

MOLECULAR BIOLOGY LAB MANUAL

The Beginning

Compiled by
Hikmet Geckil
Department of Molecular Biology and Genetics
Inonu University
Malatya, Turkey



Courtesy of
Julie B. Wolf, UMBC

Beginning Molecular Biology Laboratory Manual

CHAPTER 1: General Laboratory Methods

CHAPTER 2: Instructions for Notebook Keeping

CHAPTER 3: Vector NTI User's Guide

CHAPTER 4: Molecular Biology Methods

- Preparation of genomic DNA from bacteria
- PCR amplification of DNA
- Restriction enzyme digestion of DNA
- Phenol/chloroform extraction of DNA
- Ethanol precipitation of DNA
- Agarose gel electrophoresis
- Transformation of E. coli by electroporation
- Preparative DNA Fragment Isolation from an Agarose Gel
- Ligations of plasmid DNA to insert DNA
- Transfection of mammalian cells using Lipofectamine
- Southern blotting
- Western Blot analysis of His-tagged proteins
- Cycle Sequencing Protocols For ABI 3100
- Colony PCR
- One Step Gene Assembly (Gene Synthesis)

CHAPTER 5: Tissue Culture Methods

- Types of cells grown in culture
- Work area and equipment
- Preservation and storage
- Maintenance
- Safety considerations
- Tissue culture procedures
- Determining cell counts
- Common Stock Solutions

CHAPTER 1: General Laboratory Procedures, Equipment Use, and Safety Considerations

- I. Safety Procedures
- II. Preparation of Solutions
- III. Disposal of Buffers and Chemicals
- IV. Equipment
- V. Working with DNA
- VI. Sterile Technique
- VII. Working with *E. coli*

I. Safety Procedures

A. Chemicals

A number of chemicals used in any molecular biology laboratory are hazardous. All manufacturers of hazardous materials are required by law to supply the user with pertinent information on any hazards associated with their chemicals. This information is supplied in the form of Material Safety Data Sheets or MSDS. This information contains the chemical name, CAS#, health hazard data, including first aid treatment, physical data, fire and explosion hazard data, reactivity data, spill or leak procedures, and any special precautions needed when handling this chemical. A file containing MSDS information on the hazardous substances should be kept in the lab. In addition, MSDS information can be accessed on World Wide Web. You are strongly urged to make use of this information prior to using a new chemical and certainly in the case of any accidental exposure or spill. **The instructor/lab manager must be notified immediately in the case of an accident involving any potentially hazardous reagents.**

The following chemicals are particularly noteworthy:

- Phenol - can cause severe burns
- Acrylamide - potential neurotoxin
- Ethidium bromide - carcinogen

These chemicals are not harmful if used properly: always wear gloves when using potentially hazardous chemicals and never mouth-pipet them. If you accidentally splash any of these chemicals on your skin, **immediately** rinse the area thoroughly with water and inform the instructor. Discard the waste in appropriate containers.

B. Ultraviolet Light

Exposure to ultraviolet light can cause acute eye irritation. Since the retina cannot detect UV light, you can have serious eye damage and not realize it until 30 min to 24 hours after exposure. Therefore, **always wear appropriate eye protection when using UV lamps.**

C. Electricity

The voltages used for electrophoresis are sufficient to cause electrocution. Cover the buffer reservoirs during electrophoresis. Always turn off the power supply and unplug the leads before removing a gel.

D. General Housekeeping

All common areas should be kept free of clutter and all dirty dishes, electrophoresis equipment, etc should be dealt with appropriately. Since you have only a limited amount of space to call your own, it is to your advantage to keep your own area clean. Since you will use common facilities, all solutions and everything stored in an incubator, refrigerator, etc. **must be labeled.** In order to limit confusion, each person should use his initials or other unique designation for labeling plates, etc. Unlabeled material found in the refrigerators, incubators, or freezers may be destroyed. Always mark the backs of the plates with your initials, the date, and relevant experimental data, e.g. strain numbers.

II. Preparation of Solutions

A. Calculation of Molar, % and "X" Solutions .

1. A molar solution is one in which 1 liter of solution contains the number of grams equal to its molecular weight. Ex. To make up 100 ml of a 5M NaCl solution = 58.456 (mw of NaCl) g/mol x 5 moles/liter x 0.1 liter = 29.29 g in 100 ml of solution

2. **Percent solutions.** Percentage (w/v) = weight (g) in 100 ml of solution; Percentage (v/v) = volume (ml) in 100 ml of solution. Ex. To make a 0.7% solution of agarose in TBE buffer, weight 0.7 of agarose and bring up volume to 100 ml with TBE buffer.

