidity will result in a failed experiment due to all of the bacterial debris fouling the PCR.

4) Incubate the Master plate for growth of the clones and then store sealed and inverted at 4ºC until the PCR results are complete. (Bacteria on Master plate are viable for only a few weeks and then must be transfered to fresh media and grown up again).
5) The suspension tubes are incubated for 5-10 minutes either in a boiling water bath or hot block at 90 to 95ºC. These tubes can be used several times if stored at -20ºC.
6) Use 1 to 2 µl of the boil in a 20 µl PCR with primers directed towards the plasmid vector flanking sequences. For pGEM you can use SP6 and T7 primers or M13 forward and reverse primers. Generally, 20 to 25 cycles is sufficient and then 5 µl of the PCR product is electrophoresed to detect which clones contain inserts and how big those inserts are. Remember to take into account the distance between the primer sites on the plasmid. For example, the SP6 and T7 primers will give a PCR product of approximately 100 bp for pGEM5Z with no insert.
7) **Special note on amplification of large inserts: if the inserts are large you can amplify upto 6 kb inserts by increasing the Taq to 5U/20 µl reaction and increasing the extension times to 3 minutes.**

NaOH-based Isolation of Bacterial DNAs for PCR Screening

These methods rely on lysis of bacteria by NaOH and then either direct PCR or purification by MagBind RXNPure Plus beads (Omega Bio-Tek). We recommend you use Biochain PurSil Beads. It is likely that other silica coated paramagnetic beads will work.

BBB- 80% isopropanol 800 mM GuSCN, 8 mM TrisCl pH8, 3.52 mM EDTA. To make add the GuSCN to sterile bottle, add 10 ml sterile water and the sterile TrisCl and EDTA. Heat to 50 ºC for 10 minutes to dissolve. Bring the volume to 20 ml and then add 80 ml of isopropanol. Note: the solution should be clear. If it is yellow the GuSCN is old and should be replaced.

1) Bacteria are suspended in 90 µl sterile water
   a) From overnight culture pellet dilute 10 µl of culture to 90 µl sterile water
   b) From air-sample with impinger pellet the bacteria by centrifugation at 4400 x g for 10 minutes. Decant well, and suspend the pellet in 90 µl
   c) From culture plate toothpick a colony then twirl the toothpick tip in 90 µl sterile water.
2) Add 10 µl of freshly diluted 1 M NaOH, and mix.
3) Incubate for 10 minutes are room temperature
4) For the PCR follow one of these two alternatives:
   a) Direct PCR

i) Dilute with 400 µl sterile water
ii) Use 2 µl in a 20 µl PCR/qPCR

b) Magnetic Bead Purification
i) Add 5 µl Biochain Beads + 100 µl of BBB and mix well (Alternative: add 100 µl MagBind bead solution). For either bead source make certain the beads are resuspended well and at room temperature.
ii) Capture on magnetic stand. While still on the stand pipet off the solution and discard
iii) Remove from stand, add 200 µl 70% ethanol and resuspend the beads
iv) Capture on magnetic stand. While still on the stand pipet off the solution and discard
v) Place the open tube and stand in a 37 ºC incubator for 5 minutes
vi) Remove from stand, add 100 µl Te and resuspend the beads.
vii) Capture on magnetic. While still on the stand pipet off the solution to a clean tube.
viii) Use 1-2 µl in a 20 µl PCR/qPCR
ix) NOTE: This preparation can be scaled up with more overnight culture and will yield ~20 ng/µl based on qPCR analyses.

RapidTip post-PCR cleanup for sequencing

After the PCR, if you want to sequence the PCR product you need to remove all the unused dNTPs, and primers before you send it for sequencing. We use RapidTip from Diffinity Genomics (part of Chiral Technologies). We buy the RapidTips from MIDSCI, but they are also available from SigmaAldrich. We employ a strategy that works well if you follow directions. This is applicable to volumes from 20-30 ul, so if you have less than 20 ul we recommend you add Te or sterile H_2O to bring the volume to at least 20 ul.

1) "Bang" the bottom of the box of RapidTips on the counter top to make certain the dry resin in the tips is down near the bottom.
2) With the sample in a PCR tube or PCR plate, set your 200 ul pipettor (possible to use multichannel when you are good at this) to 100 ul and secure a RapidTip.
3) Pipet the entire sample up into the RapidTip so that all of the liquid encounters the resin (passes above the lower barrier), but not so high as to hit the upper absorbent barrier in the tip.
4) Expel the sample back into it's tube or plate.
5) Repeat steps 3 and 4 one time
6) Pipet the entire sample up into the RapidTip so that all of the liquid encounters the resin (passes above the lower barrier), but not so high as to hit the upper absorbent barrier in the tip and then transfer the sample to a clean 1.5 ml tube (you can use 0.5 ml but they are harder to label). We have found it essential to use a new tube because if you remain in the PCR tube you will have residual excess primers on the walls of that tube that will continue to contaminate your sample.
7) In the new tube pipet the sample up and down at least 3 more times so that the sample has passed over the resin at least 5 times.
8) Quantify your sample by Hoechst flourimetry and proceed with submission for sequencing (add the sequence primer and send to the core lab).

Post PCR Purification using Paramagnetic Beads

Citation: DeAngelis MM, Wang DG, Hawkins TL. Solid-phase reversible immobilization for the isolation of PCR products. Nucleic Acid Res. 1995;23(22):4742-3. PubMed PMID: 8524672
Explanation and procedure:

http://core-genomics.blogspot.com/2012/04/how-do-spri-beads-work.html
The SPRI method employs a carboxyl coated magnetic particle manufactured by PerSeptive Diagnostics, Cambridge, MA. (cat no #8-4125). This company may not exist anymore. An alternative is BioMag carboxyl (84125-10) from PolySciences or Spherotech (CM-05-10H)
HybBuffer: 2.5 M NaCl 20% PEG 8000
WashBuffer: 70% ethanol 10 mM TrisCl pH7.6
ElutBuffer: 10 mM Tris-acetate pH 7.8; or H_2O; or 10 mM TrisCl 0.1 mM EDTA (Te)
Solid-phase reversible immobilization for the purification of PCR products (96-well format).

1. Wash 10 mg/ml carboxyl coated magnetic particles three times with 0.5 M EDTA pH 8.0). You can then store in HybBuffer.
2. For each PCR reaction (50 ul), add 10 µl of washed particles and 50 µl of HybBuffer.
3. Mix well and incubate at room temperature for 10 min.
4. Place the microtitre plate on a magnet for 2 min and wash the particles twice with 150 µ1 of WashBuffer.
5. Air dry for 2 min, then resuspend the particles in 20 µl of ElutBuffer and incubate at room temperature for 5 min.
6. Magnetically separate the particles. Collect the eluate for testing and sequencing.

Size Selection using Paramagnetic Beads

Follows up on the preceding section and is based on this source:
https://research.fhcrc.org/content/dam/stripe/hahn/methods/mol_biol/SPRIselect%20User%20Guide.pdf

1. You can select for larger DNAs by reducing the ratio of HybBuffer
2. Do two rounds of selection with a higher amount of HybBuffer during the first round to select for smaller DNAs and then a lower amount to select for higher weight DNAs
3. Capture using First Capture, then elute in 50 µl of ElutBuffer and do the Second Capture and elute in 50 µl of ElutBuffer. Size ranges are approximate
4. If you are using MagBind or Amp-Pure beads then just use the volumes for DNA and HybBuffer because those beads are already in HybBuffer.

DNA (µl)	Beads (µl)	First Capture HybBuffer (µl)	Second Capture HybBuffer (µl)	Final Size Range
50	10	45	25	175-1300
50	10	42.5	28	200-700
50	10	40	30.5	230-660
50	10	38.5	32	260-575
50	10	37.5	33.5	280-540

Microsatellite amplifications for genotyping

For amplification from genomic DNA for fluorescent detection use 10-50 ng genomic DNA in a 10 µl PCR. Primers are added at 0.2 µM final concentration. For vertebrate genomes, begin with 40 cycles. After PCR the samples are mixed with 5 µl of 90% formamide, 1x gel buffer (0.5xTBE or 1xTEB) and 0.05% bromophenol blue (do not add Xylene Cyanol because it fluoresces in the 600nm range and leaves a splotch at 80 bp). Heat denature the samples (90ºC 5 min) , snap cool in ice water and load 1-2 µl on either a 6% or 8% (depending on size of amplicon) denaturing

acrylamide gel. Electrophorese an appropriate amount of time and then scan. For scanning on the Typhoon, separate the glass plates, the gel is suspended above the platen using two PVC spacers of 3 mm. Place the spacers on the platen and then invert the gel plate onto the spacers. Set the Scanner software to scan +3mm above platen, choose fluorescence mode, set the scan wavelengths for the appropriate dye (see Table below). Generally the PMTs are set to 800V for maximal sensitivity.

Table 2. Extinction coefficient (EC), with maximum Absorbance and Emission wavelengths for common fluorophores

Dye[1]	Ab_{max} (nm)	EC (λ/M·cm)	Em_{max} (nm)	Dye[1]	Ab_{max} (nm)	EC (λ/M·cm)	Em_{max} (nm)
Acridine	362	11,000	462	Erythrosin	529	90,000	553
AMCA	353	19,000	442	Fluorescein	492	78,000	520
BODIPY FL-Br_2	531	75,000	545	6-Fam	494	83,000	518
BODIPY 530/550	534	77,000	554	Tet	521	-	536
BODIPY TMR	544	56,000	570	Joe	520	71,000	548
BODIPY 558/568	558	97,000	569	Hex	535	-	556
BODIPY 564/570	563	142,000	569	LightCycler 640	625	110,000	640
BODIPY 576/589	575	83,000	588	LightCycler 705	685	-	705
BODIPY 581/591	581	136,000	591	NBD	465	22,000	535
BODIPY TR	588	68,000	616	Oregon Green 488	492	88,000	517
BODIPY 630/650	625	101,000	640	500	499	78,000	519
BODIPY 650/665	646	102,000	660	514	506	85,000	526
Cy2™	489	150,000	506	Rhodamine 6G	524	102,000	550
Cy3™	552	150,000	570	Rhodamine Green	504	78,000	532
Cy3.5™	581	150,000	596	Rhodamine Red	560	129,000	580
Cy5™	643	250,000	667	Rhodol Green	496	63,000	523
Cy5.5™	675	250,000	694	Tamra	565	91,000	580
Cy7™	743	250,000	767	Rox	585	82,000	605
Dabcyl	453	32,000	none	Texas Red	595	80,000	615
Edans	335	5,900	493	NED	546	-	575
Eosin	521	95,000	544	VIC	538	-	554

1-As activated NHS-ester with a linker arm. (From IDT web site)
NOTE: terminal G-residues can quench some fluorophores (ex. HEX)

Preparation of Taq Polymerase

References: Pluthero, F. G. 1993. Rapid purification of high-activity *Taq* DNA polymerase. Nucleic Acid Res. 21:4850-4851. doi 10.1093/nar/21.20.4850

Louwrier, A. 1999. Nucleic acid removal from *Taq* polymerase preparations using an aqueous/organic biphasic system. BioTechniques 27:444-445.

NOTES: Do not add DTT or PMSF until right before use. Dilute NP40 and Tween 20 can go bad at room temp after a few years, they may be light sensitive or oxidize. Store dilute, autoclaved detergents at 4ºC.

Solutions	Buffer A (/100ml)	Lysis Buffer (/100ml)	Storage Buffer (/L)	Column Buffer (/10ml)
50% (2.77 M) Glucose	50 mM (1.8ml)			
1 M Tris pH 7.9	50 mM (5ml)	10 mM (1ml)	50 mM (50ml)	10 mM (0.1ml)
2.5 M KCl		50 mM (2ml)	50 mM (20ml)	50 mM (0.2ml)
0.25 M EDTA	1 mM (0.4ml)	1 mM (0.4ml)	0.1 mM (0.4ml)	1 mM (40µl)
300 mM PMSF (52 mg/ml in isopropanol)		1 mM (0.333ml)	0.5 mM (1ml)	1 mM (33µl)
dry DTT			1 mM (153mg)	
40% Tween 20		0.5% (1.25ml)		
10% NP40		0.5% (5ml)		
80% Glycerol			50% (625ml)	
H_2O	(92.8ml)	(90ml)	(304ml)	(9.7ml)

1) Grow pTaq O/N in L-Amp (80 µg/ml) (culture volume dependent on method- see below)
2) Grow culture to mid log phase and induce with IPTG. I have tried three alternatives with roughly equal results
   a) method 1
      i) Dilute 5 µl of overnight to 10 ml in L-Amp (80 µg/ml) at 37ºC grow to late log phase (about 4-5 hours)
      ii) Dilute 5 ml to 1 L in fresh L-Amp at 37 ºC for 3 hours to mid log phase
      iii) Add 100-200 mg IPTG (final 0.25 to 0.5 mM) and grow 6 to 12 hours at 37 ºC
   b) Method 2
      i) Dilute 50 ml of overnight stationary culture into 1L in L-Amp (80 µg/ml) at 37ºC grow for 2 hours
      ii) Add 100-200 mg IPTG (final 0.25 to 0.5 mM) and grow 3-4 hours at 37 ºC
   c) Method 3
      i) Dilute 0.25ml of overnight culture to 50 ml in L-Amp (80 µg/ml) at 37ºC grow for 6 hours to late log phase
      ii) Dilute to 1 L in fresh L-Amp at 37 ºC for 2 hours to mid log phase
      iii) Add 100-200 mg IPTG (final 0.25 to 0.5 mM) and grow overnight (12-15 hours at 37 ºC
3) Pellet cells at 8 krpm 10 min. at 4 ºC
4) Resuspend in 50 ml Buffer A then add 200 mg Lysozyme
5) Incubate 15 min. at 30 to 37 ºC
6) Add 50 ml Lysis Buffer.
7) Note on lysis buffer: NP40 and Tween20 result in 2x higher level of enzyme activity isolated

but the detergents cause the ammonium sulfate precipitate to "float." I have tried TritonX100 but the "floaters" are much less organized. If you leave out the detergent and just add TE+PMSF you don't get floaters but your yield is about 50%

8) Incubate 60 min at 75 ºC with occasional swirling in a glass container. If you use a polypropylene container you will likely lose all or most of your activity (why? I don't know but trust me it is a problem).
9) Centrifuge at 14 krpm in JA20 at 20 ºC for 10 minutes
10) Transfer supernate (discard pellet) to sterile beaker with spin bar,
11) At room temperature, with stirbar mixing to keep crystals suspended, slowly add 30 g of $(NH_4)_2SO_4$ crystals for every 100 ml of supernate.
12) Transfer to 42ml Oakridge tubes
13) Spin 14 krpm in JA20 at 20ºC for 10 min.
14) Carefully decant liquid while saving any pellet. There should be a floating skin on top (most of the Taq is in the floaters)
15) Add 2.5 ml column buffer to each tube, spin in table top swinging rotor at app 1000xg for 1-2 minutes to spin floaters to bottom of tube. Place tubes in shaking 37ºC incubator for about 10 minutes to dissolve pellet. Pool suspensions. For a crude Taq Polymerase contaminated with sheared DNA you can proceed to <u>dialysis and storage</u>. Taq prepared in this manner should be about 100 U/µl and contain about 200 to 300 ng/µl sheared *E. coli* DNA
16) To remove contaminating DNA at this point use biphasic extraction. You can use heparin sepharose column chromatography but you will lose about 80-90% of the activity. Therefore the prefered method for speed and yield is biphasic extraction. Both heparin column purification and biphasic extraction give high quality enzyme but the biphasic extraction is faster, cheaper and gives a higher yield .
17) <u>Biphasic extraction</u> : A. Louwrier (1999) Biotechniques 27:444-445. Taq extracted by this method is suitable for RAPD or ddRT-PCR analyses. Biphasic extraction is easier than chromatography, and appears to be very clean of contaminating DNA, yields are also higher than chromatography.
    a) At room temperature add sterile saturated K_2HPO_4 to 10% (0.11 volumes) and mix well
    b) Add an equal volume of room temperature 95% ethanol and mix well. (note: the published procedure calls for room temp but I have done this at 4ºC. The precipitate is noticeably larger at 4ºC but the yield is still high
    c) Spin 5 minutes at about 2000 x g at room temperature (or 4ºC)
    d) The upper organic layer (about 90% of volume) contains the Taq and the lower layer contains the DNA. The lower layer looks like a precipitate; most of the volume will be the upper ethanol layer. I do the ammonium sulfate precipitation first and then the biphasic separation but with the concentration so high the lower layer is more of a gel than a layer. For some reason there still appears to be a lot of ADNA@ in these preps when you assay by Hoechst fluorescence (200 ng/µl). However, if you test some of this stuff on an agarose gel you see NO DNA staining with ethidium. If you do that with the crude Taq you see a big smudge of DNA.
    e) Collect the upper layer and proceed to the dialysis step.
18) <u>Dialysis and storage</u>
    a) Some things I have found to work
        i) For biphasic extraction, before proceeding to dialysis store at 4ºC for an hour or so, then spin again to get another smaller pellet
        ii) For biphasic extraction you can Speedvac© off the ethanol over about 4 hours
            (1) divide into 2 each 15 ml conical disposable tubes
            (2) place in speedvac with tube lids partially unscrewed

(3) start centrifuge then vacuum
(4) after 3-4 hours the volume should be about 25-30% and easier to dialyze

iii) to save on glycerol I have used Storage buffer without glycerol for the first 2 dialsysis steps, then used Storage buffer with glycerol for the final dialysis step. This saves on glycerol and the volume of the dialysate is not as reduced (enzyme not as concentrated). Word of Caution: sometimes using glycerol only in the final dialysis the final concentration is lower and the Taq prep will freeze at -20ºC.

b) Dialyze 3x for 2-3 hours against 25-50 volumes of Storage Buffer using 3500 to 3500 to 8000 M.W. cutoff membrane at 4ºC. Usually the bag really collapses through loss of water so don't worry, the membrane didn't leak.
c) Collect dialysate
d) Aliquot at 0.5 ml in screw cap microfuge tubes and freeze at -85 ºC for storage

10x Taq Polymerase Reaction Buffer

1) Do a biphasic extraction of BSA.
   a) Dissolve BSA (fraction V, Tissue culture grade; e.g., Intergen 3305-01) in sterile deionized water at 20-30 mg/ml. Perform the biphasic extraction.
   b) At room temperature add sterile saturated K2HPO4 to 10% (0.11 volumes) and mix well
   c) Add an equal volume of room temperature 95% ethanol and mix well.
   d) Spin 5 minutes at about 2000 x g at room temperature.
   e) The upper organic layer (about 90% of volume) contains the BSA and the lower layer contains any trace DNA contaminant.
   f) Dialyze against sterile water.
   g) Quantify by Biorad DC assay. Store aliquots frozen at -20ºC
2) Prepare sterile autoclaved solutions for the following:
   a) 1 M TrisCl pH 8.3
   b) 0.5 M $MgCl_2$
3) Mix the components to produce 10x Taq Polymerase buffer at 500 mM TrisCl pH 8.3, 10 mM $MgCl_2$, 3 mg/ml BSA.
4) Aliquot and store frozen at -20ºC.

PHOTOGRAPHIC TECHNIQUES

Autoradiography

Xray film should be handled only under safe light conditions. Prefered low intensity amber light although red light can work if time is minimized. Do not handle film with latex gloves or dirty oily fingers. Wash your hands, rinse and dry before handling film or cassettes. BE VERY CAREFUL TO NOT CONTAMINATE THE FILM OR CASSETTES WITH DEVELOPER OR FIXER. Developer spots dry to form brown spots while fixer dries as a white crust. Clean up spills or dribbles with a wet sponge or towel. NEVER handle the film box or cassette in the vicinity of the dark room sink or dip tanks. Keep the developing area and film handling area separate. If you touch any of the developing tanks or sink then be sure to rinse off your hands and dry them before leaving the sink area to handle the film, handle the cassette or touch anything else. Leaving a dirty dark room is grounds for capital punishment or at least notification of your parents. Getting developer or fixer on the box of film means certain painful death from the people who's experiments you ruin. No one enjoys bringing an ARG out of the freezer after 10-14 days exposure and finding big ugly finger prints in the middle when they develop it.

1) Exposure is in light tight cassettes with the gel, or blot pressed securely against the film. Metal cassettes usually have a foam or felt lining that presses the film against the gel or blot. For paper cassettes you should clamp the cassette between sheets of thin plywood using bulldog clips.
2) Intensifying screens increase the signal by absorbing radiation that misses the film and then phosphorescing which exposes the film. The screen is placed on the opposite side of the film from the blot or gel during exposure. A screen will generally increase the signal strength by about 5 to 10 fold but will result in fuzzier bands or exposure signals.
3) Exposure is usually best at <-60ºC which will maximize signal. Wrap the cassette in a plastic bag to prevent frost or condensate accumulation on the cassette. Label for easy identification with your name and start and anticipated stop dates for the exposure.

Steps 4 through 7 must be under safe light conditions.

4) To develop the film remove from the cassette (don't need to warm up just remove the film and return the cassette to the wrapping plastic bag.
5) The film is immersed in Xray developing solution for several minutes (development is complete within 5 minutes based on the freshness of the solution). Old developer turns dark brown and then needs to be replaced.
6) Dip the film in water or stop bath (app. 0.5 to 1% acetic acid)
7) Immerse film in fixer until the foggy background is completely gone from the film. Usual times are a few minutes for fresh fixer. Once the film has been completely immersed in the developer for a few seconds it is safe to turn on the regular room lights.
8) Rinse the film well with tap water and then deionized water. Hang to dry. Caution: the film emulsion is quite soft until dried and will scratch easily.

To Amplify or Intensify an ARG Signal:

Reference: Richard L. Mitchell @ Salk Inst.(198?) Focus 8:3 (BRL Gibco)

All operations in room light at room temp. Use Kodak Chromium Intensifier (Bleach Bath and Clearing Bath)

a) soak ARG in H_2O for 10'
b) Soak in Bleach Bath for 5'
c) Rinse with H_2O

d) Soak in Clearing bath 2'
e) rinse with H_2O
f) Redevelop by immersion in Dektol 1:3
g) Intensification process may be repeated to build up the image

GROWTH & HANDLING OF BACTERIAL CULTURES

General Guidelines

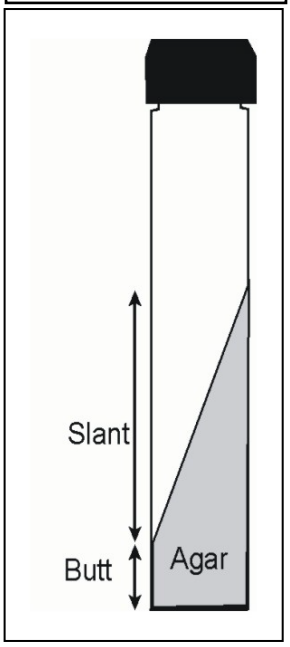
1) Most molecular experiments or cloning operations involve manipulation of micro-organisms. These versatile hosts are the life-blood of your experiment. This means that in order to be a good molecular geneticist you must also be a good microbiologist. You must understand the needs and properties of bacteria such as *E. coli* and how to handle organisms and solutions in a sterile manner. Contamination or abuse of your micro-organisms or solutions can lead to lost or failed experiments.
2) Work in a clean area. The best place is in a laminar flow or culture hood but most operations are fairly safe out on a clean lab bench. Before beginning work you should wipe down the work area to clean up dust and possible contaminants. I recommend using a dilute solution of bleach (about 10% bleach). Pour or squirt enough of the dilute bleach onto the counter top and then use a damp paper towel to wet (just moist) the entire area. Dry the area with a clean paper towel before beginning work. Don't lean on the counter top or the remaining small amounts of bleach will get on your clothing and eat holes in it.
3) Learn how to work around a Bunsen burner and how to properly flame bottles and tubes. You pass the unopened tube/bottle top through the flame. You do NOT hold it in the flame. Passing through the flame will instantly kill surface bacteria, and burn-off lint that might be on the surface. After passing the unopened bottle/tube through the flame, remove the lid and then pass the opened bottle/tube top through the flame. If you work *near* a flame then the flame creates an updraft which will carry airborne bacteria up and away, rather than letting them settle into your work/solutions/medium. If you are melting plastic on the lids or openings then you are not flaming, you are holding the item in the flame, not passing it through.
4) Clean your micropipettors. Dampen a paper towel with 10% dilute bleach and wipe down the outside of the tip barrel and the end of the pipettor that goes into the pipet tip. Do this before you start your microbiological work. Be VERY careful when pipetting bacterial solutions because if you pull the solution into the pipet tip too fast you can create a small aerosol within the tip that will carry bacteria INSIDE your pipettor where they can multiply and then contaminate all your future experiments.
5) Sterility of solutions for microbiological work is absolutely essential. If there is any doubt throw it out. Swirl the bottle to look for turbidity which could be bacterial growth. Sometimes Tryptic Soy Broth will have a natural precipitate, but not in Luria Broth. To maintain sterility never insert anything such as a pipet or micropipettor into a stock bottle of microbiological solution. This includes growth media, and solutions used to treat culture cells (i.e., $CaCl_2$ for making competent cells, 80% glycerol for making frozen cultures, and growth supplements; items stored for weeks or months at 4 ºC for multiple uses). These solutions should only be used in a clean area away from dust and drafts. Open the bottle and pour what you need into a sterile container. You can then pipet from this "working" solution with the remainder being thrown out. Don't work with the stock bottles around your cultures or DNAs as this increased the odds of contaminating the stock and ruining subsequent experiments.