3. **"X" Solutions.** Many enzyme buffers are prepared as concentrated solutions, e.g. 5X or 10X (five or ten times the concentration of the working solution) and are then diluted such that the final concentration of the buffer in the reaction is 1X. Ex. To set up a restriction digestion in 25 μ l, one would add 2.5 μ l of a 10X buffer, the other reaction components, and water to a final volume of 25 μ l.

B. Preparation of Working Solutions from Concentrated Stock Solutions .

Many buffers in molecular biology require the same components but often in varying concentrations. To avoid having to make every buffer from scratch, it is useful to prepare several concentrated stock solutions and dilute as needed. Ex. To make 100 ml of TE buffer (10 mM Tris, 1 mM EDTA), combine 1 ml of a 1 M Tris solution and 0.2 ml of 0.5 M EDTA and 98.8 ml sterile water. The following is useful for calculating amounts of stock solution needed: $C_i \times V_i = C_f \times V_f$, where C_i = initial concentration, or conc of stock solution; V_i = initial vol, or amount of stock solution needed C_f = final concentration, or conc of desired solution; V_f = final vol, or volume of desired solution

C. Steps in Solution Preparation:

1. Refer to a laboratory reference manual for any specific instructions on preparation of the particular solution and the bottle label for any specific precautions in handling the chemical. Weigh out the desired amount of chemical(s). Use an analytical balance if the amount is less than 0.1 g. Place chemical(s) into appropriate size beaker with a stir bar. Add less than the required amount of water. Prepare all solutions with double distilled water. When the chemical is dissolved, transfer to a graduated cylinder and add the required amount of distilled water to achieve the final volume. An exception is in preparing solutions containing agar or agarose. Weigh the agar or agarose directly into the final vessel. If the solution needs to be at a specific pH, check the pH meter with fresh buffer solutions and follow instructions for using a pH meter. Autoclave, if possible, at 121 deg C for 20 min. Some solutions cannot be autoclaved, for example, SDS. These should be filter sterilized through a 0.22 μ m or 0.45 μ m filter. Media for bacterial cultures must be autoclaved the same day it is prepared, preferably within an hour or two. Store at room temperature and check for contamination prior to use by holding the bottle at eye level and gently swirling it. Solid media for bacterial plates can be prepared in advance, autoclaved, and stored in a bottle. When needed, the agar can be melted in a microwave, any additional components, e.g. antibiotics, can be added and the plates can then be poured.
2. Concentrated solutions, e.g. 1M Tris-HCl pH=8.0, 5M NaCl, can be used to make working stocks by adding autoclaved double-distilled water in a sterile vessel to the appropriate amount of the concentrated solution.

D. Glassware and Plastic Ware .

Glass and plastic ware used for molecular biology must be scrupulously clean. Dirty test tubes, bacterial contamination and traces of detergent can inhibit reactions or degrade nucleic acid. Glassware should be rinsed with distilled water and autoclaved or baked at 150 degrees C for 1 hour. For experiments with RNA, glassware and solutions are treated with diethyl-pyrocabonate to inhibit RNases which can be resistant to autoclaving. Plastic ware such as pipets and culture tubes are often supplied sterile. Tubes made of polypropylene are turbid and are resistant to many chemicals, like

phenol and chloroform; polycarbonate or polystyrene tubes are clear and not resistant to many chemicals. Make sure that the tubes you are using are resistant to the chemicals used in your experiment. Micro pipet tips and microfuge tubes should be autoclaved before use.

III. Disposal of Buffers and Chemicals

1. Any uncontaminated, solidified agar or agarose should be discarded in the trash, not in the sink, and the bottles rinsed well.
2. Any media that becomes contaminated should be promptly autoclaved before discarding it. Petri dishes and other biological waste should be discarded in Biohazard containers which will be autoclaved prior to disposal.
3. Organic reagents, e.g. phenol, should be used in a fume hood and all organic waste should be disposed of in a labeled container, not in the trash or the sink.
4. Ethidium bromide is a mutagenic substance that should be treated before disposal and should be handled only with gloves. Ethidium bromide should be disposed of in a labeled container.

Dirty glassware should be rinsed, all traces of agar or other substance that will not come clean in a dishwasher should be removed, all labels should be removed (if possible), and the glassware should be placed in the dirty dish bin. **Bottle caps, stir bars and spatulas** should not be placed in the bins but should be washed with hot soapy water, rinsed well with hot water, and rinsed three times with distilled water.

IV. Equipment

A. General Comments

It is to everyone's advantage to keep the equipment in good working condition. As a rule of thumb, don't use anything unless you have been instructed in the proper use. This is true not only for equipment in the lab but also departmental equipment. Report any malfunction immediately. Rinse out all centrifuge rotors after use and in particular if anything spills. Please do not waste supplies - use only what you need. If the supply is running low, please notify either the instructor/lab manager **before** the supply is completely exhausted. Occasionally, it is necessary to borrow a reagent or a piece of equipment from another lab. Except in an emergency, notify the instructor.

B. Micropipettors

Most of the experiments you will conduct in this laboratory will depend on your ability to accurately measure volumes of solutions using micropipettors. The accuracy of your pipetting can only be as accurate as your pipettor and several steps should be taken to insure that your pipettes are accurate and are maintained in good working order. Each pair of students will be assigned a set of pipettors and upon receipt, they should be labeled with the students' name. They should then be checked for accuracy following the instructions given by the instructor. If they need to be recalibrated, do so. We have two different types of pipettors, Rainin pipetmen and Oxford benchmates. Since the pipettors will use different pipet tips, make sure that the pipet tip you are using is designed for your pipettor. **DO NOT DROP IT ON THE FLOOR.** If you suspect that something is wrong with your pipettor, first check the calibration to see if your suspicions were correct, then notify the instructor.

C. Using a pH Meter

Biological functions are very sensitive to changes in pH and hence, buffers are used to stabilize the pH. A pH meter is an instrument that measures the potential difference between a reference electrode and a glass electrode, often combined into one combination electrode. The reference electrode is often AgCl 2. An accurate pH reading depends on standardization, the degree of static charge, and the temperature of the solution.

Operation of Orion PerpHecT pH Meter

- Expose hole on side of electrode by sliding the collar down. Make sure there is sufficient electrode filling solution in the electrode (it should be up to the hole). If not, fill with ROSS filling solution only (**Do not use any filling solution containing silver (Ag)**).
- Ensure that sample to be pHed is at room temperature and is stirring gently on the stir plate.
- Calibrate the pH meter with the two solutions that bracket the target pH - 4 and 7 or 7 and 10 as follows:
- Press the CAL key to initialize the calibration sequence. The last calibration range will be displayed (e.g. 7-4). Press YES to accept or use the scroll keys to select a different range. Press YES to accept.
- The number 7 will light up on the left hand side of the screen indicating that the meter is ready to accept the pH 7 standard buffer. Rinse off electrode and place in fresh pH 7 standard buffer solution. The READY light will come on when the value has stabilized. Press YES to accept the value.
- The number 4 (or 10) will light up next indicating that the meter is ready to accept the pH 4 (or 10) standard buffer solution. Rinse off electrode and place in fresh pH 4 standard buffer solution. The READY light will come on when the value has stabilized. Press YES to accept the value.
- SLP will be displayed. The meter will then go MEASURE mode.
- Rinse electrode and place into sample. The READY light is displayed when signal is stable.

D. Autoclave Operating Procedures

Place all material to be autoclaved in a autoclavable tray. All items should have indicator tape. Separate liquids from solids and autoclave separately. Make sure lids on all bottle are loose. **Do not crowd large number of items in tray- in order for all items to reach the appropriate temperature, one must allow sufficient air/steam circulation.**

1. Make sure chamber pressure is at 0 before opening the door.
2. Place items to be autoclaved in the autoclave and close the door. Some autoclaves require that you also lock the door after it's closed.
3. Set time - typically 20 minutes.
4. Temperature should be set at 121 deg C already, but double-check and change if necessary.
5. Set cycle: If liquid, set "liquid cycle" or "slow exhaust". If dry, set "dry cycle" or "fast exhaust" + dry time.
6. Start the cycle. On some autoclaves, the cycle starts automatically at step 5. On others, turn to "sterilize".
7. At the end of the cycle, check that: a. the chamber pressure is at 0; b. the temp is <100 deg C
8. Open door.
9. Remove contents using gloves and immediately tighten all caps.