Short Term Storage

1) **Slants or Stabs** screw cap tubes containing agar solidified growth media.

A) For most *E. coli* slants are constituted of L-broth, for yeast use YPD, for either use 1.5% agar. The medium is boiled to melt the agar, thoroughly mixed and then, while molten, dispensed at about 4.5 to 5 ml into screw cap 16 mm glass culture tubes. The tubes are capped and autoclaved to sterilize. After autoclaving the tubes are inclined to angle the agar surface to about 1/2 to 2/3 up the side of the tube leaving about a 1/2 inch `butt' of agar solution along the bottom of the tube. Whole racks of tubes can be slanted to solidify. Allow to completely cool to room temp and then make sure all caps are on tight. Stabs are like slants but are usually only 1 ml of medium in a screw cap 1 dram vial and are allowed to solidify standing upright.

Figure 7. A Culture Slant



B) Store at 4ºC until use. Slants should have a small amount of visible liquid on the butt surface signifying a moist slant; if the cap is not on snuggly this will dry out and you shouldn't use dry slants. For some cultures that require antibiotics the antibiotic (Tet or Amp) can be added to the small amount of liquid, then angle the slant to distribute the solution and allow the drug to diffuse into the agar by incubating at an angle for 30 min. at 37ºC before inoculation.

C) **Inoculate** with a sterilized loop. Collect a small amount of culture (broth or colony), mix the culture into the liquid on the slant and then use the loop to smear the liquid using circular movements over most of the surface of the slant. For stabs the loop containing the culture is `stabbed' down into the agar to the base of the tube. Slants thus have growth on the agar surface while stabs grow in the stab wound. Stab cultures grow slower, dry out slower, but are more difficult to collect cells from. Stabs are a good way to mail cultures as the vials are smaller and less subject to breakage than larger slant tubes. Incubate the inoculated tubes at the appropriate temperature (usually 37ºC). *E. coli* slants should never be incubated more than 24 hours. Store the grown up slants at 4ºC.

D) **Transfer slants regularly**, bacteria and yeast die during storage at 4ºC. Death is slow, but increases with storage time. Therefore, you MUST transfer the cultures routinely every 30 to 40 days. If slants are stored longer than that then mass death will leave only odd variants of the original culture. These variants do not have the same properties as the parent culture, grow strangely, and if it was a plasmid bearing strain will give very poor yields, if at all. Make TWO slants for each culture. Label one `Work' and one `Stock'. Only use the work slant during the month of storage for your experiments. The Stock slant is ONLY used for the transfer to make the two new slants for the next month. After the new Stock and Work slants are grown up then throw out the old slants. By making a Stock slant that is only used to make the new slants then you minimize the possibilities of contamination of your culture stock.

2) Streak Plates

A) Several different clones can be stored temporarily on the same plate. It is very easy to store up to around 20 different colonies or clones on a single plate. Do not try this with bacterial stock strains since cross contamination is possible.

B) Clones are usually transfered with sterile toothpicks. Use the cheap flat toothpicks not the round sharp pointy ones which will tear the agar too easily. Toothpicks are sterilized by autoclaving a bunch in a 50 ml beaker (so the tops just stick out of the beaker where you can get a hold of them without sticking your dirty fingers into the beaker) capped with a double or triple layer of foil.

C) Using a permanent marker, label the bottom of the plate either with clone numbers or otherwise to indicate the area for each clone. (See Figure in Chapter on Plasmids **Colony Picking Grids for Master Plates**)

D) The sterile toothpick is touched to the colony or clone to streak and then the same tip and side of the toothpick that touched the colony is drug lightly across the surface of the new plate for approximately 5 to 10 mm directly over the marking on the bottom. Only one single pass to make one streak is necessary. The impression and `bruise' on the surface of the new plate will be visible.

E) After all streaks are made incubate the plate overnight for growth.

F) Plate cultures should be stored at 4ºC inverted in plastic sweater boxes or labeled zip lock bags. Unfortunately, condensation will accumulate in the bag or box and can run or fall into the upturned lid leading to contamination. You can seal the edges of the plate with a strip of parafilm to guard against this possibility.

G) If you use plates for short term storage then you should transfer your cultures every few weeks and routinely examine for accumulation of condensation. Use a paper towel to remove accumulated condensation before it ruins your plate. The safest solution is if the culture or clone *may* be valuable, **THEN FREEZE IT FOR LONG TERM STORAGE**

Long Term Storage

One of the most convenient ways of storing bacteria or yeast is by frozen storage at -70ºC or lower in glycerol.

1) Grow the organism to late log phase (stationary phase also works).

2) Mix the culture with an equal volume of sterile 80% glycerol (autoclaved to sterilize). Other cryoprotectants may work such as 25 to 50% Bovine Serum, or 50% powdered milk, or 7.5% DMSO but the glycerol method appears to work for most microorganisms and is very inexpensive.

3) Aliquot the glycerol-culture mixture at 1 ml into several screw cap sterile plastic tubes (cryo tubes or microfuge). Don't use glass as it will likely shatter upon freezing.

4) Label the tubes well but only use permanent markers and only use labeling material designed for use at ultra cold temperatures. Most regular lab tape or paper labels will fall off in the ultracold.

5) Transfer the tubes to the ultra cold (generally into a labeled box or other storage system). **Do NOT quick freeze in a dry-ice ethanol bath or liquid nitrogen, the normal freezing rate is best.**

6) To recover the culture, remove a tube from the ultra cold freezer and warm by rolling between your thumb and fingers to thaw. Pipet 20-100 µl of the culture into 1 to 10 ml of medium. Return the tube to the ultracold, most bacterial and yeast cultures can be frozen and thawed several times (4 or 5 at least). After that many cycles you should plan on freezing more tubes.

7) Incubate the inoculated broth for several hours or overnight for growth.

Notes on Media for Bacterial Cultures

Media:

- TSB/TSA- Tryptic Soy Broth/Agar
- LB- Luria Bertani medium
- THYE- Tom Hewitt 1% yeast extract
- BHI- Brain Heart Infusion
- CO- ChromAgar Orientation
- CS- ChromAgar Staphylococcus

1. *E. coli* grows well in LB broth or agar
2. Staphylococcus species will grow far better in TSB (tryptic soy broth)
3. You can distinguish many different genera and species based on colony color, sheen, and size using CO. For Staphylococci also use CS.
4. For growth of *Enterococcus cecorum* it will grow under aerobic conditions at 37 ºC on CO, TSB, BHI or THYE. It grows better in 5% CO_2. On any of these media this organism seems to wear out with subsequent passages. Adding 5% chicken serum (Gibco) seems to be essential for extended passage and yields much more luxurious growth. The best is TSB+5% chicken serum, based on final turbidity of overnight growth.

Antibiotic Selection and Growth

Most bacterial plasmid vectors are selected for based on antibiotic resistance conferred by a vector based gene such as Amp^r or Tet^r. The episome (plasmid) is only necessary for cell growth when the drug is present. Even though most modern plasmid vectors are so-called `high-copy' plasmids present in 2-20 copies per cell, this is only under drug selection. In the absence of the selective drug some cells will be produced that lack the plasmid. In the absence of the antibiotic these cells are not inhibited and may actually be at an advantage as they do not have plasmid replication/expression as a drain on the `cellular economy.' Therefore, after prolonged culturing in the absence of the selective drug much of the culture may be cells that lack the vector. Most antibiotic resistances are based on inactivation of the drug. Ampicillin resistance is by secretion of a β-lactamase that cleaves the lactam ring in the ampicillin molecule. Growth of even the resistant cells does not occur until sufficient ampicillin has been inactivated to fall below the inhibitory levels. Ampicillin is bacteriostatic, and is only bacteriocidal upon long term exposure. The best example is from selection of Amp^r colonies from a plasmid transformation. If lots of cells are plated the β-lactamase from the resistant cells will inactivate the ampicillin in the medium and you can see smaller colonies arising in a halo around the larger colony. These smaller `satellite' colonies are not resistant, they represent cells that stayed viable long enough until the bacteriocidal drug levels had decreased sufficiently to allow growth.

1) **Large scale culturing** of plasmid carrying bacteria should be a multi-step process involving stages of drug selection. That is to say, never grow large amounts of culture from very small starting inoculations. For maximal plasmid yield the final culture should be from inoculation of fresh antibiotic medium at no less than a 1 to 100 dilution of an actively dividing culture. To grow 10 ml of culture from a small inoculum you should first inoculate 0.2 to 1 ml of drug containing medium. Incubate with shaking for several hours until growth is very evident, then add 10 ml of fresh antibiotic medium and continue the shaking incubation for several hours or overnight. To grow a 1 liter culture start a 10 ml culture in

drug medium in the morning and in the afternoon when turbidity is very evident dilute into 1 liter of prewarmed drug-containing medium; continue shaking overnight to harvest in the morning.

2) **Prewarmed medium** is used for culturing to speed the growth process and to avoid a temperature shock for the culture that may induce a lag phase. If you inoculate a culture into cold medium and then place in a shaker incubator the medium may take several minutes to even an hour to equilibrate to 37ºC. During this time the bacteria will be induced to alter their membrane components and change gene expression patterns to adapt to the colder environment. Once the culture medium warms up they will have to re-adapt to that environment. Therefore, actively dividing cultures are happiest if any dilutions or introductions to new medium are into pre-warmed medium. Even recovery of cultures from cold storage will be faster if the inoculation is into 37ºC broth rather than 4ºC broth. One word of caution about pre-warming your media in a water bath, **MOST WATER BATHS ARE FILTHY DISGUSTING CESSPOOLS FULL OF BACTERIA, YEASTS AND FUNGI**, many of which are resistant to the antibiotics. If you use a water bath to warm your media then make sure the water bath contents do not get near the bottle lid or openings, after removal from the bath dry the bottle surface well and wash your hands. Otherwise, you may end up culturing organisms from the water bath rather than what you wanted to. Consider using fresh water in the bath or else warm the media incubation for sufficient time in the shaker incubator.

3) **Aeration** of most cultures is important for maximal growth. To get a good oxygen supply to the entire culture then you must have good shaking movement and the correct **vessel volume and geometry**. Straight wall vessels such as tubes should be incubated with shaking at an angle of 30 to 45º. The angle causes the solution to `slosh' up the side and then fall back trapping gases for exchange. The mixing also keeps the cells suspended in the medium. For flasks the shaking accomplishes the same mixing and aeration. If the medium volume is too great for the vessel then mixing will be insufficient and the bottom of the culture will become somewhat anaerobic. Also, if the medium volume is too great or the agitation is too violent then the medium will be splashed up near the opening of the vessel (lid) which means contamination is more likely to occur and/or spillage of the culture.

Table 3: Recommended Vessel and Culture Volumes to Maintain Good Mixing and Aeration

Medium volume	Recommended culture container
0.1 ml	10 ml slip cap tube
	2.2 ml microfuge tube
	1.5 ml microfuge tube inclined to horizontal
1 ml	10 or 15 ml slip cap tube
	15 ml conical screw cap tube
10 ml	40 to 50 ml Oakridge centrifuge tube or conical screw cap tube
	100 ml Reagent bottle
20 ml	100 ml Erlenmeyer flask or 100 ml Reagent bottle
50 ml	250 ml Erlenmeyer flask or 250 ml Reagent bottle
100 ml	500 ml Erlenmeyer flask or 250 ml Reagent bottle
250 ml	1 liter Erlenmeyer flask or 1 liter Reagent bottle
500 to 1200 ml	2800 ml Fernbach flask

Appropriate covers for † flasks are cotton stoppers covered with a single layer of aluminum foil, metal slip cap flask closures, screw caps for screw cap flasks or a double or triple layer (to minimize tears) of aluminum foil. The closure should cover the neck for 1-2

inches below the top

4) **Long term growth**: it is generally not a good idea to over incubate your culture. Petri plates will dehydrate at 37ºC even when inverted. Bacterial cultures should never be incubated more than 36 to 48 hours at 37ºC as they exhaust the medium and accumulate metabolic byproducts which may be somewhat toxic leading to cell death. The same goes for liquid cultures. Once the culture has reached maximum turbidity (stationary phase) it is not a good idea to continue incubation as the culture will start to die with cell lysis, etc., leading to loss of plasmid yield. In most cases, liquid cultures should not be incubated more than approximately 24 hours.

Densitometry for Estimation of Bacterial CFU/ml

This method must be empirically calibrated for each isolate/species

Establishing a bacterial densitometric curve

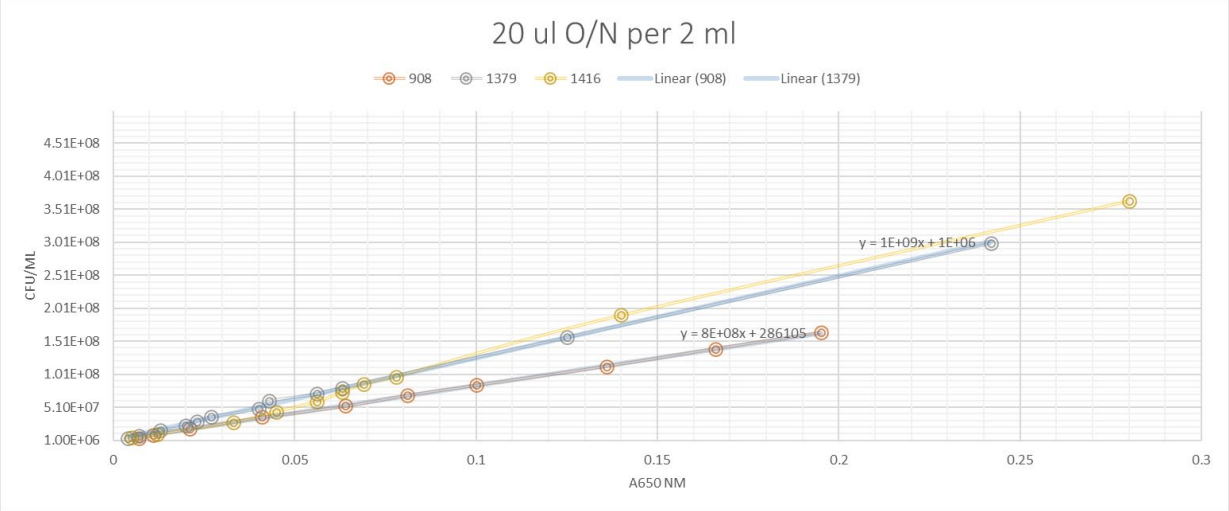
1) Grow the bacteria overnight in broth (Tryptic Soy, or Luria).
2) You need several 9 each 13 mm glass tubes that don't have scratches or smudges on them.
3) Turn on the GenSys UV spectrophotometer to warm up and auto-calibrate.
4) Pipet 2 ml of medium into each tube. The medium could be RPMI, DMEM, TSB, etc. It could also be water if the bacterium is not sensitive to pure water.
5) Add 2, 4, 8, 12, 20, 40, 80 and 120 ul of overnight culture to different tubes (10^{-2} dilution)
6) Use the other tube as the blank to "zero" the spectrophotometer at 650 nm
7) Record the absorbance of the diluted bacteria tubes in the chart
8) Do a series of 10^{-1} dilutions (100 ul +900ul) in broth of the overnight bacterial culture to plate triplicate plates to determine the viable CFU/ml in the overnight. For most overnight cultures the stationary culture will be between $2x10^9$ to $2x10^{10}$ CFU/ml, so best to plate triplicates at 10^{-7}, and 10^{-8}
9) Then compute the number of CFU/ml in the absorbance measurements
10) Use Excel to generate a graph of A650 vs CFU/ml. If you have Excel generate a straight line for the datapoints you can capture the y=ax+b formula for that line

Bacteria (ul)	Final Volume	Abs 650 nm	Computed CFU/ml
2	2002		
4	2004		
8	2008		
12	2012		
20	2020		
40	2040		
80	2080		
120	2120		

Using a bacterial densitometric curve

11) Grow the bacteria overnight in broth (Tryptic Soy, or Luria). The CFU/ml will likely be higher in Tryptic Soy broth)
12) You need two 13 mm glass tubes that don't have scratches or smudges on them.
13) Turn on the GenSys UV spectrophotometer to warm up and auto-calibrate.
14) Pipet 2 ml of medium into each tube. The medium could be RPMI, DMEM, TSB, etc. It could also be water if the bacterium is not sensitive to pure water.
15) Add 20 ul of overnight culture to one of the tubes (10^{-2} dilution)

16) Use the other tube as the blank to "zero" the spectrophotometer at 650 nm
17) Read the absorbance of the diluted bacteria tube.
18) Use the formula or graph for that species to estimate the CFU/ml in the 10^{-2} dilution tube
19) Example graph for *S. agnetis* 908 and 1379



20) Notice that 908 and 1379 have different slopes so that at equal A650 the CFU/ml of 1379 is twice that of 908. Excel computes the formula for 908 as $y=x10^9+10^6$, where y is the CFU/ml and X is the A650
21) Typically, an overnight of 908 in TSB will be between $1x10^{10}$ and $1.5x10^{10}$ CFU/ml (so the 10^{-2} dilution should read between 0.075 and 0.14 A650.
22) You can then make a dilution series such that a 10^{-6} dilution should be about 1000-1500 CFU. Note that this method is only about 50% accurate so that the actual CFU/ml could vary by 0.5x or 2x.
23) Note that if you do the dilution series you need to know whether the strain you are using is sensitive to the diluent. We have found that *S. agnetis* 908 loses viability if you do the dilutions in pure H_2O.

BACTERIAL AND PLASMID MOLECULAR WORK

Cloning Restriction Fragments into Plasmids

Calf Alkaline Phosphatase (CAP) Treatment: this procedure removes 5' terminal phosphate groups. These substituent groups are required for ligation of DNA ends (sticky ends). Removal of the plasmid 5' phosphates requires that plasmid ends be ligated to some other DNA possessing 5' phosphate groups. CAP treatment is timed and balanced for the ratio of plasmid ends to enzyme units. Over treatment can chew off plasmid sticky ends making the plasmid prep worthless for cloning. CAP treated vectors should be tested for CFU/µg (colony forming units per µg of plasmid) when ligated with and without insert DNA. With plasmids cut with only a single enzyme (identical plasmid ends) expect 100-1000 CFU/µg without insert and 2-3 times as many CFU/µg with insert. For plasmids cut with two enzymes (different plasmid ends) expect 10-100 CFU/µg without insert and about 10 times as many CFU/µg with insert. The ratio of CFU with and without insert is thus indicative of the number of colonies that contain plasmids with inserts.

1) Restrict the plasmid with the appropriate enzyme(s). Be sure and test an aliquot of 50ng on a gel vs. uncut plasmid to ascertain that the DNA is cut to completion and gives a single band that migrates distinct from the super-coiled plasmid band
   A) You may then choose to extract and precipitate the DNA prior to CAP treatment. This gives the most reliable dephosphorylation but you may be able to get reasonable dephosporylation by going directly to CAP treatment. To do this after restriction is complete, dilute with 1 volume of 2x CAP buffer, add the appropriate amount of enzyme (see below) and treat for one hour. Then go to the digestions as outlined below.
2) Extract once with $CHCl_3$-IAA and EtOH precipitate
3) Redissolve EtOH pellet in 10 µl Te
4) Volume of CAP treatment should be calculated to be about 50-200 ng plasmid/µl. Add 10x CAP Buffer and H_2O to appropriate volume. 10x CAP Buffer is 500 mM Tris pH 9.0, 10 mM $MgCl_2$, 1 mM $ZnSO_4$, 10 mM Spermidine.
5) Dilute CAP enzyme to 1 Unit/µl in 1x CAP Buffer.
6) Add 1 Unit diluted CAP enzyme for every picomole (pM) of plasmid ends to treat.

Ligatable pMoles of Plasmid Ends

$$pM\ of\ ends = \frac{(number\ of\ ends/plasmid) * (picograms\ of\ plasmid)}{\left(660 \frac{pg}{pM \cdot bp}\right) * (plasmid\ size\ in\ bp)}$$

7) Incubate at 37° for 60 min.
8) Dilute at least 2 fold with TE and add 1/20th volume 10% SDS.
9) Incubate at 65° for 5-10 min.
10) Extract twice each with phenol then $CHCl_3$-IAA. Use TE to backextract organic phases.
11) Add NH_4OAc to 2.5 M and EtOH precipitate.
12) Redissolve EtOH pellet in appropriate volume of TE and quantitate.
13) Test ligate with and without an appropriate insert. Transfect into a suitable *E.coli* host and mark the stock plasmid according to the ratio of colonies plus and minus insert.

Ligation of Plasmids and Inserts

Follow manufacturers buffer conditions. Normally 50 ng of plasmid is ligated with a 2-fold molar excess of insert in 20 µl using 1 to 2 Units of T4 Ligase. NOTE: T4 DNA Ligase is not the same as *E. coli* DNA Ligase. Also, some suppliers use different Unit definitions which can make about a 100 fold difference. Promega and BRL use the unit definition referenced here.

1 x Ligation buffer: 50 mM Tris pH 7.6, 10 mM $MgCl_2$, 1 mM rATP, 1 mM DTT, 5% (w/v) PEG 8000. Or use buffer concentrate as provided by enzyme supplier, typically provided as 5x or 10x stock.

1) Ligations may be more effective for staggered ends if the ligation reaction is incubated first for 30 minutes at room temp then overnight at 15º.

2) Most ligations can be transfected after 4 to 5 hours incubation at 15º if the highest possible yield is not important.

3) After 15º ligation store at 4ºC or freeze at -20ºC. After 24 hours at 4º or freezing transfection yields will typically be reduced by 50%.

$CaCl_2$ Transformation of E. coli with Plasmid DNA

Do not let your $CaCl_2$ stock solution become contaminated, only pour from the bottle. If transfection frequency declines then replace the $CaCl_2$ stock.

Preparation of Frozen Ca-Shocked E. coli

Works well with E. coli TB1, KW251, LE392, JM109, and XL-1 Blue

1) Transfer 1 ml of an overnight culture into 50 ml of 37º L-broth. Grow for 2 hours at 37ºC (app. OD of 0.3-0.6). Place on ice 10 min.

2) Spin 10 min. 3000 rpm (1300 x g), 4ºC. Resuspend the pellet in 5 ml cold 0.1 M $CaCl_2$ and spin 5 min. 3000 rpm, 4ºC.

3) Resuspend the pellet in 2.5 ml cold 0.1 M $CaCl_2$ and incubate for 2 hours at 0ºC.

4) Add 600 µl cold sterile 80% glycerol (makes app. 15% glycerol).

5) Aliquot at 50 µl in microfuge tubes on ice. Transfer to a -70 or -85ºC freezer to freeze. Do not use an ethanol bath as this reduces viability and thus transfection frequencies. Store at <-70ºC. Competence should be stable for at least 6 months. Expect efficiencies of 5-20 x 10^6 cfu/µg vector.

6) To use: thaw an aliquot on ice, add 450 µl cold 0.1 M $CaCl_2$ and incubate for 15 min. on ice. Use immediately at 100 µl per transfection (see below).

Preparation of Fresh Calcium Shocked E. coli

1) Inoculate 10 ml of L broth with a small amount of cells from a reasonably fresh slant (<1 month old). Warm to 37ºC and incubate with good aeration at 37º. The faster the growth rate the better the transfection frequency; therefore temp. and good aeration are essential (not more than 10 ml/50 ml tube and incubate on a good angle).