E. Operating Instructions for Spectrophotometer - Pharmacia Ultraspec

- To measure the absorbance of a solution in the short-wave range (<300 nm) use the quartz cuvettes. Disposable plastic cuvettes are available for reading in the visible range.
- Turn the spectrophotometer on - the switch is on the right in the back.
- Allow the instrument to calibrate. Do not open the chamber during this time. The deuterium lamp is OFF by default. To read absorbance in the UV range, turn the deuterium lamp on as follows after the machine has completed its calibration: Depress the function key until Fn5 is displayed. Press the mode key until d2on is displayed. Press enter. For best accuracy, the deuterium lamp should be warmed up for 20 minutes.
- Press the function key until Fn0 is displayed. Press enter. Using the up or down arrow keys, enter in the desired wavelength.
- Prepare a reference cuvette containing the same diluent as your sample. Prepare your sample.
- Place the reference cuvette in cell #1 and place your samples in cells #2-6.

Press the cell key until cell #1 is in position. Press the Set Reference key to blank against the appropriate buffer. Press the cell key to advance to read the next sample.

V. Working with DNA

A. Storage.

The following properties of reagents and conditions are important considerations in processing and storing DNA and RNA. Heavy metals promote phosphodiester breakage. EDTA is an excellent heavy metal chelator. Free radicals are formed from chemical breakdown and radiation and they cause phosphodiester breakage. UV light at 260 nm causes a variety of lesions, including thymine dimers and cross-link. Biological activity is rapidly lost. 320 nm irradiation can also cause cross-link, but less efficiently. Ethidium bromide causes photo oxidation of DNA with visible light and molecular oxygen. Oxidation products can cause phosphodiester breakage. If no heavy metal are present, ethanol does not damage DNA. Nucleases are found on human skin; therefore, avoid direct or indirect contact between nucleic acids and fingers. Most DNases are not very stable; however, many RNases are very stable and can adsorb to glass or plastic and remain active. 5 E C is one of the best and simplest conditions for storing DNA. -20 deg C: this temperature causes extensive single and double strand breaks. -70 E C is probable excellent for long-term storage. For long-term storage of DNA, it is best to store in high salt (>1M) in the presence of high EDTA (>10mM) at pH 8.5. Storage of DNA in buoyant CsCl with ethidium bromide in the dark at 5 E C is excellent. There is about one phosphodiester break per 200 kb of DNA per year. Storage of λ DNA in the phage is better than storing the pure DNA. [ref: Davis, R.W., D. Botstein and J.R. Roth, A Manual for Genetic Engineering: Advanced Bacterial Genetics. Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y. 1980.]

B. Purification.

To remove protein from nucleic acid solutions:

1. Treat with proteolytic enzyme, e.g., pronase, proteinase K
2. Purify on a silica-based column such as a Qiagen PCR Prep Column
3. CsCl/ethidium bromide density gradient
4. Phenol Extract. The simplest method for purifying DNA is to extract with phenol or phenol:chloroform and then chloroform. The phenol denatures proteins and the final extraction with chloroform removes traces of phenol
5. Purify on silica-based column such as Qiagen Brand columns (<http://www.qiagen.com>)

C. Quantitation.

1. Spectrophotometric. For pure solutions of DNA, the simplest method of quantitation is reading the absorbance at 260 nm where an OD of 1 in a 1 cm path length = 50 μ g/ml for double-stranded DNA, 40 μ g/ml for single-stranded DNA and RNA and 20-33 μ g/ml for oligonucleotides. An absorbance ratio of 260 nm and 280 nm gives an estimate of the purity of the solution. Pure DNA and RNA solutions have OD 260/OD 280 values of 1.8 and 2.0, respectively. This method is not useful for small quantities of DNA or RNA (<1 μ g/ml).
2. Ethidium bromide fluorescence. The amount of DNA in a solution is proportional to the fluorescence emitted by ethidium bromide in that solution. Dilutions of an unknown DNA in the presence of 2 μ g/ml ethidium bromide are compared to dilutions of a known amount of a standard DNA solutions spotted on an agarose gel or Saran Wrap or electrophoresed in an agarose gel.