2) When the culture reaches an OD_{600} of about 0.6 (early log phase) spin @ 2500 rpm 5' 4ºC (5 krpm in JA20 or 4000 in JS13). Gently decant and then using gentle trituration with a pipet resuspend the cells in 5 ml of 4ºC 100 mM $CaCl_2$.

3) Place on ice for 20 min (can go up to an hour), then centrifuge as in step 2. Decant and resuspend by gentle trituration in 1 ml of 4ºC 100 mM $CaCl_2$.

Note: The pellet in step 3 should be very loose and look like a crescent or a circle at this point. If it is more of a single dot in the bottom of the tube then you probably overgrew the culture and you had better start over.

4) Depending on the strain of E. coli used, incubate the Ca-shocked cells on ice for the prescribed period before introducing the DNA for transfection.

Strain dependence for Ca-Shock times

LE392 Must go for >12 hr and <36 hr; best=24 hr expect 10^6 cfu/µg form I pBR322

JM103 Use anytime from 0 to 4 hrs; by 24 hr no good; expect >10^6 cfu/µg form I M13

JM109 same as LE392- not very good for plaque selection of M13

TB1 Use anytime from 0 (10^6 cfu/µg) to 24 hr (2 x 10^6 cfu/µg) excellent host for pUC clones in color selection.

K802 Can use anytime from 0 to 24 hrs but yields 3x more colonies at 24 hrs (10^6 cfu/µg).

KW251 Best after O/N Ca shock; 3 hr=3.4 x 10^5 cfu/µg; O/N=1.2 x 10^6 cfu/µg

Transfection- use either freshly prepared Ca-shocked cells or frozen competent cells (freshly thawed and diluted as per the instructions) see above

5) In a 2.2 ml microfuge tube (use 2.2 ml for best culture aeration later) gently mix:
   100 µl of Ca-shocked (frozen or fresh) cells
   25 ng of ligated DNA (or less) in 10 µl or less of buffer (ligation buffer OK)
   Remember to include a control tube of uncut plasmid to test the transfection frequency of the Ca-shocked cells (generally 5 to 10 ng of uncut plasmid).

6) Incubate on ice for 40 minutes (not much more or less).

7) Heat shock at 42° for 2 minutes (often accomplished using H_2O in the well of a temperature block, this avoids using a large water bath which might contaminate your sample).

8) For M13 go directly to plaqueing-Otherwise- Add 200 µl 37°C L-broth and incubate the microfuge tube on its side (horizontal) at 37°C with moderate agitation for 30' to allow expression of the drug resistance. (same time works for Tet^r or Amp^r selection). The tubes must be horizontal to improve aeration and mixing. Mixing can either be in a foam block in a clip on the shaker incubator or on a horizontal roller apparatus but must be at 37°C.

9) Plate an appropriate amount of the transfection mix on selective medium (See Table). For uncut plasmids expect 5x10^5 to 1x10^6 CFU/µg of plasmid. Thus, for 10 ng of plasmid (ca. 1x10^4 CFU) in a final volume of 300 µl (30 CFU/µl) plate about 5 µl. Ligated plasmids often give about 1x10^4 CFU/µg. From a ligation of 25 ng plasmid in 300 µl expect 1 to 5 CFU/µl. So plate 50 to 100 µl per plate.

10) For plating use fresh plates (<2-3 days old). Spot the sample and use a EtOH flamed glass spreader. Remember to cool the spreader briefly on the plate before spreading the sample as the cells are extremely heat sensitive. Do not use more than 100 µl of cells on any one plate (especially for Amp selection). If too many cells are plated then the drug will be diluted out by cell density and many satellite (non-resistant) colonies will develop.

11) For recombinant plasmids allow about 20 hours for good colony development, non-recombinants may appear sooner. For color selection of LacZ interuptions (blue/white selection on Xgal:IPTG) the colonies may grow very slowly due to the metabolic byproducts from the breakdown Xgal. This inhibition is especially severe for XL-1 Blue. After 18 hours growth the blue colonies will be pinpoint size while the white colonies will be only slightly larger.

Electroporation for Transformation of E. coli

Need: Culture Broth, Cold Sterile H_2O, cold sterile 10% Glycerol, Bio-Rad Gene Pulser™ and electroporation cuvettes (or equivalent), sterile culture tubes, etc.

Preparation of Cells

1) Start overnight culture of *E. coli* or other bacterium to be transformed. Usually 10 ml

culture in 40-50 ml tube. Grow with good aeration (shake) at 37ºC overnight.

2) Next AM: dilute the overnight culture into 1 to 10 into pre-warmed 37 ºC L-broth. Plan on 10 ml culture for each transfection or ligation treatment to be electroporated. Incubate about 2-5 hours with shaking at 37ºC until the cells are in mid to late log phase. Some cultures grow faster than others and Dana Heiny says that a 1:10 dilution of *E. coli* DH10B takes at least 5 hours, so don't question that. While the culture is growing check on supplies and precipitate your ligated DNAs (see below).
3) When the culture has reached a fair turbidity, pellet the cells from the culture at 5 to 6000 rpm (2-3000 x g) for 5 minutes at 4ºC. Discard supernatant.
4) Resuspend the pellet in 1/10th volume (1 ml for every 10 ml of culture) cold sterile distilled water. Spin at 3000 x g for 5 minutes at 4ºC to pellet bacteria. Decant and discard supernate.
5) For an additional two times: Resuspend bacteria in the same volume as above in cold sterile water and pellet. These three total H_2O washes are to remove free electrolytes.
6) Resuspend pellet(s) in the same volume as above in sterile cold 10% glycerol, pellet as above and resuspend in 100 µl of 10% glycerol for every 10 ml of starting culture.
7) The cell suspension should be thick but not gummy. Add additional 10% glycerol as needed to provide sufficient volume (100 µl for each transformation) for the number of electroporations. Electrocompetent *E. coli* can be frozen in this 10% glycerol and stored at -80ºC for at least 6 months. Aliquot at 200 to 300 µl in microfuge tubes and freeze at -85ºC. Do not freeze in dry ice-ethanol or ethanol bath as the cells have higher viability if frozen at a slower rate by direct placement at -85ºC. To use: remove from ultracold and roll the tube between thumb and forefinger until starting to thaw. Transfer to ice and hold on ice <30 min for use.
8) Hold the 100 µl aliquots of bacterial suspension on ice until use but no more than an hour or so.

Preparation of DNA and Electroporation

Salts in the DNA solution will lead to unwanted arcing or sparking and thus ineffective electroporation. Therefore the DNA is precipitated and dissolved in water. T**he described procedure is for a electroporator cuvette with a 1 mm gap.**

1) While the log phase cells are growing precipitate DNA from ligations: Add 1/10 volume 3M NaOAc and mix. Add 3 volumes of cold 95% EtOH. Place in ice water for 30 minutes or -20ºC for 1 hour. Spin at 4ºC at 11,000 rpm for 15-20 minutes. Decant and discard supernate. Add 100 µl cold 70% EtOH. Centrifuge for an additional 1-2 minutes and decant. Repeat with a second 70% rinse, spin, and decant. Invert tube to drain, dry in centrifugal evaporation system (Speedvac).
2) Resuspend DNA in 10 µl sterile ultrapure water to yield about 5 ng/µl vector DNA. If desired, use 1 µl for quantitation by fluorometry or run 1 µl on an agarose gel. Use 5 µl for electroporation. Store on ice until use. The remainder can be stored at -20ºC as a back up in case the electrotransformation fails.
3) Put electroporator cuvettes and cuvette holder IN ice to pre-cool.
4) Turn on switch on back of BIO-RAD Gene Pulser™. Set **Pulse Controller** to a resistance of 200 Ohms. Set **Capacitance** to 25 µFD. On front panel set **Voltage** on digital display to 1.75 kvolts. Then press **Time Constant** button so digital display reads 0.0.
5) Add 100 µl electrocompetent bacterial cells to electroporator cuvette and add 1-10 µl DNA. Mix and hold on ice for about 1 to several minutes until electroporation.
6) Remove cuvette holder from ice to bench top and place cuvette in cold holder.
7) Slide holder on track so that cuvette is positioned between electrodes.
8) Hold in two red buttons on front of Gene Pulser™ until "beep" sounds, then release. Do

not be alarmed by display which may read **Chg** while charging. Record **Time Constant** on digital display after beep sounds. For *E. coli* a reading of 3-4.5 is considered good, low readings indicate significant arcing and may predict poor efficiency.

9) Slide out cuvette holder. Add 900 µl L-Broth to cuvette, tirturate gently to rinse the cells out from between the electrode plates, then transfer the suspension into an empty, sterile culture tube. OK to hold for a few minutes at room temperature while other electroporations are performed.

10) Repeat procedure with each ligation treatment. **DON'T FORGET A CONTROL UNCUT PLASMID SAMPLE. For the positive control use 5 to 10 µl of an uncut plasmid.**

11) For expression of drug resistance, place cell suspensions in shaker at 37 C for 30 to 45 minutes.

12) Spread aliquots of transformed culture on selective media.
For generation of plasmid genomic libraries spread 50 to 100 µl/plate. For most ligations for cloning restriction fragments or PCR fragments spread one plate each at 50 and 200 µl, spin the remaining app. 750 µl in a microfuge tube at 3-4000 x g to pellet the cells, decant most of the broth supernate leaving app. 100 µl, vortex the tube and plate all on another plate. For the positive control uncut plasmid plate 20 µl and 100 µl. For small uncut plasmid controls into most strains of *E. coli* expect 10^7 to 10^8 cfu/ug of input plasmid. Ligations will be 10-1000 times lower in efficiency. Incubate inverted plates at 37ºC for 16 to 48 hours.

Reusing Electroporator cuvettes

Electroporator cuvettes can be reused several times (but not advised for making comprehensive cDNA or genomic libraries because of chance of trace contamination with other plasmids). After use rinse with 10% bleach (don't soak in bleach or it will `craze' the plastic) then wash with soapy water, then rinse well with tap water followed by deionized water, rinse with ethanol and then store in 95% ethanol. To use, remove the cuvette and cover using ethanol rinsed forceps. Rinse the cuvette and cover with fresh 95% ethanol then lay the cuvette and cover on a paper towel in a laminar flow hood up near the screen to air dry. Once thoroughly dry place the cover on the cuvette and then use.

TSS Competent Cells

Based on: Chung, Niemela, and Miller (1989) PNAS 86:2172-2175.

We have only a little experience with this method. Initial results are highly promising, but this hasn't repeated for everyone. How long the frozen cells remain viable or competent is still not certain. TSS may need to be prepared fresh each time, that isn't known yet and still needs to be determined.

1) To prepare TSS:
   - A) Dissolve 1 g tryptone, 0.5 g NaCl, 0.5 g yeast extract, and 20g PEG 8000 in 70 ml H_2O
   - B) Adjust the pH to 6.5 with HCl then bring the final volume to 91 ml and autoclave for 15 minutes at 121ºC slow exhaust.
   - C) Cool to room temperature then add 5 ml pure DMSO and 4 ml of autoclaved sterile 0.5 M $MgCl_2$ or $MgSO_4$.
   - D) Store at 4ºC until use. How long the solution is good for is not known.

2) Grow *E. coli* strain to low turbidity, early log phase, 0.3 to 0.4 A_{600}

3) Pellet cells at 4ºC at 1000 x g for 10 minutes.

4) Decant, then resuspend pellet in 1/10th the original culture volume in ice-cold TSS.

5) Aliquot cells and use immediately or freeze at -85°C, and store at that temperature.
6) To use TSS competent cells:
   A) Mix 100 µl of ice cold TSS competent cells with 1 to 5 µl of ligated DNA in microfuge tube on ice. Also prepare positive control with 1 to 5 ng of an uncut plasmid DNA.
   B) Incubate for 30 minutes on ice.
   C) Move to room temp then add 900 µl of 37°C L-broth
   D) Insert tubes into foam block or other holder to hold tube securely.
   E) Place tubes on horizontal while shaking at 37°C for 30 minutes to one hour.
   F) Plate on selective media according to the table
      a) For the uncut plasmid expect 5 to 10 x 10^6 cfu/µg plasmid. For 5 ng of input plasmid this computes to 25 to 50 cfu/µl of cell suspension so for the control plate 5 and 50 µl
      b) For ligation mixes expect 10^4 to 10^5 cfu/µg of vector. If we assume you used 5 µl of a 10 µl ligation that contained 25 ng of vector, then you transformed about 12 ng of vector and thus should expect around 100 to 1000 total transformants in your 1ml of cell suspension. Therefore, I suggest you plate 50 and 150 µl of the suspension. The remainder should be centrifuged at 1 to 2000 x g for 5 minutes, decant most of the broth off leaving around 50 to 100 µl in the tube. Resuspend the pellet in this remaining liquid by trituration, then spread all of this on a single plate.

SELECTIVE MEDIA FOR SOME VECTORS

vector	drug concentration	notes
Amp^R		
pBR322	75-100 µg/ml	
pUC13	75-100 µg/ml	75 µg/ml for selection of inserts (especially large ones) otherwise 100 µg/ml
pGEM	50-100 µg/ml +XGal+IPTG	For some reason the pGEM vectors numbered 5 or above do not give as high resistance to Amp as the earlier versions. This is especially true when plated in the presence of XGal+IPTG. There is strain dependence as well since XL1 Blue is more sensitive than XL1 Blue MRF'. If you are using these vectors and have problem with getting colonies or they are real small then consider lowering the Amp level to 50 or 60 µg/ml ampicillin.
Tet^R		
pBR322	15 µg/ml	If your tetracycline stock is showing a yellow precipitate do not use for transformant selection as this indicates an inhibitory 'breakdown' product.
Chlor^R		
pBELOBAC	15 µg/ml	
pLI50	20 ug/ml	Gram+/Gram- shuttle vector

Isolation of Plasmid DNA

Solutions needed: L-broth, GTE, 150 mM NaOH 1% SDS, 5 M KOAc pH 4.8

Large Scale Isolation of Plasmid DNA

Cleared Lysate Method: This method is used for preparing native (non denatured) plasmids and is only useful for preparation of small plasmids (<10 kbp). The protocol may be scaled up or down with no adverse affects. For Larger plasmids or for the highest possible yields try the Alkaline SDS method.

Growth

NOTE: be sure to review and follow the procedural notes (see above) relating to culture volumes, growth and antibiotic selection or else yields will be very low.

1) Using a staged growth (turbid small culture diluted into fresh 37ºC medium) grow to late log phase at 37ºC

NOTE: chloramphenicol amplification can be used to amplify some restricted growth plasmids but does not work with most modern vectors (pUC, pGEM, pBluescript, etc.). To chloramphenicol amplify grow the cells to late log phase (not stationary) then add chloramphenicol to 200 µg/ml. Incubate >12 hr 37ºC with shaking. **If your plasmid requires chloramphenicol amplification** then maximize your yield of plasmid by waiting to add the chloramphenicol until the culture is very turbid, i.e., **Late log phase**.

2) Collect cells by centrifugation 5' 8000 rpm 4ºC.

VOLUMES INDICATED ARE FOR EACH 100 mls OF CULTURE:

3) Resuspend the cell pellet in 4 ml GTE per 100 mls of culture.

4) Warm to room temp. and add 1 ml of a fresh, 10 mg/ml lysozyme solution in the same buffer, mix gently and incubate 20-30' at room temp

5) Add 200 µl 10% SDS mix very gently, let stand 5' at room temp and then add 400 µl 6 M KOAc pH 7, mix very gently and incubate on ice 30'

6) Centrifuge for 30' at 12 krpm at 4ºC.

7) Transfer the supernatant to a new tube and extract with 50:50 phenol:$CHCl_3$-IAA and then twice with $CHCl_3$:IAA

8) Add 1/10th vol 6 M KOAc and 2 volumes of Cold EtOH for 30' at -20ºC then spin 20' at 10 krpm at 4ºC

9) Discard supernate, redissolve pellet in 200 µl TE (10 mM TrisCl pH 7.5 1 mM EDTA) and transfer to a microfuge tube

10) Add 100 µl 7.5 M NH_4OAc and incubate 20' on ice

11) Spin 20' at 10 krpm, transfer the supernate to a new tube and add 1 ml 95% EtOH 30' -20ºC to precipitate.

RNase T1 treatment of this DNA yields restrictable plasmids.

To purify form I DNA treat with RNase T1 and then run on a CsCl gradient.

Alkaline SDS Method: Only acceptable method for large plasmids (>10 kbp). Lysis Volumes/Culture Volume ratios may be altered slightly with no appreciable affect, however the ratios between GTE, NaOH-SDS and KOAc must remain the same. Not recommended for size screening as some plasmids become partially denatured as snap back supercoiled DNA, which alters gel migration until the DNA is renatured or purified on CsCl gradients.

VOLUMES ARE PER 100 ML OF CULTURE (although volumes may be varied, i.e. decreased proportionately 50% with some decrease in yield). Theory for this method is that the lysozyme partially digests the bacterial cell wall creating spheroplasts which are lysed by the SDS. The

alkaline conditions for lysis denature proteins inactivating nucleases, partially hydrolyze RNAs, and denature DNAs. Genomic DNAs are partially sheared upon lysis and denature into single strands while plasmids remain as duplex circles which can not denature but instead ball up. Acidic KOAc is added to neutralize the solution. The rapid neutralization causes the sheared genomic DNA to form snap back duplex tangles or networks, while the small duplex circular plasmids are excluded. The SDS-protein components are precipitated as potassium salts of SDS are insoluble. The K-SDS-protein precipitates onto or along with the genomic DNA and the complex is removed by a relatively low-speed centrifugation. The supernate contains small RNAs and the plasmids which are precipitated with isopropanol. Isopropanol is used for speed and to save volume. The pellet is redissolved and extracted once with 50:50 phenol:$CHCl_3$-IAA. Longer RNAs and some proteins are then precipitated in the cold as insoluble NH_4 salts. This leaves a solution of plasmid DNAs contaminated with a sizable amount of short RNAs and a small amount of genomic DNA which are ethanol precipitated. At this point you must decide how clean you need to get your plasmid and how much time you want to spend on it. For the cleanest plasmid you should RNAse treat to reduce the RNA contaminants and then purify super-coiled plasmids on a CsCl gradient. As an alternative to expensive and time consuming CsCl gradients, the plasmids and trace genomic DNA can be selectively precipitated from RNAsed plasmid preparation using PEG. PEG precipitation is somewhat wasteful as precipitation is not quantitative but is fast and inexpensive.

Growth

NOTE: be sure to review and follow the procedural notes (see above) relating to culture volumes, growth and antibiotic selection or else yields will be very low.

1) Using a staged growth (turbid small culture diluted into fresh 37ºC medium) grow to late log phase at 37ºC

NOTE: chloramphenicol amplification can be used to amplify some restricted growth plasmids but does not work with most modern vectors (pUC, pGEM, pBluescript, etc.). To chloramphenicol amplify grow the cells to late log phase (not stationary) then add chloramphenicol to 200 µg/ml. Incubate >12 hr 37ºC with shaking. **If your plasmid requires chloramphenicol amplification** then maximize your yield of plasmid by waiting to add the chloramphenicol until the culture is very turbid, i.e., **Late log phase**.

2) Collect cells by centrifugation 5' 8000 rpm 4ºC.

VOLUMES INDICATED ARE FOR EACH 100 mls OF CULTURE:

3) Resuspend the cells in 12 ml (per 100 ml of culture) GTE made 2 mg/ml with lysozyme (more lysozyme may be added up to 4 mg/ml if the culture is a capsule former)

A) (note: cells may be stored frozen in 50 mM glucose 25 mM Tris then add EDTA and lysozyme upon thawing).

4) Mix and incubate at 10-15' at 37ºC. The enzyme digests the cell walls making the cells osmotically sensitive protoplasts that should appear whiter (not yellow anymore). Besides color change a good indication is that the protoplasts should aggregate and tend to settle out of solution during the incubation leaving a clear layer of buffer above.

5) Add 20 ml 150 mM NaOH 1% SDS while gently swirling the protoplast suspension. Mix thoroughly by inversion (not shaking) to disperse into **CLEAR** suspension. Incubate 5' at room temp.

6) Add 15 ml 5 M KOAc pH 4.8, mix by inversion

7) Centrifuge 10' 6-8000 x g @ 4ºC and collect supernatant. Try to avoid the flocculent white solids some of which will be floating on the surface. For large preps easiest to decant through gauze.

8) Precipitate nucleic acids by adding an equal volume of isopropanol (2 propanol). Do NOT leave to long in ispropanol (or at -20º) as this precipitates contaminating salts, and low MW RNAs

and DNAs.

9) Centrifuge >8000 x g 10' 4ºC, invert to drain thoroughly. You can remove some of the excess alcohol by briefly drying *in vacuo*, but do NOT over dry as the pellet will be hard do dissolve.

10) For each 100 ml of culture: Dissolve in 0.5 ml 10 mM Tris 1 mM EDTA pH 7.5 (TE). Some debris will not dissolve but, if you don't give enough time to dissolve the DNA you will lose it at the extraction step.

11) Extract one time with 50:50 Phenol:$CHCl_3$:IAA. Extraction is by inversion mixing for several minutes followed by room temp centrifugation (>1000 x g for 5-10').

12) Add 1/2 vol 7.5 M NH_4OAc and incubate 5' on ice

13) Spin 5' at >8000 x g 4ºC, transfer the supernate to a new tube and add 2.5 vol 95% EtOH.

NOTE: this is the first step in this isolation procedure where the preparation may be stored (overnight or longer). If you start an isolation you must take it this far.

14)Collect DNA by centrifugation >7000 x g 4ºC and rinse with 70% EtOH.

This DNA should be mostly plasmid contaminated with small RNAs. Further purification should involve RNAse digestion and then fractionation on CsCl gradients or PEG precipitation.

YIELD: 1 L= 1 to 2 mg.

RNase Treatment

RNAse digestion can use RNAse T1 and/or RNAse A. RNAse T1 is easier to get rid of by Phenol-$CHCl_3$ extraction. Presence of either will not inhibit restriction enzymes, or transfections. RNAseA will have contaminating enzymatic activities as it is generally a less pure enzyme. For cloning operations where DNA ends must remain intact we suggest use of RNAseT1 to avoid trace exonucleases that may contaminate the RNAseA. If the plasmid is being prepared for production of RNA transcripts then do NOT use RNAseA unless you plan to first protease treat and then phenol extract to remove the RNAseA which is difficult to completely remove. RNAse A is prepared by dissolving in TNE, place in boiling H_2O bath 20' then bring to boil over flame, aliquot and store at -20ºC

12) For 100 ml of culture:

- A) Dissolve in 100 µl TE then add 5 M NaCl to 100 mM (2 µl/100 µl volume), add RNase T1 to 2 U/µl or RNAseA to 10 µg/ml. Incubate at 37ºC for 30'.
- B) Extract with 50:50 Phenol:$CHCl_3$:IAA then with $CHCl_3$:IAA.
- C) Add 1/10th volume 3 M NaOAc and 2.5 volume cold EtOH, 4ºC 5 min.
- D) Pellet DNA 5-10 min 10,000 rpm in cold, 70% EtOH rinse, dry and then proceed to PEG precipitation or CsCl gradient.

PEG Precipitation and Fractionation

For precipitation of nucleic acids >1000 bp. Reference: Lis, J.T. (1980) Meth. Enzymol. 65:347-353. Plasmids purified by PEG precipitation are virtually free of small RNAs (if preceeded by an RNAse treatment) and are suitable substrates for restriction digestion, sequencing, mammalian cell transformations or even as cloning vectors. These plasmid preparations will contain a variable amount of genomic DNA depending on the extent of shearing in the original NaOH-SDS lysis step. Contaminating genomic DNA will appear as a band of >20 kbp on 0.7% agarose gels. For most routine sub-cloning operations this not a big problem. These precipitations are not quantitative, percent recovery decreases rapidly with amounts less than 10 to 20 µg. Losses of 50% are not unusual.

1) Dissolve the DNA in 1 ml TE for every original liter of culture, add an equal volume of 13% PEG (mw 6-8000) 0.8 M NaCl, incubate on ice 20'. Collect DNA by centrifugation 10' 10-

12000 x g at 4ºC, decant, add 70% EtOH (app. same volume as before decant) spin 1-2 minutes (same speed at 4ºC), decant, repeat 70% rinse and spin. Speedvac to dry and dissolve in 100 µl Te per liter of starting culture. Quantify by fluorimetry.

Cesium Chloride Purification of Plasmid DNA

Super-coiled plasmids can be purified by equilibrium density centrifugation in CsCl the presence of Ethidium bromide.

1) DNA is dissolved or diluted to according to the following table (for preps derived from greater than a 1 L culture run 2 gradients to avoid overloading)

Rotor	TE	CsCl	EthBr	45 krpm	Tube
vTi65.2	4 ml	4 g	200 µl	20 hr	1/2x2", quick seal
vTi65.1	0	10	500	20 hr	5/8x3", quick seal
AH875	7	7	350	36 hr	5/8x3", thickwall

2) Add CsCl to DNA solution and dissolve then add 10 mg/ml EthBr stock, transfer to ultracentrifuge tube

3) For fixed angle rotor tubes top with mineral oil (little or no air bubble), cap and spin. For vertical rotors do not top with mineral oil instead seal in quick seal tubes filling all the way to the bottom of the neck to prevent air bubbles, seal and squeeze the tube to ensure the seal is good

4) All centrifugations are at 20ºC **NEVER IN THE COLD**

5) Harvest **Form I band** (lower band in middle of tube) with a hypodermic, dilute 3x with TE, add 1/10th vol 3 M NaOAc and 2.5 vol 95% EtOH. -20ºC 1 hour. DNA pellet is redissolved in 500 µl TE, transferred to microfuge tube and extracted once or until no color with H_2O saturated-Isoamyl alcohol (mix well for several minutes for good extraction of the Ethidium Bromide from the DNA). Collect aqueous, add 1/10th vol 3 M NaOAc and EtOH ppt.

Methods for Small Scale Preparation and Screening of Plasmids

For an alternative procedure for screening plasmids by PCR see the chapter on PCR.

Method 1: Small Scale Prep for Sequenceable Plasmids:

Note: This procedure yields very clean plasmid DNA free of RNA. Plasmids prepared in this manner may be sequenced, restricted, and or used for transient expression in mammalian cells. The procedure may be scaled up to accommodate 100 ml cultures.

1) Pick colonies onto a master plate and inoculate 10 ml overnight cultures containing appropriate antibiotic.

2) Harvest overnight cultures, 3-6000 x g, 5 min. Drain cell pellet and resuspend in 1 ml TE on shaker. Transfer to microfuge tube spin 3 min @ 3-6000 x g to pellet cells. Drain on paper towel.

3) Add 135 µl GTE and vortex until resuspended. Add 15 µl 10 mg/ml lysozyme in GTE. Flick to mix. Set at 37ºC 15 min.

4) Add 300 µl 1% SDS, 150 mM NaOH (made fresh). Invert and flick to mix. Place @ room temp 5 min.

5) Add 225 µl 5 M KOAc pH 4.8. Invert tube several times to mix well.

6) Spin full speed in cold 5 min. Decant supernate to clean 1.5 ml tube avoiding white pellet. Add 2 µl 5 µg/µl RNase A. Set at 37ºC for 30 min.

7) Extract once with phenol:chloroform (interface should be clean). Transfer aqueous to new tube.

8) Add 1 volume of Isopropanol. Pellet DNA 10,000 rpm 5' in cold, rinse with cold 70% EtOH, dry and resuspend pellet in 100 µl TE. Plasmids may be digested for restriction mapping or testing at this point. To further purify for sequencing do step 9.
9) Add an equal volume (100 µl) 13% PEG 8000 0.8 M NaCl. Place in ice water 10 min. Spin down DNA (10 min 10-12000 rpm) in cold, decant, rinse 2x with app. 500 µl cold 70% EtOH. Dry and resuspend pellet in 30 µl Te. [for ref. see: alkaline-SDS prep above]
10) Quantify DNA by fluorometry or gel electrophoresis. Include original vector or undeleted parent plasmids as size markers.

Method 2: Sequence-able Plasmids FAST:

For 10 ml cultures (may be scaled up to 20 ml culture but poor lysis and yield results) and plasmids <10 kbp where you just need some sequence as fast as possible. More RNA contamination than Method 1 but usually good enough for good sequence data.
1) Pellet culture, resuspend in 1 ml TE, transfer to microfuge tube, pellet cells and decant well.
2) Add 100 µl TE (or 50 mM TrisCl pH 7.5 10 mM EDTA) and vortex to resuspend.
3) Add 350 µl 150 mM NaOH 1% SDS, mix well by shaking
4)Incubate 5' at 37°.
5) Add 260 µl 3 M KOAc pH 5.6, mix well by shaking.
6) Spin 2' @ 10 krpm in cold (angle rotor or swinging bucket rotor; room temp spins are OK).
7) Decant Supernate into 1.5 ml tube
8)Extract with equal vol 50:50 Phenol:$CHCl_3$
9) Centrifuge and transfer upper aqueous to new tube.
10)Precipitate with equal vol Isopropanol, pellet 5' at 11 krpm (room temp or at 4°C), decant, add about 0.5 ml cold 70% EtOH, spin 1', decant, drain on paper towel
11)Dry in speedvac.
12)Redissolve in 90 µl TE+20 µg/ml RNAseA. Incubate 30' at 37°.
13)For better quality sequence extract 1x with $CHCl_3$-IAA
9) Add 10 µl 3M NaOAc pH 5.5, mix, add 250 µl cold ethanol, mix well, spin 5' @ 11 krpm (room temp or 4°C), decant, add about 200 µl cold 70% EtOH, spin 1', decant well, dry and redissolve in 30 µl Te.

Note: the original procedure called for a 30' RNAse treatment with less RNAse A, followed by a 40% isopropanol precipitation at room temp. This is a border line precipitation for larger nucleic acids and very susceptible to minute volume changes. Experience proves that this amount of RNAse treatment and ethanol precipitation removes sufficient small RNAs to give good quality sequencing and still has high plasmid yields.

YIELD = 200-1000 ng/µl

Method 3: Rapid Small Scale Prep for Screening:

For 1.5 ml of culture and plasmids <10 kbp. This is a quick way to obtain restrictable, fairly clean, plasmid DNA
1) Pellet 1.5 ml of culture and resuspend in 400 µl TE+1.25 M NaCl.
2) Add 500 µl of 50:50 Phenol:$CHCl_3$-IAA that has been pre-equilibrated first with 1 M Tris pH 8 and then with 1 M NaCl.
3) Mix by shaking for 15 sec. and place on ice for 20'.
4) Spin in angle rotor at 4° for 15 min.

5) Collect supernate and add 1 ml EtOH on ice for 20 min.
6) Pellet DNA, rinse with 70% EtOH and dry.
7) Dissolve pellet in TE containing 2.5 µg/ml RNAseA.

1 µl of this solution should contain about 100 ng of plasmid DNA contaminated with some chromosomal DNA and very little RNA. This DNA should be restrictable with a variety of enzymes.

Method 4: Fastest Small Prep:

For 1.5 ml cultures and plasmids <10 kbp. Yields are high and DNA is clean enough for most enzyme digestions. More RNA than Method 3 but higher yield. Up to 12 samples can be easily processed at one time. Ref: Zhou, C., Yang, Y., and Jong, A.Y.(1990) BioTechniques 8(2):172-3.

1) Grow 1.5 ml *E.coli* cultures to late log or stationary (very turbid). Pellet cells in microfuge tube 2' 6 krpm. Decant most of broth off leaving about 50 µl behind.
2) Vortex tube to resuspend cells in remaining broth.
3) Add 300 µl freshly prepared TENS (TE + 0.5% SDS + 0.1 M NaOH) and vortex briefly to mix. Cells should lyse and solution become viscous but cloudy. Don't over vortex.
4) Add 100 µl 3 M KOAc pH 5.4, vortex briefly.
5) Spin 4' 10 krpm room temp.
6) Pour supernate into fresh tube and fill tube with -20ºC 95% EtOH. Invert to mix and spin 5' 12-13 krpm 4ºC or room temp. Decant, add cold 70% EtOH, spin a few minutes, invert to drain and then speed-vac to dry.
7) Add 20 µl TE+10 µg/ml RNAseA, vortex and incubate 10' 37º.
8) should yield 3-6 µg plasmid DNA at 1-200 ng/µl. Restriction digests may be aided by addition of spermidine to 1 mM.

Method 5: Alkaline Lysis for Small Preps:

5 ml of culture or a large colony

1) Pellet cells in microfuge tube, resuspend in 50 µl H_2O, add 200 µl 100 mM Tris pH 12.8 50 mM EDTA 1% SDS, mix and incubate 30' 37ºC
2) Add 50 µl 2 M Tris pH 7 and mix well (Should be viscous)
3) Add 30 µl 30% NaCl and extract 2x with phenol (3% NaCl sat.)
4) Extract 2x with $CHCl_3$:IAA
5) Add 30 µl 3 M NaOAc pH 5.4 and 1 ml cold EtOH, -20ºC >60'
6) Centrifuge 8' 4ºC, decant, add 1 ml cold 70% EtOH, spin again, decant, dry and redissolve in 50 µl TE.

This DNA will contain a fair amount of RNA contamination and is seldom used. Plasmid will require further purification by RNAse treatment/PEG ppt. or gel purification (See **Gel Purify**). YIELD: 5 ml culture= 500 ng Ref:(1979) NAR 7(6):1513

PCR Screening of Bacterial Clones

This method works for screening colonies for inserts and may also work for yeasts. There have been reports in the literature that some types of toothpicks may be inhibitory to subsequent PCR. I (DR) have not had that problem but sterile pipet tips also work and have not been reported to be a problem. This protocol works fine on *E. coli* and *Staphylococci,* but has been problematic on

Enterococcus cecorum. The best toothpicks are the "flat" and you can sterilize by autoclaving in a 100ml beaker covered with a quadruple layer of foil to cover.

1) Appropriate colonies are toothpicked onto a master plate which is incubated overnight.
2) A sterile toothpick or pipet tip is then used to sample a small amount of the colony into 50 µl of sterile H_2O.
   A) You can toothpick into the water in either a PCR plate, or snap cap tube that can fit your PCR machine (0.5 ml?).
   B) The sample is best from the "edge" of the colony and you just need to "touch" the colony, don't take very much or you will have too much bacterial debris in the PCR which can be inhibitory
   C) Insert the toothpick into the water and leave in the tube while you pick all the colonies. This can help keep track of which wells have been used on a PCR plate.
   D) Don't forget to pick a negative control (either off of the control plasmid transformation, or a blue colony in a LacZ-XGal screen).
   E) When all colonies have been sampled, twirl the toothpick in the water and discard the toothpick.
3) Place the sealed tubes or plate into PCR machine programmed for 100 ºC (heated lid) for 10 minutes, then cool to room temp.
4) Optional: Spin the tubes at 10000 rpm at room temperature for 5 minutes.
5) Transfer 1 to 2 µl of the supernate into a PCR reaction containing the appropriate primers. PCR primers can either be to plasmid sequences flanking the cloning site (SP6 and T7 primers or M13 forward and reverse primers), rDNA primers, or specific to the insert.
6) PCR is a standard PCR using appropriate times and temperatures specific to the primers and insert size. For SP6-T7 20mer primers use 0.5-0.8 pmoles/µl primer and 45ºC annealing temperature. Generally, amplification is for 20 cycles. For 16S Bact8F x Bact968R use 50 ºC anneal for 25 cycles.
7) After the PCR run an aliquot (2-5 µl) of the product on a gel with size markers. All potential positives should be compared to the negative control to verify that amplification is specific to the clone.

Induction of single strand DNA from Phagemid clones

Titer R408 helper phage on JM109

1) Grow 2 ml culture of JM109 or XL1 Blue cells containing plasmid overnight in Minimal medium + Amp
2) Transfer 1 ml of cell suspension into 3 ml of L-amp at 37ºC
3) Add 1 x 10^9 pfu R408 cells (approximate MOI of 10x)
4) Incubate 3 hours at 37ºC
5) Spin twice at 8 krpm 5 min saving supernate to remove cells
6) Heat to 55ºC for 30 minutes to kill remaining cells
7) Store at 4ºC

Yields are apparently higher from XL1 Blue than JM109

<u>To test for packaged phagemid use JM109</u>

8) Grow JM109 to mid log phase in minimal medium at 32ºC (have to use JM109 as R408 and phagemids don't titer well on XL1
9) Mix 0.1, 1.0 and 10 µl of supernate with 50 µl aliquots of JM109 cells
10) Also mix supernate with Lbroth (no cells) for background. Expect app. 20 cfu/µl

background Amp resistant colonies

11) Incubate 15 min at 37°C

12) Spread on L-Amp

Yields for pGEM plasmids in JM109 500 cfu/µl or in XL1 750 cfu/µl

To isolate single strand phagemid DNA

Use any protocol for purification of ssDNA from M13 supernates

13) Re-centrifuge the supernatant, collect supernate, add 225 µl/ml 25% PEG 6000 3 M NaCl and let stand room temp for 15'.

14) Pellet phage 10 krpm for 30' 4°C, decant, invert for several minutes to drain and wipe the sides dry with a Kimwipe.

15) Gently resuspend the pellet in 500 µl 10 mM Tris pH 7.5, 1 mM EDTA, 20 mM NaCl and transfer to microfuge tube.

16) Phenol extract twice then twice with $CHCl_3$:IAA

17) Add NaOAc to 0.3 M and 1 ml EtOH -20°C 1-2 hours

18) Pellet DNA, rinse with 1 ml cold 70% EtOH and dry

19) Redissolve in 20-40 µl Te. Determine concentration by running on gel or measure using fluorimetry assuming 1/10th efficiency in reading single strand DNA.

ExoIII Deletions of Double Stranded Plasmids:

This procedure is adapted from Stratagene Bluescript sequencing system and from Henikoff, GENE 28:351-359, 1984. NaCl addition to ExoIII from Tomb and Barcak (1989) BioTechniques 7(9):932-933.

Solutions:

2x Exo buffer: 100 mM TrisCl, pH 8; 10 mM $MgCl_2$; 20 µg/ml tRNA

5x Mung Bean Buffer: 150 mM NaOAc pH 5.0: 250 mM NaCl, 5 mM $ZnCl_2$; 25% Glycerol

Notes:

i) ExoIII activity is temperature dependent. Approximately 400 bp/min will be digested at 37°C, 230 bp/min at 30°C and 125 bp/min at 23°C. However, samples taken at any given time point will result in plasmids deleted to various lengths. Another way to slow ExoIII down is with NaCl. If the Exo reaction is run at 30°C but in 50 mM NaCl deletion rate is ca. 125 bp/min.

ii) Plasmid DNA used in this procedure must be clean (CsCl purified if possible) to ensure complete digestion with restriction enzymes. Your cloning strategy must place a 3'-overhang restriction site proximal to the primer (to protect the primer from ExoIII activity), and a 5' or blunt end restriction site proximal to the insert (to allow exoIII deletion of the insert).

iii) The choice of enzymes to protect is somewhat limited and a 4 base 3' overhang is preferred. 2 base 3' overhangs have been found (*Sst*II) which do not entirely protect; various suppliers or friends have indicated that the following enzymes are insufficient for complete protection: *Sst*I, *Apa*I, *Sst*II, *Pst*I

iv) An alternative method for end protection is to gap fill a 5' overhang using α-[phosphorothioate]-dNTPs.

a) Cut first with the site to protect, phenol and then $CHCl_3$ extract and EtOH precipitate.

b) Suspend in 50 µl Klenow Buffer (50 mM TrisCl pH 7.8, 5 mM $MgCl_2$, 1 mM DTT, 10 µg/µl BSA) made 400 µM phosphorothioate- dNTPs (Promega).

c) Add 5 U Klenow (for app. 30-50 µg 8-10 kbp plasmid) and incubate for 20' at room temp.

d) Dilute to 50 µl with TE, add EDTA to 5 mM, SDS to 0.1%, and heat to 65°C for

10'.

e) Extract with phenol, phenol:$CHCl_3$, and $CHCl_3$ and EtOH ppt.

f) Proceed with second digestion.

v) Protection by gap filling with phosphorothioates does a great job of protection but over exposure to the Klenow (or to much enzyme) results in a significant fraction of the plasmids which are substituted a fair distance from the site of gap filling. This may render the site for the secondary restriction non-susceptible to digestion. This will result in a substantial level of plasmid which is not deleted by the ExoIII-Mung bean treatment. Sites more distal to the gap-filled end are much more susceptible.

vi) An alternative to steps 8 through 12 is to directly ligate the deleted plasmids without extraction. Mix 3 µl of treated DNA with 2 µl 100 mM Tris pH 8.0 (to neutralize Mung Bean Buffer) then add 15 µl of (4 µl 5x Ligation Buffer, 10 µl H_2O, 1 µl T4 Ligase) then transfect into *E. coli* TB1 or KW251. Expect low yield of colonies: 30 to 60 from transfection of 10 µl from the ligation. But, this should be enough colonies. The remaining 30 µl of DNA may be frozen for extraction, ligation and transfection later if you need more colonies from a few troublesome time points. This can speed everything up, has worked for DDR and is recommended by Promega.

Procedure:

1) Double digest **TO COMPLETION** the plasmid DNA at the protected site; see note iii. If using phosphorothioate protection see note iv and v. Determine number of time points, time point interval, reaction temperature and amount of ExoIII nuclease (based on molar amount of DNA: 700 U/pmole susceptible end) required to span your insert at selected temperature.

2) Allow 0.5 µg double digested plasmid DNA for each time point. Prewarm DNA in 1x ExoIII nuclease buffer containing 20 mM 2-mercaptoethanol. Total volume is based on a DNA concentration of 100 ng/µl. (ie. For 10 timepoints prewarm 5 µg DNA in 50 µl.)

3) Set tempblock to 68º. For each ExoIII digestion time point set aside a microfuge tube containing 35 µl 1x Mung Bean nuclease buffer.

4) To the prewarmed DNA solution add 700 U/(pmole susceptible end) ExoIII Nuclease. This must be determined separately for each plasmid and more ExoIII may be added with no appreciable problems.

5) Remove 5 µl aliquots at determined intervals to tubes containing 1x Mung Bean Nuclease buffer. Heat tubes 15' at 68ºC. Hold on ice until all points are collected and heated.

6) Dilute Mung Bean Nuclease in 1x Mung Bean buffer to 1 U/µl. Add 3 U per tube. 30' @ 30ºC.

7) Remove 5 µl sample for gel electrophoresis. (Compare time-point samples with original vector and undeleted parent plasmid to determine extent of ExoIII and efficiency of Mung Bean Nuclease activities.)

8) Stop Mung Bean Nuclease reaction (see note vi) by extraction with 1 vol phenol:chloroform. Transfer aqueous layer and extract with chloroform.

9) Transfer aqueous layer and add 1/10th vol 3 M NaOAc.

10) Precipitate in 2 vol ice cold EtOH >1 hr. Rinse with 70% EtOH and dry.

11) Redissolve in 10 µl TE.

12) Ligate plasmids and transform *E. coli* strain TB1 according to your favorite protocol.

Preparation of T-tailed cloning vectors for PCR products

This procedure is adapted from:

N. Hadjeb and G. Berkowitz, (1996) *Preparation of T-overhang vectors with high PCR product cloning efficiency*. **BioTechniques** 20(1):21-22.

Vectors prepared in this manner are excellent for color selection cloning of PCR products. **NOTE**:

T-tailed cloning will only work on PCR products where neither PCR primer has a T as its 5' most base. This cloning strategy is based on the terminal adenylation activity of Taq Polymerase where most products receive a 3' terminal A residue (unless they already have one). It will likely not work with PCR products made with other thermally stable DNA polymerases. Preparation of the vector depends on 'forcing' Taq DNA Polymerase to add a single T-residue to the 3' end of the vector. The T-tailed vector DNA is then ligated and plasmid which remains un-ligated is gel purified.

1) Prepare the vector and purify as well as possible, PEG precipitation is good but CsCl gradient purification is better.
2) Digest 50 µg of plasmid with a blunt cut enzyme such as EcoRV, run a 50 ng sample on a mini gel to confirm complete digestion.
3) $CHCl_3$ extract the plasmid and ethanol precipitate using standard methods
4) Redissolve the pellet in:
   50 µl 10 x Taq Polymerase buffer (1 or 1.5 mM $MgCl_2$)
   50 µl 2 mM dTTP (final 200 µM)
   400 µl H_2O
   add
   50 U Taq DNA polymerase
   incubate at 72ºC for 2 hours
5) Extract with phenol:$CHCl_3$ and then $CHCl_3$
6) Ethanol precipitate
7) Redissolve in:
   200 µl BRL 5x Ligation buffer
   10 ul 100 mM rATP
   780 µl H_2O
   then add
   50 Units T4 DNA Ligase
   incubate 30 minutes at room temp and then at 15ºC overnight
8) $CHCl_3$ extract and ethanol precipitate
9) Redissolve the sample in 50-80 µl of loading buffer. Electrophorese on a 0.7% low melting point agarose mini gel with cut plasmid as a marker. Electrophorese at low voltage (20V) to avoid smearing.
10) Excise the band that runs to the same position as cut plasmid. Purify from the gel by β-agarase digestion (see section: **Recovering Samples from Gels)**. Purification and removal of digested agarose oligosaccharides can be facilitated by use of Microcon™-50 spin filters (Amicon, Inc., Beverly, MA):
    A) Suspend the sample in 200-500 µl of Te (<500 µl per filter) in the filter units upper chamber.
    B) Spin at 10,000 rpm in angle microfuge rotor for 8 min. Discard solution in lower collection tube.
    C) Add additional Te to the upper chamber and spin at same speed for 5 min.
    D) Invert filter chamber into new collection tube and spin at 1000 x g for 3 minutes. Recover liquid from collection tube and add 5 µg Glycogen.
    E) Extract once with $CHCl_3$:IAA, add NaOAc and Ethanol precipitate
    F) Note: remaining agarose fragments apparently quench fluorometric quantification based on Hoechst dye binding. However, agarose contamination will not adversely affect ligations and transformations. Quantification can be either by estimation based on samples run on mini gels or by UV spectrophotometry.

11) Test the quality of the T-tailed plasmid by test ligations with and without a PCR fragment. Expect a few hundred cfu/µg without insert (but all blue) and a significant percentage of white colonies (e.g., 60-70% white) with the test insert

Isolation of Staphylococcus DNA.

Adapted from: D W Dyer and J J Iandolo. (July 1983). Rapid isolation of DNA from Staphylococcus aureus. Appl. Environ. Microbiol. 46:283-285

Note on Lysostaphin: If you buy lysostaphin from Sigma-Aldrich (#L7386) the 3 mg/ml solution will be about 2000 U/ml. If you buy from BioVendor (RP179269) the specific activity is 6.5 times higher so the 3 mg/ml is around 13000 U/ml

1) Grow an overnight culture of Staphylococcus species in tryptic soy broth (depending on isolate).
2) Dilute 0.5 ml to 40 ml in pre-warmed broth in culture flask.
3) Incubate 4 hours at 37 ºC to mid log phase.
4) Transfer to centrifuge tubes and pellet bacteria at 8000 rpm at 4 ºC for 5 minutes. Decant supernate into flask and bleach before discarding.
5) Resuspend the pellet in 2 ml TES (30 mM TrisCl pH 8.0, 5 mM EDTA, 50 mM NaCl), transfer to 5 ml snap cap tube.
6) Pellet at 6000 rpm at 4 ºC for 5 minutes. discard supernate as above.
7) Resuspend pellet in 2.5 ml TES
8) Add 3 or 18 µl of 3 mg/ml lysostaphin (see note above), mix by inversion, and incubate at 37 ºC for 40-60 min.
    a. For plasmid isolation go to standard NaOH-SDS protocol here; lyse with 4ml 150 mM NaOH 1%SDS (see Alkaline SDS Method, step 5), the volume of 2 M KOAc will be 3 ml.
9) Add 125 µl 10% SDS, mix by inversion, and heat at 50 ºC in a water bath for 15 min.
10) Add 12.5 µl of 20 µg/ml RNAseA, mix by inversion, and incubate for 30 min at 37 ºC.
11) Add 12.5 µl of 20 µg/ml pronase, mix by inversion, and incubate for 30 min at 37 ºC.
12) Extract two times with 50:50 phenol:$CHCl_3$-IAA, then once with $CHCl_3$-IAA. (centrifuge at >3000 rpm for each).
13) Add 250 µl 3M NaOAc, mix well, divide into 2 tubes, and add 2.5 vol 95% ethanol, mix well, incubate on ice for 5-15 minutes
14) Pellet DNA at 8-10,000 rpm for 15 min at 4 ºC. Decant and discard supernate. Rinse each tube with 1 ml cold 70% ethanol, drain, then speedvac dry.

Redissolve in 300-700 µl Te, quantify and evaluate quality on gel. May need further purifications depending on needs (see NanoSep Cleanup for NGS).

Isolation of Enterococcus cecorum DNA.

Note on Lysozyme: Lysozyme stocks can be made up in advance by dissolving in GTE at 50 ug/ml and stored frozen at -20 ºC.

1) Grow a starter culture of 1 ml in TSB+5% chicken serum with shaking at 37 ºC in 5% CO_2
2) Dilute the overnight to 20 ml same medium (split to 2 oakridge or conical 50 ml tubes and grow overnight in same conditions
3) Pool the two aliquots and pellet the cells at 8000 x g 5 min. Decant off supernate
4) Resuspend the cell pellet in 0.7 ml GTE and transfer to 2 ml snap cap tube. This suspension can be stored frozen until ready for isolation

5) Add 200 ul of 50 ug/ml Lysozyme in GTE. Mix well
6) Incubate 30 min at 37 ºC
7) Add 40 µl 10% SDS, mix by inversion, and heat at 50 ºC in a water bath for 15 min.
8) Extract two times with 50:50 phenol:$CHCl_3$-IAA, then once with $CHCl_3$-IAA. (centrifuge at >3000 rpm for each).
9) Add 1/10th volume 3 M NaOAc, and divide to two 1.5 ml microfuge tubes. Add 3 volumes of cold 95% ethanol. Mix by inversion until the DNA knot has dehydrated and is visible (usually takes several minutes)
10) Pellet DNA at 8-10,000 rpm for 15 min at 4 ºC. Decant and discard supernate. Rinse each tube with 1 ml cold 70% ethanol, drain, then speedvac dry.
11) Redissolve in 300 ul TE+100 mM NaCl
12) Add 3 µl of 10 µg/ml RNAseA, mix by inversion, and incubate for 30 min at 37 ºC.
13) Add 3 ul 10% SDS and mix well
14) Extract two times with 50:50 phenol:$CHCl_3$-IAA, then once with $CHCl_3$-IAA. (centrifuge at >3000 rpm for each).
15) Add 1/10th volume 3M NaOAc, mix well, add 2.5 vol 95% ethanol, mix well by inversion for several minutes.
16) Pellet DNA at 8-10,000 rpm for 15 min at 4 ºC. Decant and discard supernate. Rinse with 1 ml cold 70% ethanol, drain, then speedvac dry.

Redissolve in 100 µl Te, quantify and evaluate quality on gel. May need further purifications depending on needs (see NanoSep Cleanup for NGS).

NanoSep Cleanup for NGS

It can be laborious to cleanup total bacterial (or plasmid) DNA to send for Next Generation Sequencing (Illumina or Nanopore) owing to the contamination with small RNA and DNA fragments and oligopeptides. A simple and fast method is purification on 100 MWCO (molecular weight cutoff) filters. We use the Pall OD100C33 spin cups.

1) The spin cups can be clogged if overloaded. They can easily handle 3 µg of partially purified DNA (see methods above).
2) Load the DNA sample into the insert cup.
3) Centrifuge at 8-9000 x g at either room temperature or 4 ºC for 10-15 minutes
4) Empty the flow-through. Be careful to not touch the bottom of the insert cup!
5) Add 200 µl Te to the insert cup and repeat steps 3 and 4
6) Repeat step 5 for a total of two rinses with Te
7) After removing the flow-through, add 50-100 µl Te to the insert cup. Place the tube and insert in a table top shaker and allow to mix for 10 minutes.
8) Recover the liquid from the insert cup
9) Quantify. Generally, you will recover about 50% of the DNA quantification for what was loaded. Example: 100 µl at 30 ng/µl loaded, and recovery is 100 µl at ~15 ng/µl.

Isolation of Staphylococcus RNA for RT-PCR

Modified from: S. J. Vandecasteele, W. E. Peetermans, R. Merckx, and J. Van Eldere Quantification of Expression of Staphylococcus epidermidis Housekeeping Genes with Taqman Quantitative PCR during In Vitro Growth and under Different Conditions. J. Bacteriol. December 2001 183:24 7094-7101; doi:10.1128/JB.183.24.7094-7101.2001

Solutions:

See Guanidinium Isothiocyanate isolation of RNA from Eukaryotic Cells

GuSCN solution: 5.4 ml sterile dH_2O, 4.7 g guanidinium isothiocyanate, 250 µl 1 M NaCitrate pH 5.2, 500 µl 10% sarkosyl, heat to 50 ºC to dissolve. Before use add 70 µl 2-mercaptoethanol per ml.

1) Overnight culture at 37 ºC in TSB (tryptic soy broth).
2) Pellet 0.5 ml at 2000 xg at RT
3) Resuspend in 4 ml prewarmed TSB and incubate with shaking at 37 ºC for 2 hours
4) Chill in ice water.
5) Pellet 3000 xg 5 min at 4 ºC
6) Resuspend in 500 µl GuSCN+2ME, and 500 µl acidified phenol (AE-equilibrated phenol)
7) Add 400 µl 0.2 mm glass beads in Safe-lock tube.
8) Process 5 min on full at 4 ºC in Bullet blender
9) Add 100 µl $CHCl_3$:IAA and 50 µl 2 M NaOAc pH 4.2, and vortex
10) In ice water for 10 min
11) Spin 10 min 10,000 xg at 4 ºC
12) Transfer upper aqueous to new tube containing 400 µl acidified phenol+100 µl $CHCl_3$:IAA, and mix well
13) Spin 10 min 10,000 xg at 4 ºC
14) Aqueous mix with equal volume isopropanol
15) Spin 10 min 10,000 xg at 4 ºC
16) Decant, add 1 ml cold 70% ethanol
17) Spin 1 min 10,000 xg at 4 ºC
18) Decant, vacuum dry
19) In 180 µl sterile deionized H_2O
20) Quantify for A260, A280 and µg/ul
21) Add 20 µl 10x RQ1 DNAse buffer
22) Mix, and add 5 U (5 µl) RQ1 DNAse Promega
23) 45 minutes at 37 ºC
24) Add 100 µl TE, and 3 µl 250 mM EDTA
25) Extract with 50:50 phenol:CHCl3-IAA, then $CHCl_3$-IAA
26) Add 1/10th volume 3 M KOAc, mix, add 1 ml 95% ethanol, mix well
27) Spin 10 min 10,000 xg at 4 ºC
28) Decant, add 1 ml cold 70% ethanol
29) Spin 1 min 10,000 xg at 4 ºC
30) Decant, vacuum dry
31) In 20 µl sterile deionized H_2O
32) Quantify for A260, A280 and µg/ul
33) First strand cDNA synthesis

Sterile dH_2O	To make 50 µl
5x MMLV buffer	10 µl
20 mM dNTP	0.5 µl
100 mM DTT	0.5 µl
Random hexamer primers 250 uM (500 µg/ml)	5 µl
RNA	5-10 µg
RNAsin Promega (40 U/µl)	0.5 µl (20 U)
MMLV RT (400 U/µl	0.5 µl (200U)

Incubate at 42 ºC for 45 minutes.

34) Heat to 70 ºC, 10 min.

35) Use 0.5 to 1 µl per PCR reaction

Electroporation of Staphylococcus agnetis

EP- 10% glycerol, 1.1 M sucrose; made by mixing sterile stocks

B2 medium- 1.0% casein hydro!ysate, 2.5% yeast extract, 0.1% K2HPO 4, 0.5% glucose, 2.5% NaCI; adjust pH to 7.5; autoclave.

1) Dilute 6 ml of an overnight culture in B2 broth into 100 ml prewarmed B2 broth
2) Grow at 37 ºC with shaking to an OD_{660} of 0.4-0.6 (about 2-4 hours.
3) Swirl the flask in an ice water slurry for 5 min.
4) Collect cells by centrifugation. All centrifugation are 2672 x g for 5 min, at 4 °C
5) Resuspend the pellet in 75 ml ice-cold sterile water, then pellet.
6) Resuspend the pellet in 50 ml ice-cold 0.5 M sucrose, incubate on ice for 30 minutes, and then pellet.
7) Resuspend the pellet in 15 ml ice-cold 0.5 M sucrose, then pellet
8) Repeat the resuspension and pellet 3, 1.5 ml and finally 0.4 ml ice-cold EP.
9) Incubate the final suspension on ice for 5 min, then aliquot at 90 µl. Use directly for electroporation or stored at –80 °C. To use rozen cells, thaw on ice for 10 min
10) For electroporation, the freshly prepared or thawed cell suspension, is transferred to a sterile 0.5 ml tube, and pelleted.
11) The majority of the supernatant is aspirated and discarded, then the cells are resuspended by gently pipetting up and down.
12) The tube is placed in a 50 °C thermocycler block for 2 min (to inactivate restriction systems), then incubated at room temperature for 2 min, then cells pelleted
13) Resuspend the pellet in 400 ul room temperature EP, then pellet the cells
14) Resuspend in 90 ul room temperature EP, and incubate at room temperature for 30 min.
15) Add DNA dissolved in sterile high purity water. Shuttle plasmid (pLI50) at 30-50 ng, or gDNA at 50-500 ng. Mix the cells and DNA gently by pipetting. Incubate at room temperature for 25 min.
16) Transfer to a 2 mm gap electroporation cuvette.
17) Electroporate: for a BioRad Gene Pulser program to 2 kV, 25 µFd, 200 Ohms, and electroporate. Record the time constant (acceptable range is 3.8 - 4.4 ms).
18) Rinse the cuvette with 900 ul 37 ºC B2 broth without antibiotics and transfer to a 14 ml sterile culture tube. Incubated 5 min at room temperature, and then at 37 ºC with shaking for 2 hours.
19) For pLI50 plate aliquots on tryptic soy agar containing 12ug/ml chloramphenicol and incubate overnight at 37 ºC for plate counts and calculation of transformation efficiency
20) For gDNA proceed with the downstream selection.

Figure 8. Colony Picking Grids for Master Plates

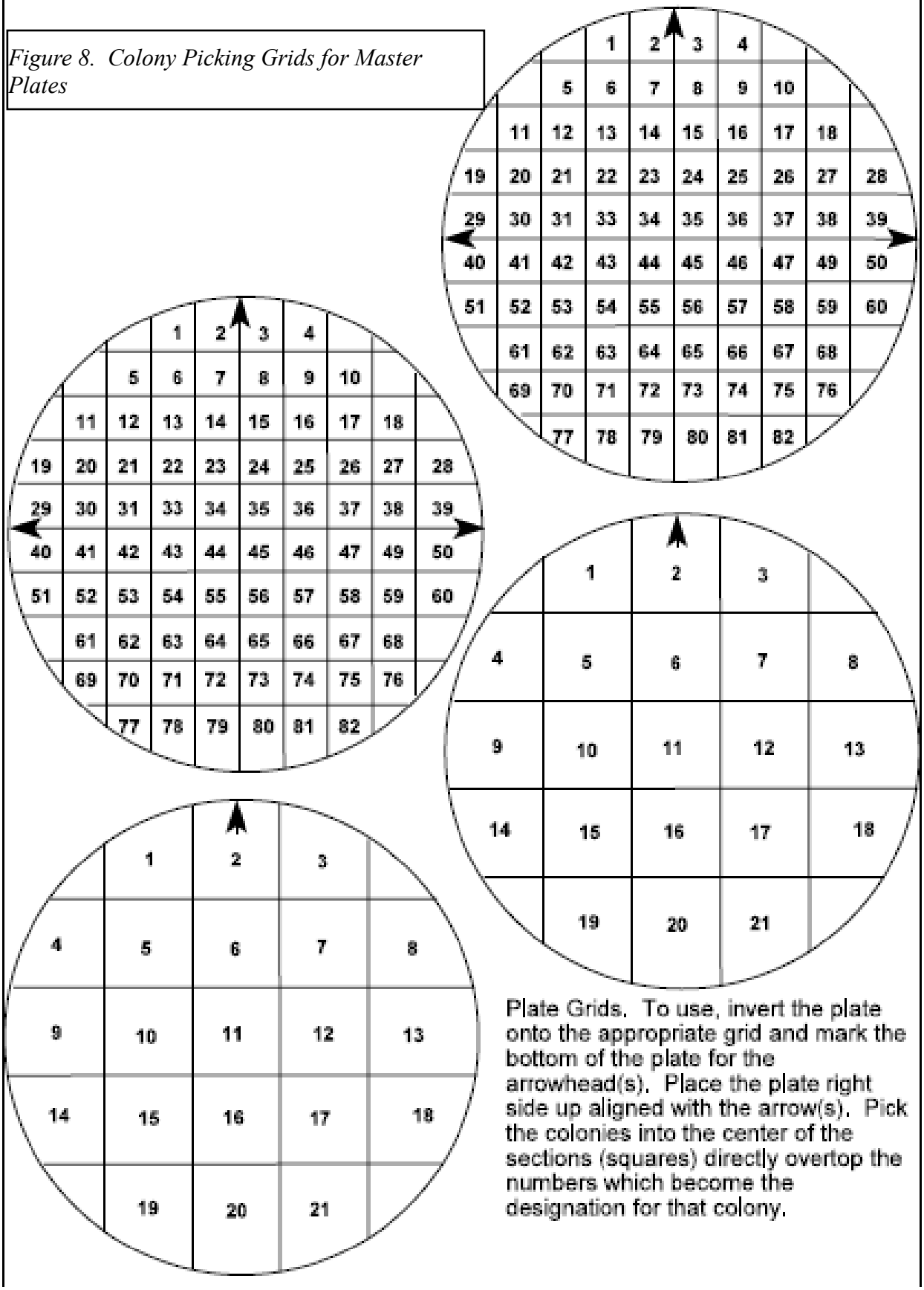


Plate Grids. To use, invert the plate onto the appropriate grid and mark the bottom of the plate for the arrowhead(s). Place the plate right side up aligned with the arrow(s). Pick the colonies into the center of the sections (squares) directly overtop the numbers which become the designation for that colony.

ANIMAL CELL CULTURE

Solutions:

1xPBS: Per Liter: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, adjust pH to ~7.4, autoclave.

HBSS (with divalent cations): 140 mM NaCl, 4 mM KCl, 1 mM $CaCl_2$, 0.4 mM $MgSO_4$, 0.5 mM $MgCl_2$, 0.3 mM Ha_2HPO_4, 0.4 mM KH_2PO_4, 6 mM D-Glucose, 4 mM $HaHCO_3$. To 800 ml H_2O sequentially add 8 g NaCl, 0.4 g KCl, 140 mg $CaCl_2$, 100 mg $MgSO_4$-$7H_2O$, 100 mg $MgCl_2$-$6H_2O$, 60 mg Ha_2HPO_4, 60 mg KH_2PO_4-$2H_2O$, 1 g D-Glucose, 350 mg $HaHCO_3$, bring to 1 L filter sterilize, store at 4 ºC

Polybrene Mediated Cell Transformation

This method has successfully been applied to Chinese Hamster and human HT1080 cells and was first reported with chick embryo fibroblasts. The method does not work for COS cells as they die (explode) in the DMSO treatment. Applicability to other cell lines can only be determined by trying. NOTE: Medium unless otherwise indicated is complete medium for the particular cells. For CHO cells this would be:DMEM+ 3 x 10^{-4} M pro-gly + 10% FCS

Plating of Cells- Day 1

1) Subconfluent monolayers are trypsinized and collected in media. Coulter count to determine cell density and plate 5 x 10^5 - 1 x 10^6 cells in 10 ml medium + 30 μg polybrene per 100 mm dish. (For CHO expect about 5 x 10^6 cells per 75 cm^2 flask). **NOTE: Cell number is critical; if plating efficiency is poor the transfection will not work. At 2.5 x 10^5/dish the efficiency is less than 1/5th that for 5 x 10^5/dish. Efficiencies for 1 x 10^6 are equal to that for 5 x 10^5 (with polybrene pretreatment) however the cells may reach confluence too early; this may not be a problem in transient assays however. To insure no loss of time be sure and visibly inspect plates for sufficient numbers of attached cells in the morning before adding the DNA mixture. The plates should be 1/8th to 1/4th confluent; if not- START OVER AND SAVE YOURSELF SOME HEADACHES LATER.**

2) Cells are allowed to sit and stretch at 37º for >12 hours. Normally cells are plated one afternoon and the DNA added the next morning. Overnight preexposure to polybrene apparently increases transfection frequencies (1-2 fold) and may give more consistent results with varying cell numbers.

DNA Introduction-Day 2

NOTE: High molecular weight DNAs (50 kbp) are not suitable for polybrene-mediated transfections as the DNA has a tendency to precipitate in the cell exposure phase (or earlier). For lambda and cosmid clones use the $CaPO_4$ method. Also, for large quantities (>20 μg) of larger DNAs (>20 kbp), polybrene will tend to complex with the DNA and precipitate. Therefore, for high concentrations of large DNA it is necessary to add the DNA to the medium and then add the polybrene. For low concentrations and small DNAs, the polybrene may be added to the medium first. Another factor is the Calf serum. Newborn and fetal calf serum has been observed to inhibit transfection significantly. Therefore, omit the serum from culture medium used during the introduction of DNA.

DNA Amount - For transient expression each plate should be transfected with the equivalent of 20 μg of a 10 kbp plasmid (i.e., 70-100 μg of a 50 kbp molecule, however see note above). For isolation of stable transformants using a drug selectable marker (e.g., pSV2Neo) use 150 to 600

ng per plate.

Serum Nucleases - the original procedure called for serum to be included in the DNA mixture but subsequent experience shows that many lots of serum may contain nucleases which greatly lower the transfection frequencies. For CHO cells the serum is NOT necessary and therefore should be left out. If your cells need serum then you need to test your lot of serum for its nuclease activity.

3) Prepare DNA-polybrene mixtures by adding to fresh medium (without serum) first DNA (diluted in PBS) then polybrene (also in PBS) to 10 μg/ml. Mix well. NOTE: Co-transfection is usually accomplished by adding co-transfected DNA at a 5-20 fold molar excess to a drug-selectable marker DNA (i.e., pSV2Neo). With pSV2Neo frequencies are approximately 2-300 transformants per μg DNA (i.e., 200 ng per dish gives about 50 transformants per dish).

4) Aspirate the medium from the cells and pipet on 3 ml DNA-polybrene mixture per 100 mm dish. It may be possible to increase the volume of medium used at this step but others have indicated that the amount of polybrene added should remain at 30 μg per 5 x 10^5 cells.

5) Place the plates on an even level at 37° for 6 hours, rocking gently to spread the medium every 1.5 hours. Transformation frequency may increase by lengthening the time of Polybrene-DNA treatment up to 12 hours.

6) In groups of 4 to 6 plates remove the medium, replace with 5 ml 30%DMSO-medium (70 ml medium + 30 ml autoclaved DMSO, **prepare the DMSO-medium mixture in advance as DMSO solvation generates heat and the mixture needs to be at room temp or it will kill the cells**) and let stand at room temperature for 4 minutes. This step is critical to transformation frequency and time of exposure to DMSO and concentration of DMSO have been optimized by others. We find it best to stagger this addition in 1' intervals, so that each dish is treated with DMSO for exactly the same time. Solution addition to each plate should be by gentle addition onto the wall of the dish so as not to wash the cells off, the DMSO will reduce their adherence.

7) Remove the DMSO-medium, replace with 10 ml medium; let stand at room temp for a few minutes; then replace with fresh medium (with FCS).

8) Return plates to 37°. **Note**: Cells do not appear adversely affected by this treatment, but continue to divide and look healthy.

Transient Expression Assay - Day 3

Analysis with an S14 gene plasmid (pGS14-9) reveals that the transient expression of a transfected gene is detectable by 12 hrs post DMSO treatment. The level of expression remains constant from 12 hrs through 60 hrs and appears to decline by 72 hrs. Therefore, isolation of RNA for analysis of the transient expression of a gene should be done any time from 12 hrs to 60 hrs however there is no advantage to going longer as the percentage of transfected-gene-RNA declines; i.e., 5 μg of RNA from 12 or 24 hrs is equivalent to 10 μg of RNA from 48 hrs.

9) Isolate cytoplasmic RNA by dumping off the medium and scraping the cells into 10 ml cold PBS. Process the suspended cells as you would for isolation of RNA (see Isolation of cytoplasmic rna from transformants below)

Stable Transformant Selection - Day 3

9) Transformants are selected by replacing the medium with drug selection medium at least 24 hours after DMSO shock treatment. Frequency may increase slightly by waiting to 36 hours before adding drug selection conditions but the cells may reach confluence by then and many of the drug selections (including G418) are not as effective on non-dividing cells.

NOTE: G418 is dissolved in PBS at 50 mg/ml and pH adjusted with NaOH to app. 7.0, then filter sterilized and stored frozen. Although working stocks can be stored at 4° for several weeks. **Concentrations: stored G418 (wet or dry) loses activity (about 50%/year). When preparing**

G418 stock solutions from a new supply (order only when you need it) be sure to ascertain the efficacy of a new stock, or stocks which have been around for 6 months or longer, by testing on susceptible cells at concentrations ranging from 100 to 2000 µg/ml. Typically, for selection of transformed CHO cells use G418 at 500 to 1000 µg/ml dry weight (this assumes G418 active ingredient of about 50%). After selection and cloning transformants can be maintained in 0.25 mg/ml G418.

10) To maintain drug selection with G418 the medium should be replaced on day 4 (post DNA addition) and then again on day 8 or 9. Medium will probably not be turning but the drugs effective concentration may become depleted if to many cells are present and dead cells will be floating in the medium.

11) After 12-14 days post transformation colonies should be visible and clonable on day 14 or 15. Don't let the colonies get to big or the frequency will be erroneous and cloning difficult because of satellite colony formation by floaters.

Calcium-Phosphate Mediated Transfection

This method appears to work well with COS cells and may be applicable to other cell lines. Inherent problems are reproducibility for Cell viability and $CaPO_4$-DNA mixtures. For reference, see: Reference: Chu and Sharp (1981) Gene 13:197-202.

Stock Solution:

2x HeBS: for 100 ml dissolve 1 g HEPES, 1.6 g NaCl, 74 mg KCl, 28 mg Na_2HPO_4, and 200 mg Glucose in Millipore filtered-Autoclaved H_2O and adjust pH to 7.05 with NaOH. Bring to vol. and filter sterilize.

1) Preparation of $CaPO_4$-DNA precipitate:
   a) Mix DNA (20 µg) in 500 µl 250 mM $CaCl_2$
   b) Add DNA-Ca solution dropwise to 500 µl 2xHeBS with swirling. Add the solutions slowly so as to get uniform mixing to form a fine particulate precipitate.
   c) Let the solution stand for at least 30 minutes at room temp before adding to the cells.
2) Preparation of the cells:
   a) Trypsinize the cells (use T+E). Leave the cells exposed to the trypsin until app 90-95% of the cells are rounded on the dish (determined microscopically); this is necessary to assure that the cells are permeabilized. Stop with complete Medium (+FCS).
   b) Quantitate with Coulter counter.
   c) Pellet the appropriate number of cells (see step #3) at room temp. at app. 500-800 x g for 5 min: Use setting #5 on IEC-CL tabletop centrifuge.
3) Resuspend the cell pellet in 0.2 ml of $CaPO_4$-DNA solution per 10^7 cells and let stand at room temp for 15'.
4) Dilute and plate the cells in complete medium+ 6.25 mM $CaCl_2$ 0.05 x HeBS.

Proceed with drug selection or transient assay.

Analysis of Stable Transformants

Generally, for analysis of stable transformants one 150 mm dish provides sufficient RNA (>20 µg) and DNA (>100 µg) for several S1 analyses or genomic blots.

Isolation of Expressed RNA from Transformants.

Use either the NIB lysis and RIB-urea method for preparation of cytoplasmic RNAs or the Guanidine Isothiocyanate procedure for preparation of total cell RNA. The latter is preferred both for its ease of use and for the quality of the RNA isolated. Both procedures are described in the chapter on RNA Isolation and Analysis.

Isolation of High Molecular Weight Genomic DNA from Nuclei

1) Nuclear pellet resuspended in 10 ml NIB and add 0.5 ml 10% SDS to final concentration of 0.5%. Incubate with occasional inversions for 10' at 50°C.
2) Bring to 37°C and add boiled RNAse A to final concentration of 100 µg/ml (0.4 ml 2.5 mg/ml). Incubate at 37°C for 30'.
3) Bring to 10 mM EDTA (0.4 ml 250 mM) incubate 10' at 50°C.
4) Bring to 37°C, add pronase to final concentration of 100 µg/ml (0.2 ml 5 mg/ml), incubate at 37°C for >8 hours.
5) Extract 3 times with phenol:$CHCl_3$-IAA. NOTE: extractions should be gentle mixing of the two solutions for app. 5'.
6) Extract 1 times with $CHCl_3$:IAA.
7) Add 1/10th vol 3 M NaOAc and 3 vol cold 95% EtOH. Invert to gently mix. Hook out the white string-like, mass of DNA with a bent pasteur pipette. Rinse the DNA in cold 70% EtOH and then place the mass of DNA in a microfuge tube and dry briefly under vacuum.
8) Redissolve DNA in 200-800 µl TE. Read A_{260}.

Hirt Extraction of Small DNAs

1) Cells growing in monolayer culture.
2) Decant off medium and rinse plate with cold PBS+CaMgGlu twice.
3) For each 100 mm dish add 4 ml of 10 mM Tris pH 8.0, 0.6% SDS, 10 mM EDTA
4) Incubate on shaker platform at room temp with gentle rocking for 30 minutes
5) Decant the viscous cell lysate into a high speed Corex tube, add 1 ml 5 M NaCl, cap with parafilm and gently invert several times to mix.
6) Incubate >12 hours on ice to precipitate chromosomal DNA and proteins.
7) Centrifuge 17-18000 x g (11 krpm, JS13; 15 krpm JA20) for 45' at 4°C.
8) Collect the supernate and extract with 50:50 Phenol: $CHCl_3$-IAA then with $CHCl_3$-IAA.
9) Add 2-3 vol EtOH to precipitate nucleic acids (no need to add NaOAc because of NaCl concentration).
10) The nucleic acid pellet is dissolved in TE, transferred to a microfuge tube and EtOH precipitated (this time add NaOac) to remove salts.

Cell Fusion Using Polyethylene Glycol

PEG-8000 Solution: Dissolve PEG-8000 in sterile DMEM without serum at 60°C to a concentration of 50% (w/w). Mix by swirling and sterilize by suction filtration through Nalgene 0.2 micron disposable filter.

1) 24 hours before inoculating dishes, feed subconfluent flasks of each parental cell line with DMEM + 10% FCS containing <u>no selective drugs!</u>
2) Inoculate 60 mm dishes with a mixture of two parental cell lines at density of 1 x 10^6 of each and incubate in DMEM + 10% FCS for 24 hrs. at 37°C, unless one or both of the parental

lines are temperature-sensitive mutants. In the latter case, incubate at the permissive temperature (i.e.33ºC) until all cells have sat and stretched on the plastic dish.
3) From this point on, do one dish at a time:
4) Aspirate the medium from the dish, tilt the dish on edge and allow the medium to drain for 30 sec. Then aspirate the remaining medium that has collected.
5) Cover the cells with 3 ml of 50% PEG solution at room temperature and incubate at room temperature for one minute without agitation.
6) Remove the PEG and rapidly rinse the dish three times with 5 ml of DMEM (no serum) + 10% DMSO.
7) Harvest cells with trypsin + EDTA and re-plate at desired cell densities in nonselective medium. For CHO and CHL cells expect approximately 0.1 - 1.0% hybrid formation (i.e. 103-104 hybrids per original 60 mm fusion dish containing 106 of each parental clone).
8) After 24 hrs re-feed plates with medium containing appropriate agents to select hybrid clones.
9) Feed plates every 7 days until hybrid clone are large enough to pick and/or score.

EMS Mutagenesis of Chinese Hamster Cells

EMS Treatment

Note: EMS is a potent mutagen and should be handled carefully. Also be careful of fumes filtering flasks should be used and contain 0.5 M NaOH. All medium should then be autoclaved and rinsed down the sink with lots of water.

1) Cells should be growing in 75 cm^2, use 6-8 sub-confluent flasks (ones that should reach confluence in 2 days). Alternatively, start with 4 flasks of confluent cells that you trypsinize and seed individual flasks with 3 x 10^6 cells each and allow the cells to set and stretch overnight (one doubling means each flask has 6 x 10^6 cells.
2) One flask should be the control for Spontaneous mutations. This flask is trypsinized and plated in 5 plates each at 10^5, 10^4, and 10^3 cells in 5 ml per 100 mm dish and 5 x 10^5 to 10^6 cells in 10 ml per 150 mm dish in medium (no drugs). After cells have attached treat as in step 6. The cell number is based on a Coulter count if using method in **1a)**, in which case all the other flasks are assumed to yield the same number of cells, or the anticipated 6 x 10^6 if using method **1b)**.
3) The EMS medium is complete medium without Serum or drugs (EMS would alkylate the serum and quench the mutagen). In the fume hood (wear gloves) add 1 µl of EMS per ml of medium final=10^{-2} M (you need 10 ml for each flask). Mix well on a stir plate (EMS is not very miscible with water), then in the sterile hood filter sterilize through a nalgene filter (0.2 micron).
4) Aspirate medium from flasks of cells to be mutagenized then rinse with PBS. Add 10 ml of EMS medium to each flask and incubate at 37ºC for 2 hours. Aspirate the medium into 0.5 M NaOH (not into the normal flask). Rinse each plate with PBS (again aspirate into NaOH solution). Then re-feed each flask with Medium+FCS. Allow cells to recover for 2 days.
5) Trypsinize each flask separately and plate as in the controls.
6) After the cells have set and stretched 4-12 hours add an equal volume of medium that is 2x the level of selective agent (i.e., 4 x 10^{-7} M emetine gives 2 x 10^{-7} M emetine final).
7) After 4-5 days re-feed with fresh medium and selective drug(s).
8) Re-feed again 6-7 days later. Removal of medium and re-feeding should be done carefully as to not dislodge cells from the dish as this will alter plate counts.
9) About 2 weeks after plating colonies should be visible and ready to clone. It is best to clone only one colony from each plate to guarantee independence. After cloning stain and count plates with few colonies to calculate mutation frequency relative to the spontaneous mutations in

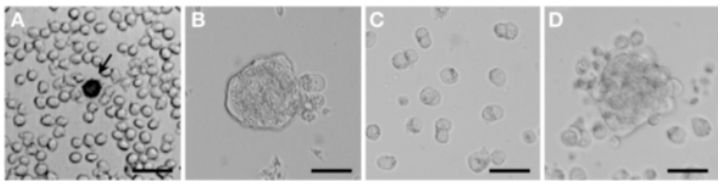
the controls.

Culture of Chicken PGCs

Choi, J. W., S. Kim, et al. (2010). "Basic Fibroblast Growth Factor Activates MEK/ERK Cell Signaling Pathway and Stimulates the Proliferation of Chicken Primordial Germ Cells." PloS ONE **5**(9): e12968.

Table 4. Medium Components for Chicken PGCs

Medium Component	Vendor	Part Number	Stock Conc	Stock Vol	Price	Final Conc	Cost per 500 ml
Advanced DMEM	Invitrogen	12491015	1x	500ml	21.70	1x	2.70
GlutaMAX-I supplement	Invitrogen	35050061	200mM	100ml	28.15	2mM	1.41
2-mercaptoethanol	Invitrogen	21985023	1000x	50ml	31.50	1x	0.31
Fetal Bovine Serum	Hyclone		100%	500ml		7.5%	0
	Invitrogen	16000044	100%	500ml	262.65		23.18
Chicken serum	Sigma-Aldrich	C5405	100%	100ml	21.50	2.5%	0.53
	Invitrogen	16110082	100%	500ml	62.70		1.65
MEM-nonessential amino acids	Invitrogen	11140050	100x	100ml	15.40	1x	0.77
Nucleosides	Millipore	ES-008-D	100x	50ml	18.00	1 x	1.80
human LIF	Sigma-Aldrich	L5283-10ug	10 ug/ml	1ml	284.00	2 ng/ml	0
	ProSpecBio	Cyt-644	10ug	dry	130.00		13.00
	Invitrogen	PHC2115	10ug		130.00		
human SCF	Sigma-Aldrich	H8416	10 ug/ml	1ml	264.50	5 ng/ml	0
	ProSpecBio	Cyt-255	10 ug	dry	130.00		32.50
	Invitrogen	PHC9464	10 ug		150.00		
human bFGF	Sigma-Aldrich	F0291-25ug	25ug	Dry	247.50	10 ng/ml	0
	ProSpecBio	Cyt-218	50ug	Dry	130.00		13.00
	Invitrogen	PHG0264	10 ug		50.00		
	Creative Biomart	FGF2-11H	100 ug	Dry	450.00		
							89.05
Accutase	Invitrogen	A1110501		100 ml	$32.80		



Culture of chicken PGCs. (A–D) Morphology of cultured PGCs. (A) Whole embryonic blood cells at day 0 in primary culture. PGCs were identified by SSEA-1 staining as indicated by an arrow. (B) PGC colonies after 10 days of culture. (C) Dissociated PGC colonies. (D) Reformation of PGC colonies (Bar = 25 mm).

Figure 9. Microscopic Images of Chicken PGC Cultures

Isolation of PGCs

1. 2 ml of whole blood cells taken from the dorsal aorta of stage 14–15 (H&H) (50–54 h of incubation) chicken embryos (mixed-sex)
2. Cultured in media in a CO_2 incubator maintained at 37°C in an atmosphere of 5% CO2 in air with 60%–70% relative humidity.
3. Recommend: change 1/3rd of media every 2-3 days.
4. After 7-14 days of growth most of the blood cells will be dead and PGC colonies formed and loosely attached to culture plate
5. Cultured PGCs are detached by gentle pipetting and disaggregated with an appropriate enzyme. They are subcultured at 3- to 4-day intervals by dissociating cell colonies using Accutase (Millipore).
6. To show migratory activity test for generation of pseudopodia, PGCs were cultured on hESC-qualified Matrigel (BD Biosciences, San Jose, CA).

Migration Assay

1. PGCs cultured for 82 days were used for the migration assay.
2. To assay migration into the germinal crescent, cultured PGCs were labeled with PKH26 fluorescent dye (Sigma-Aldrich)
3. Transferred into the subgerminal cavity of stage X embryos.
4. Eggs were sealed with Parafilm and incubated until stage 6 (24 h of incubation).
5. Embryos were cut away from the yolk with the aid of filter paper and microdissecting scissors and then the number of fluorescent PGCs in the germinal crescent of excised embryos was counted under a fluorescence microscope (IX-70, Olympus, Tokyo, Japan).
6. To assay migration into the gonads, PKH26-labeled PGCs were injected into the dorsal aorta of stage 14–17 embryos.
7. Eggs were sealed with Parafilm, then incubated until stage 30.
8. Gonads from the recipient embryos were retrieved,
9. The number of fluorescent PGCs in the gonad was counted under a fluorescence microscope (IX-70, Olympus).

Production of Germline Chimeric Chicken

1. PGCs cultured for more than 50 days were used for germ cell transfer.
2. A small window was made at the pointed end of the recipient egg,
3. 2 ul (containing approximately 3,000 cells) was injected into the upper portion of the dorsal aorta of the stage 13 WL embryo (50 h of incubation) using a micropipette.
4. The window was then sealed twice with Parafilm, and the egg was incubated with the pointed end down until hatching.

5. Putative germline chimeric chickens that reached sexual maturity were then testcrossed by mating with KO (i/i) chickens of the opposite sex.
6. Donor PGC-derived offspring could be identified based on their color: donor PGC-derived progeny (i/i) had black feathers, whereas the progeny (I/i) from endogenous WL PGC (I/I) had white feathers.

Freezing PGCs

1. Each vial should contain 500,000 to $1x10^6$ PGC's/0.5ml
2. Resuspend and remove PGC's from feeder layer
3. Transfer to a 15ml polypropylene tube & using a hemacytometer count the total # of PGC's
4. Pellet the PGC's @200g for 5min., discard the supernatant
5. Resuspend the PGC's in 0.25mls 20% FBS in complete media for each $0.5\text{-}1x10^6$ cells (e.g. if you have 4 million cells resuspend in 1 ml)
6. On ice, add dropwise w/swirling an equal volume of cold 20%DMSO in 20%FBS in complete media Transfer cell suspension to labeled cryovials immediately
7. Place cryovials in a cold Nalgene Cryo 1ºC freezing container for give a rate of 1ºC/min freeze Place the container in a -80ºC freezer overnight
8. Next day transfer to liquid nitrogen cell storage tank

Culture of Chicken HTC immortalized macrophage

References:

N.C Rath, M.S Parcells, H Xie, E Santin, (2003) Characterization of a spontaneously transformed chicken mononuclear cell line. Veterinary Immunology and Immunopathology, Volume 96 (1-2):93-104.

Medium:

RPMI-1640 medium (no glutamine) containing 1× concentration of antibiotic–antimycotic solution, 2 mM glutamine (1x GlutaMax), 1 mM Na-pyruvate, 50 μM β-mercaptoethanol, and 5% FBS or 2% chicken serum (v/v). You can also just use 10 % FBS. Very important to buy endotoxin free certified serum.

1. In low densities the HTC cells grow as loose monolayers but tend to detach from the bottom as growth progresses, form aggregates, and continued to grow in suspension.
2. The cells are passaged at a ratio of 1:10 every 5 days with fresh medium.
3. To passage use Cell Dissociation Buffer
4. To Freeze use RPMI complete medium containing 30% FBS and 10% DMSO.

Enzyme Free Cell Dissociation

Cell Dissociation buffer:

1% BSA, 1mM EDTA, 1mM EGTA, in PBS, pH 7.4; Sterile filter, store at 4 or -20 ºC.

1. Prewarm all reagents.
2. Remove all medium from the culture vessel.
3. Rinse the cells with a balanced salt solution without calcium and magnesium, such as PBS. Gently rock the vessel for 30 seconds and remove the buffer.
4. Add the Cell Dissociation Solution (about 5 ml/75 cm^2 flask) and rock the vessel to bathe the cell monolayer.
5. Incubate 5-10 minutes.

6. Sharply tap the vessel against the palm of your hand to dislodge the cells. Strongly adherent cells may require additional time to become dislodged.
7. Add complete growth medium to the cells and pipet repeatedly to dissociate clumps.

Freezing of HTC Macrophage Cells

1. Collect cell suspension using Cell Dissociation buffer or similar Enzyme Free Cell Dissociation.
2. Gently pellet the cells at 500-1000 x g for 5' at room temperature.
3. Resuspend the cell pellet in RPMI complete medium (all supplements including antibiotic-antimycotic) that has been made 30-50% endotoxin free fetal bovine serum, and 10% DMSO (dimethyl sulfoxide).
4. Aliquot to freezer vials
5. Put the vials in a sealed foam box at -80 ºC overnight
6. Transfer to liquid nitrogen.
7. To recover, thaw rapidly.
8. Centrifuge as in step 2
9. Resuspend in complete RPMI and seed into a flask.

Purification and culturing of chicken peripheral blood monocytes (PBMC)

Solutions

Alsever's Solution- 0.05 g Citric acid (Anhydrous), 0.8 g Sodium Citrate (dehydrate), 2.05 g d-Glucose, 0.42 g NaCl, ultrapure H_2O to total volume of 100 mL. Autoclave at 118 °C for 15 minutes liquid cycle and store at 4 °C. Makes 2.6 mM Citric acid, 27.2 mM NaCitrate, 113 mM glucose, 71.8 mM NaCl, pH 6.0.

Ficoll Hypaque: GE Healthcare 17-1440-02

References

Drechsler Y, Tkalcic S, Saggese MD, Shivaprasad HL, Ajithdoss DK, Collisson EW. A DNA vaccine expressing ENG and GAG offers partial protection against reticuloendotheliosis virus in the Prairie Chicken (*Tympanicus cupido*). Journal of Zoo and Wildlife Medicine. 2013;44(2):251-61. doi: 10.1638/2011-0229R1.1.

Dawes ME, Griggs LM, Collisson EW, Briles WE, Drechsler Y. Dramatic differences in the response of macrophages from B2 and B19 MHC-defined haplotypes to interferon gamma and polyinosinic:polycytidylic acid stimulation. Poult Sci. 2014;93(4):830-8. doi: 10.3382/ps.2013-03511.

Enrichment of PBMC from blood

1) Blood is collected in vacutainer with EDTA.
2) 3 mL of blood is mixed with an equal volume of Alsever's solution.
3) 3 mL of this mixture is layered over an equal volume of Ficoll-Hypaque (density 1.077 or 1.083). Need a tube with small diameter to make the interface in step 5 easier to see and collect.
4) Samples are centrifuged for 35 min (400 × g, 23°C; brake off)
5) After centrifugation, the opaque interface containing the mononuclear cells is collected.
6) Isolated cells are washed twice in 5 mL of PBS (400 × g, 10 min, 23 ºC),
7) Resuspend in 1 ml RPMI+10%FBS, counted, and viability confirmed based on the exclusion of 0.1% trypan blue dye (≥90%). .

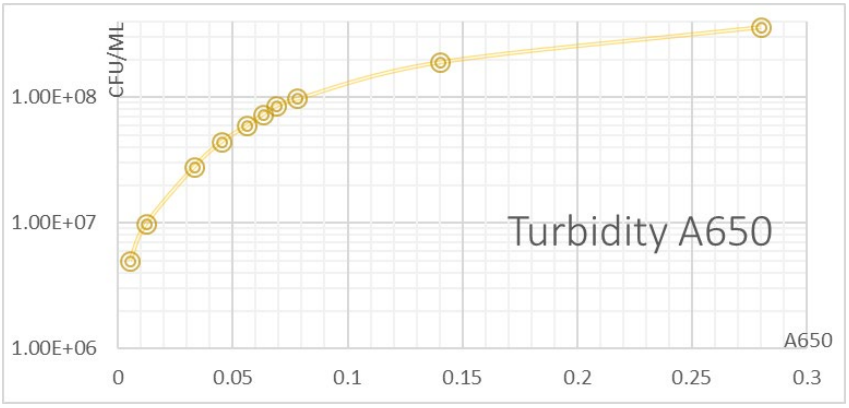
Adherent PBMC Culture

1) PBMCs ($5x10^7$ cells/mL) are incubated (37°C/5% CO2) for 3 h in each well of a 12-well plate containing RPMI w/o Phenol Red supplemented with 10% heat-inactivated fetal bovine serum; 0.1 mM/mL nonessential amino acids, 2 mM L-glutamine, 55 µM/mL 2-mercaptoethanol, 50 U/mL penicillin, and 50 µg/mL streptomycin.
2) Medium is aspirated and well is rinsed with warm PBS to remove nonadherent cells
3) Medium is replenished and cells are incubated for 24 to 48 h to facilitate monocyte adherence and complete removal of thrombocytes, nonadherent lymphocytes and other semi-adherent cells.
4) Medium is aspirated and well rinsed with warm PBS, then well is refed with fresh medium
5) Monocytes are cultured for 6 d to allow maturation and differentiation of cells, with medium changes occurring every 3 to 4 d, thus ensuring that optimal nutrient requirements are met.

Quick Phagocytosis Assay

Modified from: Recent human-to-poultry host jump, adaptation, and pandemic spread of Staphylococcus aureus. BV. Lowder, CM. Guinane, NL. B Zakour, LA. Weinert, A Conway-Morris, RA Cartwright, AJ Simpson, A Rambaut, U Nübel, JR Fitzgerald. Proceedings of the National Academy of Sciences Nov 2009, 106 (46) 19545-19550; DOI:0.1073/pnas.0909285106
RPMI: RPMI 1640 1x(Nonessential amino acids; pyruvate; Glutamax; 2-mercaptoethanol)

1) HTC cells growing in RPMI +10% FBS +Pen/Strep. Need 2 75 cm^2 flasks at ~50% confluence
2) For all the following work RPMI is RPMI
3) Overnight culture of bacterial cultures in LB or TSB
4) Aspirate HTC medium, and rinse with 5-8 ml PBS per flask
5) Add 5 ml CellStripper, and scrape cells with a lifter or scraper
6) Collect cells to 15 ml centrifuge tube. Mix by inversion, and sample for Countess system (10 ul trypan blue + 10 ul of cells). Count for viable cell count and % viable
7) Pellet HTC at 500xg 10 min at 20C. Decant and resuspend at $5x10^6$ cells/ml in RPMI +1% FBS.
8) If using purified PBMCs start here with O/N bacterial cultures and the PBMC at $5x10^6$ cells/ml in RPMI +1%FBS
9) Dilute bacterial cells 20 ul of cells + 2 ml of RPMI +1%FBS. Read A650 nm. Calculate cell density using the Turbidity A650 chart. Read the estimated CFU/ml.
10) For different cultures you need to convert the estimated CFU/ml to the actual CFU/ml for that culture. Multiply the estimated CFU/ml by the conversion value.



Species	Isolate	Conversion
S. agnetis	908	1x
S. agnetis	1379	0.5x
S. aureus	1510	4x
S. agnetis	1416	?
S. aureus	1302	?

11) Dilute the RPMI diluted bacteria to 10^6 CFU/ml in RPMI + 1%FBS
12) Control: 350 ul of RPMI +1% FBS in 2.2 ml snap cap microfuge tube
13) Experimental: 350 ul of HTC in RPMI +1%FBS at $5x10^6$ cells/ml in 2.2 ml snap cap microfuge tube
14) Add 350 ul of 10^6 CFU/ml bacteria in RPMI +1%FBS to the Control and the Experimental
15) Add 60 ul of chicken pooled plasma to the Control and Experimental.
16) Mix gently but thoroughly.
17) For both the control and experimental, aliquot each at 200ul in each of 3 wells in a 48 well culture dish. Swirl the plate to distribute across the well bottom.
18) Place the dish in the culture incubator at 37C 5% CO_2 for 2 hours
19) Add 800 ul of sterile dH_2O to each well and triturate a few times with the pipettor. This represents a 10^{-1} dilution of the bacterial culture.
20) Dilute each lysate at $2x10^{-1}$, 200 ul of the lysate with 800 ul of sterile dH_2O in a 1.5ml snap cap microfuge tube
21) Plate in duplicate or triplicate, 40 ul of this dilution on petri plates in (TSA or LB). The overall dilution is $8x10^{-4}$ of the starting 10^5 CFU, so expect 40 to 160 colonies per plate on the controls.

RNA ISOLATION & ANALYSES

RNA Stabilization

Reference: De Wit P, Pespeni MH, Ladner JT, Barshis DJ, Seneca F, Jaris H, Overgaard Therkildsen N, Morikawa M and Palumbi SR (2012) The simple fool's guide to population genomics via RNA-Seq: an introduction to high-throughput sequencing data analysis. Molecular Ecology Resources 12, 1058-1067.

This is a substitute for RNALater

For 1 liter RNAStorage buffer:

623.3 ml of autoclaved, MilliQ water
466.7 g Ammonium sulfate Stir until dissolved
Add 16.7 ml of 1 M Sodium Citrate (autoclaved)
Add 26.7 ml of 0.5 M EDTA (autoclaved)
Adjust to pH 5.2 using concentrated H_2SO_4 (about 13 drops= 0.66 ml) check pH using pH paper
Store at RT

To use:

1. Collect fresh tissue samples and work quickly to get them into the storage solution
2. Cut into strips or pieces ≤0.5 cm
3. Immerse in ≥ 5 volumes of RNAStorage buffer
4. Place on ice or store overnight at 4 ºC to allow permeation of the tissue
5. Samples may be stored at RT or 4 ºC for week
6. For long term storage store at -20 ºC
7. To process, remove tissue from RNAStorage buffer and directly process using Trizol or Guanidinium isothiocyanate method.
8. Khaloud Alzahrani has successfully purified nuclei from tissues stored at -20 ºC in RNAStorage buffer by Dounce homogenization.

Guanidinium Isothiocyanate Method

Adapted from Chomczynski and Sacchi, Anal. BioChem. 162:156-159, 1987). For details, see Current Protocols in Molecular Biology (Red Book) Chp. 4.2.4 - 4.2.8.

Solutions:

Denaturing Solution: 4 M Guanidine Isothiocyanate, 25 mM Sodium Citrate pH 7, 0.5% Sarkosyl, 0.1 M 2-mercaptoethanol. **NOTE: I have found that you can substitute NaCitrate at pH 5.2 and actually get cleaner RNA (less DNA contamination).**

For 100 ml mix: 47.26 g GuSCN, 2.5 ml 1 M Na·citrate pH 7.0, 5 ml 10% sarkosyl, 54 ml H_20, heat at 65ºC for 10'. Final volume should be 100 ml. To use, add 0.35 ml 2-mercaptoethanol per 50 ml stock. (This working solution is stable at room temp for 1 month.)

2 M Sodium Acetate pH 4: 16.42 g NaOAc (Anhyd) + 40 ml depc-H_2O.

Adjust to pH 4.0 with glacial acetic acid and adjust final volume to 100 ml with DEPC-H_2O.

AE buffer: 50 mM NaOAc 10 mM EDTA pH 5.3 to 5.4; adjust pH with HOAc

AE buffered Phenol: Thaw the phenol, mix with AE buffer, Pull off aqueous, Repeat several times. Store frozen with a layer of AE buffer on top.

DEPC: Diethyl pyrocarbonate; some people use this to treat solutions before autoclaving but I have

never found it to be necessary if you handle the solutions and RNA quickly and with forethought

NOTE: One of the biggest problems in RNA analyses is overhandling RNA. The fewer steps between isolation and first strand synthesis the better. RNA is very labile; although RNAse inhibitors can be added they don't eliminate degradation. RNA is more sensitive to degradation by freeze-thaw or pH.

For Adherent Tissue Culture Cells: (For one 100 mm plate)

1) Aspirate media. 1 ml Denaturing Solution (see above) and rock dish to lyse cells. Tilt dish to collect lysate and transfer to 15 ml Corex tube. Repeat 2 more times with 1 ml of Denaturing solution each time and pool the lysates with the original.
2) Triturate lysate several times using Pasteur Pipet.
3) Add 0.3 ml (1/10th vol) 2 M NaOAc pH 4 and mix by inverting the tube several times.
4) Add 3 ml AE-buffered phenol and vortex. Add 0.6 ml $CHCl_3$:IAA and vortex again. Incubate in ice water bath for 15 min.
5) Spin for 20 min at 10,000 x g at 4ºC (9000 rpm on JA20 rotor).
6) Using Pasteur pipette, transfer aqueous (upper) phase to clean 15 ml Corex tube without disturbing the interphase.
7) Add 3 ml isopropanol, mix and incubate at -20ºC for at least 30 min. This is a good place to leave the preps overnight.
8) Centrifuge at 10,000 x g, 4ºC, 10 min. and discard supernatant.
9) Dissolve pellet in 300 µl Denaturing solution and transfer to 1.5 ml microfuge tube. Rinse Corex tube with additional 300 µl of Denaturing solution and pool with first aliquot in microfuge tube.
10) Add 600 µl isopropanol. Vortex and allow to precipitate at -20ºC for >30 min. Collect precipitate in cold microfuge (4ºC) by centrifugation for 10 min at 10,000 x g.
11) Resuspend pellet in 1 ml 75% ethanol. Vortex. Incubate 15 min at room temperature to redissolve residual GuSCN. Centrifuge in microfuge for 5 min at 10,000 x g and discard supernatant.
12) Dry pellet in Speed-Vac for 5 min. and then redissolve RNA in 200 µl of depc-H_2O (37ºC, 15 min). Quantitate in 10 mM NaOH. A_{260}/A_{280} should be between 1.8 and 2.0.

Bead Homogenization of Tissues for Trizol or GuSCN Isolation of RNA

1) Transfer the tissue to a SureLok microfuge tube or screwcap microfuge tube ON ICE. Tube must be compatible with the Bullet Blender and should be pre-loaded with app, 200-300 µl volume of metal beads (2 mm) for the Bullet Blender. For tougher tissues like muscle use the UFO beads.
2) Add 1 ml of TRIZOL reagent or 0.5ml GuSCN denaturing solution, per 50 to 100 mg of tissue.
3) Process for 5 minutes at full with Bullet Blender in 4 ºC coldbox.
4) For GuSCN see post homogenization treatment in next section (should be step 6). For Trizol proceed with Step 5.
5) Add 200 µl of $CHCl_3$:IAA per 1 ml of TRIZOL Reagent. Close tube and mix vigorously.
6) Incubate at room temperature for 2 to 3 minutes.
7) Centrifuge at 10-12,000 x g for 15 minutes (swinging bucket is best) at 2 to 8ºC. The centrifugation separates the sample into lower red, phenolchloroform phase, an

interphase, and a colorless upper aqueous phase. RNA is in the upper, aqueous phase.

8) Transfer upper aqueous phase carefully without disturbing the interphase into a fresh tube. Measure the volume of the aqueous phase (The volume should be about 60% of the volume of TRIZOL Reagent used for homogenization).
Add an equal volume of isopropyl alcohol.
9) Incubate samples at room temperature for 10 minutes and centrifuge at 10-12,000
10) x g for 10 minutes at 2 to 4ºC. The RNA precipitate, often invisible before
11) centrifugation, forms a gel-like pellet on the side and bottom of the tube.
12) Decant off the supernatant. Add 1 ml cold 70% ethanol and shake the tube to mix.
13) Centrifuge at no more than 10-12,000 xg for 5 minutes at 2 to 8ºC.
14) Repeat washing procedure once more. Dry sample in SpeedVac
15) Dissolve pellet in TE or Te, depending on your next steps
16) Quantify by NanoVue or other spectrophotometry.
17) Record A260, A280, and calculate A260/A280 which should be >1.6.

Tissue-tearer Homogenization of Tissues for GuSCN extraction

1) Wash hand-held "tissue tearer" (Virtis or other brand).
   A) Immerse probe (motor running) in soapy water, then running tap water. Do not immerse the entire probe, just the part that will be exposed to the processed tissue.
   B) Immerse probe (motor running) in 100 mM HCl, then 100 mM NaOH, then four changes of deionized H_2O. This step is only necessary to eliminate any traces of "carry over" of genomic DNA, and is not really necessary for RNA isolations.
   C) Finish in 95% EtOH, and then run in air to dry.
   D) Be certain to clean the probe after usage and make certain it is completely dry inside the probe before storage.
2) Large pieces of frozen tissue will not homogenize or thaw rapidly. They can first be broken by smacking with a hammer (tissue in folded aluminum foil or plastic wrap). .
3) Transfer tissue pieces to 25 or 50 ml beaker that has been previously weighed. Record the weight of the beaker + tissue. Calculate the weight of tissue.
4) Add at least 5ml GuSCN solution per gram of tissue. Homogenize with cleaned tissue-tearer until all tissue is homogenized.
5) Pour homogenate into centrifuge tube.
6) Add 1/10th volume of 2 M NaOAC pH 4.2, mix well
7) Add 1 volume (same volume as GuSCN in step 4) AE buffered phenol and mix well.
8) Add 1/5th volume (1/5th the volume of GuSCN in step 4) $CHCl_3$:IAA and mix well.
9) Incubate in ice water bath for 15 min.
10) Spin for 15-20 min at 10,000 x g at 4ºC (9000 rpm for JA20 rotor).
11) Transfer aqueous (upper) phase to clean centrifuge tube avoiding the interface.
12) Extract one more time with equal volume of 4 parts AE-Phenol and 1 part $CHCl_3$:IAA. Spin 10 minutes as in Step 10
13) Transfer upper aqueous to new tube.
14) Add an equal volume of isopropanol, mix well and incubate in ice water bath for 15 minutes.
15) Centrifuge at 12,000 x g, 4ºC, 15 min. Decant and leave inverted to drain for several minutes on a paper towel.
16) Dissolve pellet in 0.5 ml GuSCN per gram of starting tissue. Make sure the pellet is fully dissolved. If not, add more GuSCN or you will lose RNA. Transfer to microfuge tube (if possible)
17) Add equal volume of isopropanol. Mix well and incubate on ice for 15 min. Collect

precipitate in cold microfuge (4ºC) by centrifugation for 10 min at 10,000 x g.

18) Decant well, add 1 ml cold 70% ethanol. Mix well to redissolve residual GuSCN. Centrifuge in microfuge for 5 min at 10,000 x g and discard supernatant.

19) Dry pellet in Speed-Vac for 5 min. and then redissolve (37 to 50ºC, 15 min with mixing) RNA in 300 to 400 µl of TE per g of starting tissue. For some tissues with high RNA levels you may need more TE to dissolve the pellet. Quantify by spectrophotometry, A_{260}/A_{280} should be between 1.8 and 2.0.

Oligo-dT Chromatography of RNA

1) Spin down RNA ppt, rinse with 70% EtOH and vacuum dry. Redissolve in TE (10 mM TrisCl 1 mM Na_2EDTA pH 7.5. Volume is dependent on the size of the column. 1 ml for 1 g column, 6 ml for 3 ml column, for BRL push columns use 1-200 µl.

2) Read A_{260} and A_{280} of 1:100 dilution in 100 mM NaOH

3) Make spectral recording from 300 to 230 nm.

4) Add 1/10th vol 5 M NaCl to sample

5) Rinse the column 3x with TE made 0.5 NaCl (1/10th vol 5 M NaCl)

6) Spin column to dry (600 x g) for push columns use pipet pump, and load with sample

7) Spin 5' at 600 x g or push with pipet pump.

8) Pass void volume back over the column

9) Wash column with 3x 1-6 ml with TE+0.5 M NaCl solution

10) Before going to elution, spin or push to be sure that column is fairly dry

11) Elute A^+ mRNA with 100 µl for push columns or 1-3 ml for spin columns of 55ºC TE.

12) Two elutions should be sufficient. For large samples the A_{260} readings may be significant.

13) Add 1/10th vol 3 M KOAc pH 5.3 (do not use NaOAc as Na^+ interferes with reverse transcriptase and in vitro translations) and EtOH ppt.

14) Redissolve in 50-500 µl TE and read A_{260} of 1:100 in 10 mM NaOH

15) Store aliquots as EtOH ppt in 500 µg aliquots using KOAc. Also, may wish to save back a 2 µg aliquot for testing in wheat germ translation (see 'Wheat Germ Translation')

16) To regenerate a column:
   a) Rinse 3x with 1-2 ml 100 mM NaOH
   b) Then rinse 2x with elution buffer and store in TE + 0.1 M NaN_3

Two-Step RT-qPCR

Some Reverse transcriptases are sensitive to sodium and favor potassium. To be certain, the RNA used for cDNA synthesis should be free of sodium ions. Therefore, be sure that the most recent ethanol precipitation was with KOAc as the salt (rather than the standard NaOAc).

Note on enzymes: This protocol works well with Promega MMLV Reverse Transcriptase, Life Technologies SuperScript II or III.

1) For each RNA sample you need to run a 20 µl first strand reaction that will be sufficient for at least 3 different genes (2 experimental and reference). You can make a cocktail of everything but the RNA and add to each RNA sample.
   4 µl 5x SS Buffer (RT buffer from supplier)
   0.2 µl 20 mM dNTPs (all four)
   0.4 µl 30 µM (pM/µl) $CT_{23}V$
   1 µl 100 mM DTT
   H_2O to make final volume 20 µl (including the RNA below)
   mix

0.2 µl (1-2 U) RNAsin (Promega) or other RNAse inhibitor
0.5 µl (100 U) Reverse Transcriptase
Mix well then add to
X µl containing 10-20 µg total RNA in Te in a 200 µl PCR tube.
Mix and incubate 20-30 min at 42 ºC
Move to ice

2) For the qPCR use 20 µl reactions and run all genes in triplicate. Make up a cocktail containing everything except the cDNA and primers, then aliquot enough for triplicate reactions for each gene (each experimental and the reference). The PCR mixture is mixed with the cDNA in a 1.5 ml microfuge tube, then pipet aliquots sufficient for triple reactions into wells in every third column in a PCR plate, then add the primers for each gene. Then mix the well by trituration with a 50 µl multichannel pipettor (set it to 50 and triturate several times from bottom to top, set to 19.5 and pipet to the other two wells. The cocktail components per reaction are:
H_2O to make final volume 20 µl accepting volumes for RNA, primers and enzymes
2 µl 10x Taq Polymerase buffer
0.2 µl 20 mM dNTPs (all four)
0.2 µl 25 mM $MgCl_2$
1 µl 20x EvaGreen dye
Mix well
4U Taq Polymerase
Mix Well
3) Aliquot to tubes for adding cDNA. Add enough cocktail for one to two extra reactions.
4) Add about 6 µl cDNA for each gene. Mix well. Extra cDNA can be frozen at -20 for use later.
5) Pipet 54 µl for each cDNA mixture into every third well in the qPCR plate, then add 6 µl of 10 mM Forward and Reverse primers. Mix well by trituration with a 50 µl multichannel pipettor so you are mixing multiple mixtures. Then set the multichannel to 19.5 µl and using the same tips pipet to the other two wells for the triplicate reactions.
6) Seal the plate and run the amplification protocol with reads only in the FAM/SYBR channel. Representative protocol is:

ºC	seconds	Cycling
90	30	Initial Denaturation
90	15	10x
55	15	
72	60	
90	15	30x
55	15	
72	60 then read	
72	180	Final Soak
90	15	High Resolution Melt curve
65	180	
75 to 90 @0.1ºC	5 sec then read	

First Strand Synthesis for qPCR

Some Reverse transcriptases are sensitive to sodium and favor potassium. To be certain, the RNA used for cDNA synthesis should be free of sodium ions. Therefore, be sure that the most recent ethanol precipitation was with KOAc as the salt (rather than the standard NaOAc).

Note on enzymes: This protocol works well with SuperScript III from Life Technologies. Other RT sources may work but may require slight adjustments. The method relies on tracer levels of fluorescent labeled nucleotide. We have used Cy3-dCTP but you may adapt for other labels and dNTPs.

7) For a 100 µl reaction add:
   - 20 µl 5x SS Buffer (RT buffer from supplier)
   - 1 µl 20 mM dNTPs (all four)
   - 1 µl 1 mM Cy3-dCTP
   - 2 µl 30 µM (pM/µl) $CT_{23}V$
   - 5 µl 100 mM DTT
   - X µl containing 100 µg total RNA in Te
   - H_2O to make final volume 100 µl
   - 0.5 µl (10 U) RNAsin (Promega) or other RNAse inhibitor

   incubate 5' @ 37ºC or 45ºC,

add: 3 µl (200 U/µl) SuperScript III (Life Technologies)

2) incubate @ 45ºC for one hour,
3) Stop the reaction with 100 µl TE+5 mM EDTA+0.4% SDS
4) Extract with Phenol-$CHCl_3$-IAA, then with $CHCl_3$-IAA, and back extract the organic layers with 100 µl TE
5) Ethanol precipitate the aqueous pool
6) Spin, 70% rinse, and dry
7) Redissolve in 100 µl Te
8) Transfer to a 500 µl microfuge tube and read absolute fluorescence using the blue module in the GloMaxJr. The tube goes into the PCR Tube Adapter in the blue module. Use a blank tube (100 µl Te in 500 µl tube) to read the background then read your cDNA sample in its tube. You must have 100 µl volume to get a reading. Read as Raw Fluoresence.

First Strand cDNA ng from Absolute Fluoresence.

$$ng\ 1st\ strand = \frac{reading - blank\ [background]}{14000\ [input]} * 80\ nmoles\ dNTP * 330\ ng/nmole$$

9) Expect 500-1000 ng total from 100 µg total RNA (100 µg contains about 5 µg of poly A+ RNA so this would be 10-20% conversion to first strand cDNA)
10) Use 1-2 µl per PCR for qPCR.

NIB-RIB RNA Isolation Method

Solutions needed: NIB, RIB, Phenol, $CHCl_3$:IAA

This method uses a nonionic detergent and hypo-osmotic conditions to lyse the cytoplasmic membrane. Intact nuclei are removed and then RNA is isolated by phenol extraction in the presence of urea, SDS and EDTA. This protocol works well with tissue culture cells, suspension cells, and some soft tissues. It generally does not work with insect larvae, or cells high in degradative enzymes.

Isolation of Cells

Cells can be harvested by trypsinization or CHO cells can be washed off by the following method:

a) add 20 ml 40 mM Tris pH 7.5, 140 mM NaCl, 0.5 mM $CaCl_2$ 1 mM EDTA to each roller bottles place on rollers at 37ºC for 10 min.

b) Roll bottle to dislodge cells and pool cells. Rinse all bottles with another 50 ml same solution.

Cell Lysis and Isolation of cytoplasmic RNA

Regardless of the method, cells should be rinsed once with cold PBS (resuspended in PBS then pelleted in a tared bottle or tube 3000 rpm 5' in the cold). Speed is critical. Move quickly from trypsinized cell population to the phenol extraction to minimize mRNA degradation

1) Add 25 ml COLD NIB per gram of cells. (This volume may be reduced, probably as low as 10 ml/g cells, no systematic optimization has been done).
2) Resuspend by gentle trituration and mix on ice to create a suspension. No more than 1 or 2 minutes.
4) Pellet nuclei 5' 5000 rpm 4ºC. During this centrifugation prepare a tube or bottle which containing one volume (equivalent to the amount of NIB used) of the following: redistilled phenol, $CHCl_3$:IAA, and RIB. DO NOT MIX, the RIB should be sitting on top of the phenol:$CHCl_3$ mixture.
5) Decant supernatant into RIB, phenol:$CHCl_3$, shake well to mix for 5' at room temp
6) Distribute to centrifuge bottles and spin 10' 8000 rpm at room temp (may be spun in cold but phenol extraction may not be as effective).
7) Collect upper, aqueous phase. If a large interface forms you should backextract the organic phase with TE and pool with the aqueous phase.
8) Extract aqueous phase with an equal volume $CHCl_3$:IAA spin 10' 8000 rpm at room temp
9) Collect aqueous phase and add 1/10th vol 3 M NaOAc pH 5.3, 2.5 vol 95% EtOH and allow to ppt >1 hour at -20ºC

Isolation of Nuclear RNA

Note: Be Sure all solutions are made up in advance and those that need to be cold are at or below 4ºC. It is essential that you move quickly to limit degradation of the nuclear and cytoplasmic RNAs.

Solutions needed:

PBS + Ca, Mg, Glucose- ice cold
TMN- 10 mM TrisCl pH 7.6, 3 mM $MgCl_2$, 100 mM NaCl
DNAseI (2 mg/ml)- highly purified DNAseI (Worthington #6330) in TMN should be made fresh can be frozen a few times but loses activity quickly
NIB, RIB, Phenol, $CHCl_3$:IAA, 10% SDS, 250 mM EDTA, autodigested Pronase (5 mg/ml)

Cell Lysis

1) Cells can be grown in any manner but should be actively dividing (sub-confluent). Can be collected by trypsinization but is better to collect by scrapping because it is faster. Collected cells should be pooled in media and cooled on ice as quickly as possible.
2) Pellet cells 5', 4ºC, 2000 x g

Note: For the following steps when the term RESUSPENDED is used this means to use gentle trituration with a pipet to suspend the cells or nuclei. This is to render the cellular components immediately accessible to the treatment to follow. Failure to disperse clumps will adversely affect yields or result in contamination of nuclear preps with cytoplasmic RNA or vice-versa.

3) Resuspend the cells in cold PBS+Ca,Mg,Glu and transfer to tared corex tube.
4) Pellet cells 5', 4ºC, 2000 x g
5) Weigh tube+cell pellet and determine cell weight. Resuspend cell pellet in ice-cold NIB 25 ml per gram of cells.

6) Mix the sample by low speed vortexing, pellet the nuclei and membranes by centrifugation at 5000 x g 5' 4ºC.
7) Cytoplasmic RNA may be isolated from the supernatant (see ABOVE)

Purification of Nuclei and Lysis
Note: Steps 8 and 9 are only necessary if you need to reduce contamination with cytoplasmic RNAs.
8) The nuclear pellet is resuspended in 8 ml cold NIB (must be resuspended well). In a 15 ml corex tube make a 4 ml sucrose cushion by mixing 666 µl 60% Sucrose and 3.3 ml cold NIB. Layer the nuclear suspension on top of the Sucrose cushion and centrifuge at 2500 rpm (600 x g), 10' 4ºC in the JS13 (swinging bucket rotor).
9) Pipet off and discard the cloudy supernatant above the sucrose cushion and rinse the tube by gently filling the tube with cold NIB, then decant off all solution.
10) Resuspend the nuclear pellet in cold NIB. The volume used should be the same as the volume used to originally lyse the cells.
11) Decant into 1 volume RIB:0.1 vol 10%SDS:1 vol Phenol:1 vol $CHCl_3$-IAA. Mix well for about 5' at room temp.
12) Centrifuge to separate the phases. It is best to use a swinging bucket rotor and spin at 8000 rpm for 5' at room temp.
13) Extract once more with 50:50 Phenol:$CHCl_3$-IAA and then once with $CHCl_3$-IAA. The nuclear lysis releases high-molecular weight DNA that make the solution viscous and hard to pipet off the aqueous phase, therefore you may need to backextract the organic phases with 10-20 ml 1x RIB to maximize recovery and don't be alarmed by the inability to not carry over some (or most) of the interface this will not interfere with the preparations quality.
14) After the extractions are complete precipitate by addition of 1/10th volume of 3 M NaOAc pH 5.4 and 3 volumes of EtOH. Store at -20ºC >1 hour.

DNAse Treatment
15) Pellet nucleic acids 10,000 x g 20' 4ºC in a swinging bucket rotor. Rinse the pellet with cold 70% EtOH and invert the tube to drain. The pellet may be briefly dried in·vacuo but only to remove visible moisture, don't over do it. If you dry the pellet to much you will never get it redissolved.
16) Resuspend the pellet in 10 ml TMN + RNAsin (or another RNAse inhibitor) per gram of starting cells. Incubate at 37º for 10-15' with gentle swirling; sufficient to disperse the pellet into small chunks (complete dissolving isn't necessary). Add DNAseI solution to 40 mg/ml and incubate 60' at 37º with occasional swirling.
17) Add SDS to 0.5%, EDTA to 5 mM, and autodigested pronase to 50 µg/ml and incubate for 30' at 37º.
18) Extract with Phenol then 50:50 phenol:$CHCl_3$-IAA then $CHCl_3$-IAA. Add 0.1 vol 3 M NaOAc and 3 vol EtOH to precipitate, -20ºC >1 hour.
19) To remove all DNA it may be necessary to repeat steps 16-18.
20) The EtOH pellet is redissolved in 200-400 µl TE (10 mM Tris pH 7.5 1 mM EDTA) and run over a 4 ml centrifuge G50 column (see **DNA Labeling**). Pool the void volume and the first rinse. Add 0.1 vol 3 M NaOAc and 3 vol EtOH to precipitate (-20º >30').
21) After centrifugation redissolve in TE and determine concentration by reading Absorbance at 260 nm of a dilution in 10 mM NaOH.
Concentration in µg/ml=A_{260} X dilution factor X 40.
YIELD: 1 mg per gram of starting cells

YEAST PROCEDURES

Solutions

Zymolyase: Purchased from ICN (cat #190123: Yeast Lytic Enzyme 100k U/mg, or cat #152270: yeast lytic enzyme 70k+ U/mg), made up to 20 mg/ml in 10 mM NaPhosphate pH 7-7.5, store at 4°C up to 1 month.

Glusulase or β-glucuronidase: (100k U/ml) purchased from Sigma G-7770 (sterile solution) stored at 4°C

Phosphate Citrate buffer: 100 mM Potassium phosphate, 50 mM Citrate, pH 5.3

NDS: 500 mM Na_2EDTA, 10 mM TrisCl pH 7.5, 1% N-Lauroyl sarcosine pH 9.5. Just before use, add proteinase K to 2 mg/ml. (Note make up 5% N-lauroyl sarcosine in 0.2 N NaOH for prep of NDS).

NDS: (alternative that has worked for ddr) 100 mM Na_2EDTA, 10 mM TrisCl pH 7.5, 1% SDS. Just before use add proteinase K to 2 mg/ml.

PBS: 10 mM potassium phosphate pH 7.2 150 mM NaCl

Preparation of unbudded single cells

This procedure is useful for preparation of uniform populations of cells for UV irradiation or germ tube induction. reference: Busbee and Sarachek (1969) Arch. Mikrobiol. 64:289-314.

1) Grow overnight culture of cells. Dilute 20 fold into 37°C YPD. Grow at 37°C for 6 hours. Pellet cells at 1-2000 x g 5' at room temp and resuspend in 1/5th volume of sterile H_2O.

2) Pipet 400 µl of cells onto a thick fresh YPD plate and spread uniformly over ALL of the surface. The plate should be uniformly covered with a thin layer of cells and not too much water.

3) Incubate <u>right side up</u> at 37°C overnight on a <u>level</u> shelf (check level). After growth NO holes or edges should be present in the lawn of cells.

4) Add 3-5 ml sterile H_2O to the plate. Using a sterile spreader resuspend the cells and pipet off the resuspended cells. Dilute small amount about 100 fold in water and check under scope. Yield per plate should be about $4x10^{10}$ cells.

Preparation of Protoplasts

reference Sarachek, Rhoads, and Schwarzhoff (1981) Archives of Microbiology 129:1-8

NOTE: If you are preparing protoplasts for isolation of DNA then substitute 1 M Sorbitol for the 0.6 M KCl osmoticum. If you use KCl then the SDS will precipitate when it is added.

1. For yeast that convert to protoplasts easily (*S. cerevisiae*): Grow yeast to stationary phase in YPD at 30°C with good aeration.

b. For yeast that do NOT converted to protoplasts as easily as *S. cerevisiae* use cells in logarithmic growth: Grow yeast to stationary phase O/N in YPD at 37°C with good aeration. Dilute app. 1:20 in pre-warmed 10 ml YPD and grow 4 hrs at 37°C to generate cells which are in logarithmic growth.

2. Wash cells 2x with sterile water by centrifugation (1000 x g 5')

3.Determine the cell density by optical means. For most yeast cells a barely visible turbidity in a 100 ml milk dilution bottle is roughly 10^6 cells/ml (10^8 cells per 100 ml). Based on the volume of cells required for this 10^8 cells calculate the number of cells in the entire sample.

4. Pellet the cells and resuspend in the appropriate buffer at 2 x 10^8 cells/ml. For glusulase

use phosphate-citrate buffer. Add 1/100th volume of 2-mercaptoethanol (makes 1% v/v) and incubate for 30 minutes at 30ºC
5. Pellet and resuspend in same volume of the appropriate buffer (no 2-mercaptoethanol) made 0.6 M KCl (use 1 M sorbitol if you are making protoplasts for HMW DNA preparation).
6.Per ml of cell suspension add either: 0.1 ml glusulase or 7 µl Zymolyase solution.
7. Incubate 1 hour at 30ºC
8. Pellet 600-1000 x g 5'. Wash once by resuspension in buffer + 0.6 M KCl and re pellet.
9.Resuspend at 2 x 10^8 cells/ml in buffer + 0.6 M KCl (if fusion is planned then include 10 mM $CaCl_2$ in this solution)
Protoplasts prepared in this manner are ready to fuse or isolate of high molecular weight genomic DNA. For DNA isolation see the chapter on **DNA: Genomic** and begin with addition of SDS and EDTA.

Isolation of Genomic DNA from Yeast Cells

Smash & Grab-

ref Hoffman and Winston (1987) Gene 57:267-272
1. Grow 10 ml yeast culture to saturation and collect cells by centrifugation (1000 x g 5').
   Note: for Candida cells, lysis is better if the cells are in log phase. Therefore, dilute an overnight 10 ml culture to 50 ml in YPD, grow for 3-5 hours and then harvest cells
2. Resuspend in TE (0.5 to 1 ml) and transfer to microfuge tube.
3. Pellet the cells by brief (a few seconds) spin in microfuge.
4. Decant off all but the last drop of liquid and vortex the tube to resuspend the cell pellet in this last drop.
5. Add 0.2 ml TE+ 2% Triton X-100, 1% SDS, 100 mM NaCl and mix. Add 0.2 ml 50:50 Phenol:$CHCl_3$ and app. 0.3 g Acid-washed glass beads (0.3-0.5 mm diameter). [glass beads can be obtained from Sigma. Wash with 1 M HCl with stirring for >30' at room temp, rinse extensively with distilled water, drain, autoclave and then dry in baking oven. Store at room temp in sealed container. Use forceps and sterile microfuge tube to measure out beads for use.]
6. Vortex for 3-4 min.
7. Add 0.2 ml TE, mix and centrifuge for 5 minutes.
8. Transfer aqueous to new tube and add 1 ml cold EtOH and mix.
9. Centrifuge 2-5 min., decant, add 0.5 ml 70% EtOH, and decant. Resuspend pellet in 0.4 ml TE+75 µg/ml RNAseA and incubate 5-10 min. at 37ºC. Add 10 µl 10 M NaOAc and then 1 ml cold EtOH.
10. Centrifuge for 2-5 min., decant, rinse pellet with cold 70% and redissolve in 50 µl TE.

Yield: 200 to 400 ng/µl.
Note: for rescue of yeast plasmids by transformation into E. coli, use 1-2 µl of the aqueous phase after step 7. For transformation into S. cerevisiae use 15 µl of the aqueous extract.

Isolation of Yeast Chromosome Markers for PFGE-

Modified from Schwartz and Cantor Cell 37:67 [1984] in CSH Yeast Course 1990. Sopme yeast which are not converted to protoplasts as easily as *S. cerevisiae* cells must be in logarithmic growth and treated with 2-mercaptoethanol to loosen their cell walls. In our experience the yields may be better for *S. cerevisiae* if they are also in logarithmic growth (the original procedure called for an O/N stationary culture).

b. Grow yeast to stationary phase O/N in YPD at 37ºC with good aeration. Dilute app.

1:20 in pre-warmed 10 ml YPD and grow 4-6 hrs at 37ºC to generate cells which are in logarithmic growth.

c. Pellet the culture (1k x g 5'), resuspend in 3 ml ET and pellet again.

d. This step is not essential for *S. cerevisiae* but is required for *C. albicans*. Resuspend in 3 ml ET + 1% 2-mercaptoethanol incubate for 10' at 30ºC.

e. Aliquot at 1 ml per tube in flat bottom 2.2 ml microfuge tubes and pellet (1k x g 3 minutes).

10. Resuspend cells in 150 µl ET and add 1 µl 20 mg/ml Zymolyase 100k (cocktail for multiple samples).

11. Place in 42ºC hot-block, add 250 µl 1.5% low melting agarose made up in 125 mM Na_2EDTA pH 7.5. (P. Hieter says any Low melt agarose will work)

12. Pipet up and down quickly and GENTLY to mix and immediately place into ice bath >10'.

NOTE: From this point on treat gently, no vortex, no centrifugation, no shaking, no rough handling of any type.

5. After agarose has solidified (a few minutes) add 400 µl 500 mM EDTA 10 mM TrisCl pH 7.5.

6. Incubate >8 hrs (O/N) at 30ºC.

7. Very gently loosen the plug with a micro-spatula and transfer to a 12x75 mm snap cap tube (Falcon) containing 400 µl NDS.

8.Incubate O/N at 50ºC.

9.Wash the plug 4x by incubation for 1 hr each in 2 ml of ET.

10.Store at 4ºC.

11.To use, slice off about 20 µl worth of plug, place into microfuge tube and heat to 50ºC to melt (may incubate while several samples are prepared)- remember no rough handling or centrifugation. Load into dry well of gel with 50 µl glass pipet, allow to solidify and then run gel.

Isolation of HMW DNA from S. pombe

SP1 50 mM CitratePhosphate buffer pH 5.6 (7.1 g/L Na_2HPO_4, 11.5 g/L Citric Acid)
40 mM EDTA

SP2 Same as SP1 but also contains 1.2 M Sorbitol

SP3

Ref: as modified by Charlie Hoffman from Methods in Enzymology (1991) 194:795-

1. Grow 10 ml overnight culture in YES at 37ºC.
2. Dilute to 100 ml in YES at 37ºC and continue shaking incubation for 2-4 hours to attain 1 to 2 x 10^7 cells/ml (mid log phase).
3. Pellet cells. Wash twice by resuspension and pelleting out of 10 ml sterile water.
4. Resuspend final pellet in 10 ml SP1. Add 20 µl 2-mercaptoethanol. Incubate 10 minutes at room temperature.
5. Pellet 5 minutes at setting 800 to 1000 x g in tabletop. Resuspend in 10 ml SP2. Add 10 mg "Lysing enzymes" from Sigma. Incubate at 37ºC.
6. After 20-30 minutes, examine cells under microscope. About 50% should have "rounded up". You may want to mix 10 µl cells with 1 µl 10% SDS and examine microscopically (look for cells to become black). At this point you may see such complete lysis of the cells that you just add SDS to the prep and scale up the potassium acetate step.
7. Add 30 ml SP3 and pellet 5 minutes at setting 8. Resuspend in 10 ml 5X TE (50 mM Tris 5 mM EDTA). Add 1 ml 10% SDS and mix well. Incubate 5 minutes at 65ºC. Check lysis under microscope.
8. Add 4 ml 3 M KOAc pH 5, mix by inversion, and incubate on ice for 30 minutes. Pellet

15 minutes at 5 to 8000 x g.

9. Pour supernatant into a new tube. Add 25 ml ethanol. Invert to mix.
10. Pellet 5 minutes at 10,000 x g. Pour off supernatant and leave tube inverted on a paper towel to drain. Dry briefly in centrifugal evaporator.
11. Resuspend gently in 3 ml TE at 37ºC. This may take a while. Swirl gently occasionally. Add 6 µl 10 mg/ml RNaseA. Incubate 1 hour at 37ºC.
12. Add 100 µl 10 M NH_4OAc and 3 ml 100% isopropanol. Mix gently by slow inversions until the DNA is one clump.
13. Hook out the clump with a 200 µl pipettor tip and squeeze dry against the wall of the tube before transferring to an Epp tube containing 200 µl 70% ethanol to rinse the clump. Pipet out the 70% ethanol. Dry under vacuum and redissolve in 200 µl TE. Quantitate and test 100-200 ng on 0.7% gel for quality.

Protoplast Fusion and Regeneration

reference Sarachek, Rhoads and Schwarzhoff (1981) Arch. Microbiol. 129:1-8

1. Mix 1 x 10^8 freshly prepared protoplasts of the two parents to fuse. Protoplasts should be in 0.6 M KCl + buffer.
2. Pellet by centrifugation 5' at 1000 x g at room temp.
3. Gently resuspend in 5 ml 40% PEG (MW 3400) + 10% $CaCl_2$ (sterile $CaCl_2$ added after autoclaving). Concentrate the fusion protoplasts by centrifugation for 1' at 200 x g. Incubate 20' at 30ºC.
4. Centrifuge 5' at 1000 x g, decant off supernate and resuspend the pellet in 0.6 M KCl + 10 mM $CaCl_2$
5. Plate on osmotically stabilized medium for regeneration of protoplasts. For *C. albicans* we have found that addition of 0.6 M KCl to minimal medium is sufficient. Other routinely use 1 M Sorbitol for *S. cerevisiae*.

Isolation of RNA by phenol freeze-thaw

modified from: Schmitt, Brown and Trumpower (1990) NAR 18(10):3091-2

1. Generate log phase yeast cells (about 10 ml of culture)
2. Collect cells by centrifugation 5' 2000 x g.
3. Resuspend in 400 µl of AE buffer (50 mM NaOAc pH 5.3, 10 mM EDTA). Phillip Stafford has found that for hard to crack cells (mycelial Candida) you should not exceed 50 µl of packed cells per 400 µl of AE in step 3. If the packed volume is greater then scale up all the subsequent volumes
4. Transfer to microfuge tube and add 40 µl 10% SDS and vortex
5. Add 440 µl phenol (pre-equilibrated with AE buffer) and vortex
6. Incubate at 65ºC for 5-10 minutes then vortex
7. Place at -80ºC for 4 hours or more to freeze solid. We don't use liquid N_2 or an ethanol bath but allow the solution to freeze slower.
8. Centrifuge at 10000 rpm for 10'
9. Collect upper aqueous phase and extract aqueous with equal volume of $CHCl_3$:IAA. Spin and collect aqueous.
10. If the next step is to make first strand cDNA then add 3 M KOAc to bring to 0.3 M. If RNA is to be purified on oligo-dT or used for northerns then use 3 M NaOAc.
11. Add 2.5 volumes of ethanol, mix and incubate at -20ºC for 1-4 hours.

12. Collect RNA by centrifugation at 10,000 rpm for 15-20' at 4ºC. Rinse pellet with cold 70% ethanol. Dry and redissolve in 20-50 µl TE. Quantitate by spectrophotometry.

yields range from 60 to 300 µg RNA per 10 ml culture. Procedure has been scaled up to 40 ml (with proportional increases in treatment volumes) for *C. albicans*.

DAPI Staining of yeast nuclei

recommended by Michael Lichten

1. Pellet cells, resuspend in 50 ul water. Add 1 ml ETOH. Alternatively, fix cells in 50% ETOH ahead of time and chill at -20 C.
2. Add DAPI to 0.1-0.2 ug/ml. Let sit 5 min.
3. Spin out, rinse 2x with water. Resuspend in a small volume (usually 20-50 ul).

Electroporation of S. cerevisiae

from Pierre Falson: we are currently use electroporation of yeast without any problem. here follow these conditions:

1. 3 weeks-less-old colony, growth in 100 ml Yeast Extract 1%, bactopeptone 1% and glucose 1%. Go to 1.3-1.5OD600nm/ml (10^8cell/ml).
2. Centrifuge in two falcon tubes two times at 5000 rpm and wash with 100 ml of sterile, cold water, centrifuge, wash with 50 ml water, centrifuge, suspend in 4 ml 1 M sorbitol (sterile, cold), centrifuge, suspend in 0.1 ml of 1M sorbitol (s, c). let the yeasts at 4ºC. Cells can be used immediadely or one or two days after.
3. Yeasts are aliquoted in 40 µl fraction, mixed with 0.1µgDNA/0.1-5µl buffer (TE). the mix is left on ice for 4 min.
4. The apparatus (Biorad) is set to 1.5kV, 25 µF (gene pulser) and 200 Ohms (pulse controler). pulse in a 2 mm chamber (time constant is 4.5 - 5 msec).
5. Add 1 ml of 1 M cold sterile sorbitol, pour on plate. colonies will appear in 48-72 h.

Alternative from Michael Lichten: we do the same thing, with the following modifications:

1. Use a 5ml overnight grown in YPD. Make it from a fresh colony. Wash the cells 3x in 5 ml water, 2x in 1M sorbitol, and resuspend the pellet of cells in whatever 1M sorbitol remains after pouring off the second wash.

2.We have found that the addition of carrier DNA (12.5 µg/transformation) enhances the yield of integrative transformants about 10x. See Schiestl and Gietz (Curr Genet 1989 Dec;16(5-6):339-46) for how to make carrier.

3. We find that plating at lower cell concentrations often helps recover transformants. If you put too many cells on a single plate, they'll eat up all the goodies.

Electrotransformation of C. albicans for REMI

Based on: Stable transformation and regulated expression of an inducible reporter construct in Candida albicans using restriction enzyme-mediated integration. Mol. Gen. Genet. vol 251, pp. 75-80.) Carol A. Kumamoto, Dept. of Molecular Biology and Microbiology, Tufts University School of Medicine, 136 Harrison Ave., Boston, MA 02111, PH: (617)-636-0404, FAX: (617)-636-0337, EMAIL: ckumamot@opal.tufts.edu

Preparation of cells for transformation by electroporation:

1. Inoculate 300 ml YPD with 150 ul of SGY-243 stationary culture in late afternoon. Grow at 30oC with vigorous shaking.
2. Next morning, take a 0.5 ml aliquot of culture and dilute 1/10 with water. Read the Klett with a Klett meter equipped with green filter, blanking against YPD diluted with water. Harvest the culture when Klett reading is 52. (Note: cell density is very important for transformation efficiency. However, somewhat lower cell densities and densities up to Klett 70 work OK.) Spin cells at 4oC for 5 min. at 5000g. Discard supernatant.
3. Resuspend cells in 300 ml ice cold water. Spin and discard supernatant.
4. Resuspend cells in 150 ml ice cold water. Spin, etc.
5. Resuspend cells in 6 ml ice cold 10% glycerol. Spin, etc.
6. Resuspend cells in 3 ml ice cold 10% glycerol. Spin, etc.
7. Resuspend cells in 0.75 ml ice cold 10% glycerol.
8. Aliquot 43-50 ul cells into eppendorf tubes and freeze at -80oC.
   (Note: Some lab members use 10% glycerol for the resuspensions in steps 3 and 4.)

From: cve@ifm.mh-hannover.de (Christian Velten)

1. You can increase the transformation rate by putting the cells after first collection for 45 min into LiAc/TE (20 ml/100 ml culture) and adding DTT (500 microl 1M DTT/20 ml) for another 15 min.
2. You can also freeze the final Sorbitol-suspension. Max. decrease of transformation rate about 10%.

Transformation by Restriction Enzyme-Mediated Integration

9. Digest 90 µg plasmid DNA with BamHI enzyme. Phenol extract and ethanol precipitate the DNA. Store the digest as an ethanol precipitate until the day of transformation. Then spin down and resuspend in 5 µl TE. (Note: Some lab members omit the phenol extraction and simply ethanol precipitate. We find that the efficiency of transformation goes down if DNA was previously resuspended and stored frozen.)
10. Use electrocompetent cells that have been frozen at -80ºC for at least 24 hrs. and allow them to warm to ice temperature.
11. To an aliquot of cells (43-50 µl), add 1 µl BamHI-digested DNA (i.e. 18 µg) and 100 units BamHI (e.g. 5 µl). Electroporate as soon as possible after additions.
12. Place the cell/DNA/enzyme mixture into a chilled 0.2 cm electroporation cuvette. Electroporate cells at 2.0 kV, 200 ohms, 25 µFd. (note: some lab members use 1.45 kV).
13. Add 0.6 ml of YPD to the cuvette and resuspend cells. Plate on 2 CM-uri plates. Incubate at 30ºC for 3-4 days. (note: although most protocols suggest the use of osmotic stabilization, we found that sorbitol causes significant loss of plating efficiency and the cells do better if osmotic stabilization is omitted.)
14. For integrative transformation by homologous recombination, we use the same methods but omit enzyme during the transformation and use somewhat less DNA.

This electroporation protocol was developed using strain SGY-243 and we are beginning to use it successfully with other strains such as CAI-4.

SPERM ASSAYS

Fluorimetric analysis of sperm for intact sperm

Solutions:

PBS- 130 mM NaCl, 20 mM KCl, 20 mM NaPB pH7.2

Dilute Hoechst 33258 from stock to 0.1 µg/ml in PBS.

Calibrate TKO fluorimeter

1. Place plastic 1cm square cuvette in fluorimeter (λexcitation 365nm; λemission 460nm)
2. Add 2 ml Hoechst-PBS. Set zero for machine.
3. Turn scale knob to full (clockwise) and then 2 full turns counter clockwise.

Measuring Intact Sperm

4. Using positive displacement pipettor, add 2 µl sperm, cover with parafilm and invert several times.
5. Allow to stand at room temp for 2-4 min.
6. Record initial reading.
7. Add 20 µl digitonin (10 mg/ml in EtOH), mix by inversion.
8. Allow to stand at room temp for 2-4 min.
9. Record Final reading.
10. %Intact= 100*(Final-Initial)/Final

Live Sperm concentration (x10^9/ml): ((0.00241*Final)-1.5)*((Final-Initial)/Final)

Semi-micro Spectrophotometric Analysis of Sperm Mobility

Solutions:

Sperm Mobility Buffer. 128 mM NaCl 50 mM TES, pH 7.4, and 2 mM $CaCl_2$. For 1000 ml: 11.46 g TES, 7.4803 g NaCl, and 0.2940 g $CaCl_2$ 900 ml diH_2O, pH 7.4 with 5 M NaOH, and bring volume to 1000 ml. Filter sterilize through 0.2 or 0.45 µM. Store at 4 ºC

5 mM TES-KCl buffer. For 100 ml: 0.1150 g TES, 0.0220 g KCl, pH 7.4 with 5 M NaOH. Filter sterilize through 0.2 or 0.45 µM. Store at 4 ºC

30% (w/v) Accudenz. For 100 ml: add 30 g Accudenz to 60 ml 5 mM TES-KCl buffer with vigorous stirring (volume should come to 87 ml). Bring to 100 ml with app. 13 ml 5 mM TES-KCl buffer. Make from sterile solutions in a sterile bottle. Store at 4 ºC

6% (w/v) Accudenz. 372 ml Sperm Mobility Buffer, 28 ml diH_2O, 100 ml 30% Accudenz. To maintain proper osmolality for the Accudenz solution the sperm mobility buffer has to be diluted to about 93% (290 to 300 mmol/kg). The final 6% Accudenz should be pH 7.4, 300 to 320 mmol/kg, and 1.025 g/ml. Make in sterile bottles from sterile solutions and store at 4 ºC. .

1. Set spectrophotometer to 560 nm.
2. Semi micro cuvette containing 610 microliters of prewarmed 6% Accudenz, (use to set baseline). It is very important to make sure the reagents are prewarmed, that the water bath stays between 40 and 41 ºC.
3. Overlay with 60 microliters of a sperm suspension containing 0.5 billion per ml. Make sure you overlay so that you have a nice clean interface,

4. Incubate the cuvette for 5 min in a water bath at 41 ºC. Use a cuvette holder and have the water level with the upper deck of the cuvet holder. You don't want them floating, but you want the whole suspension surrounded by warm water.
5. After 5 min, gently wipe off the water, place back in the spec, wait 1 min, then read the absorbance.

Histological analysis of testis tissue

Bouin's Fixative- A buffered solution of formaldehyde, acetic acid and picric acid. Wear gloves and make in the hood; aldehydes are carcinogenic. After use, store in waste container in hood. Do not pour waste fixatives down the drain. 75 ml Picric acid (saturated aqueous), 25 ml 40% Formalin, 5 ml Glacial Acetic Acid Working Notes: This mixture keeps well at 4ºC, but some people persist in making the solution up immediately before use. Alternative formulation: 85 ml Picric acid (saturated aqueous), 10 ml 40% Formalin, 5 ml Glacial Acetic Acid. Reference: Bouin (1897) Arch. d'Anat. Micr., 1: 225.

1. Euthanize chicken by CO_2 asphyxiation
2. Mist feathers with spray bottle of dilute soapy water to minimize stickiness of down and feather
3. Make incision through skin at base of sternum proceeding caudad towards each side at base of rib cage. Pull skin up over the head.
4. Flex the legs down at hip to dislocate hips to lay legs flat
5. Make incision under tip of sternum and extending up either side of rib cage. Grasp legs and push rib cage up above head
6. Use fingers to enter body cavity from chickens right pushing liver out of way, until right testis is visible on back wall of abdominal cavity. Tear away overlying membranes, gently slide fingers under testis and use curved scissors to cut away connective tissue under fingers between fingers and body wall.
7. Use sharp curved scissors to cut epididymis from surface of testis. Place epididymis in Bouin's Fixative
8. Repeat testis removal for left testis. Remove and discard epididymis.
9. Record weights for each individual testis. Use a single edge razor blade to slice a "wedge" of testis tissue from the mid-section of one testis. The wedge should be no thicker than 2mm and no more than 1 cm on a side. Add to Bouins Fixative from step 7.
10. Be sure that all tissue is immersed in Bouin's and incubate overnight at 4ºC.
11. Decant Bouin's into collecting jar for hazardous waste disposal. Replace with 10% formalin. Incubate additional 24 hours at 4ºC.
12. With sharp scalpel slice epididymis in cross section into multiple slices of app. 2 mm.
13. Cut app. 5 mm square piece of testis section.
14. Mark ID number in pencil on Mounting cassette. Place epididymis and testis sections in cassette, close and cover in 70% Ethanol. Take cassettes in 70% ethanol to David West for HE staining, embedding and sectioning.

Beltsville Poultry Semen Extender

BPSE pH 7.5	mM	1 L	500 ml
Potassium Phosphate Dibasic*	53	9.29g	4.65
Sodium Glutamate		8.67g	4.34
Glucose	27.7	5.00g	2.5
Sodium Acetate · 3 H2O	19	2.59g	1.295
HEPES		2.03g	1.015
Potassium Citrate		0.64g	0.32
Potassium phosphate monobasic	4.77	0.65g	0.325
Magnesium Chloride · 6 H2O	1.67	0.34g	0.17
Note: Osmotic pressure = 330 (mOsn/kg · H2O) Mix all chemicals → pH to 7.5 (May not filter sterilize well → aliquot into 15 mL sterile tubes)			

No index entries found.