D. Concentration.

Precipitation with ethanol. DNA and RNA solutions are concentrated with ethanol as follows: The volume of DNA is measured and the monovalent cation concentration is adjusted. The final concentration should be 2-2.5M for ammonium acetate, 0.3M for sodium acetate, 0.2M for sodium chloride and 0.8M for lithium chloride. The ion used often depends on the volume of DNA and on the subsequent manipulations; for example, sodium acetate inhibits Klenow, ammonium ions inhibit T4 polynucleotide kinase, and chloride ions inhibit RNA-dependent DNA polymerases. The addition of MgCl₂ to a final concentration of 10mM assists in the precipitation of small DNA fragments and oligonucleotides. Following addition of the monovalent cations, 2-2.5 volumes of ethanol are added, mixed well, and stored on ice or at -20 °C for 20 min to 1 hour. The DNA is recovered by centrifugation in a microfuge for 10 min (room temperature is okay). The supernatant is carefully decanted making certain that the DNA pellet, if visible, is not discarded (often the pellet is not visible until it is dry). To remove salts, the pellet is washed with 0.5-1.0 ml of 70% ethanol, spun again, the supernatant decanted, and the pellet dried. Ammonium acetate is very soluble in ethanol and is effectively removed by a 70% wash. Sodium acetate and sodium chloride are less effectively removed. For fast drying, the pellet can be spun briefly in a Speedvac, although the method is not recommended for many DNA preparations as DNA that has been over dried is difficult to resuspend and also tends to denature small fragments of DNA. Isopropanol is also used to precipitate DNA but it tends to coprecipitate salts and is harder to evaporate since it is less volatile. However, less isopropanol is required than ethanol to precipitate DNA and it is sometimes used when volumes must be kept to a minimum, e.g., in large scale plasmid preps.

E. Restriction Enzymes.

Restriction and DNA modifying enzymes are stored at -20 °C in a non-frost free freezer, typically in 50% glycerol. The enzymes are stored in an insulated cooler which will keep the enzymes at -20 °C for some period of time. The tubes should never be allowed to reach room temperature and gloves should be worn when handling as fingers contain nucleases. Always use a new, sterile pipet tip every time you use a restriction enzyme. Also, the volume of the enzyme should be less than 1/10 of the final volume of the reaction mixture.

VI. Sterile Technique

1. All media, including plates, liquid media and top agar must be autoclaved immediately after it is prepared. It is best to prepare media in several small bottles, only opening one at a time. Check the bottle for contamination **before** you use it by gently swirling it and looking for cloudy material in the center. **Always** grow up a small amount of broth alone when growing cells overnight. A small amount of contamination is not always evident until the media is incubated at 37 °C.
2. Use a flame on inoculating loops and on the lips of media bottles before and after pipetting from them. Never leave a media or agar bottle open on the bench and don't take an individually-wrapped pipet out of its protective wrapper until you are ready to use it (i.e., don't walk across the room with an unwrapped pipet). Always use a fresh, sterile pipet or pipet tip when pipetting culture media, and **never** go back into a media bottle or cell culture with a used pipet.
3. To prevent wide-scale, untraceable contamination, **each** person should have his own stock of liquid culture media, top agar, plates, 100% glycerol, glycerol stocks of cells, etc. and **don't share**.
4. Overnight cultures should be grown only from a single colony on a fresh plate or from a previously-tested glycerol stock that was grown from a single colony. To prepare an overnight culture from a glycerol stock, take an individually-wrapped 1-ml pipet and a culture tube of media to the -80 °C freezer. Quickly remove the cap from the freezer vial containing the glycerol stock, scrap a small amount of ice from the surface of the culture, replace the cap on the freezer vial, and place the pipet into the culture tube. Sufficient

numbers of bacteria are present in the ice in order for the culture to grow to saturation in 16 hours. **Never let the glycerol stock thaw.**

5. **Think about what you are doing. The best defense is common sense.**

VII. Working with *E. coli*

A. Small Scale Cultures

Experiments using *E. coli* cells should always be done on fresh cultures, either from a freshly streaked plate or from a glycerol stock. To grow a small scale *E. coli* culture, prepare 3-5 ml of LB (or appropriate broth - include antibiotic if the culture contains a plasmid) in two sterile 50 ml tubes.

(Note: smaller tubes can be used but the culture will not be appropriately aerated and hence will not grow well **and is not recommended**). Inoculate one tube with a single colony from a fresh plate or a scraping from a glycerol stock. The second tube is used as a broth control. Incubate both tubes at 37 deg C, shaking vigorously overnight. Inspect the tubes the next morning. The broth control should be clear and the inoculated culture should be very turbid. Make a note of any debris found in the tubes and only incubate longer if the culture is not dense. Do not allow cells to overgrow. Use immediately. For some applications, cells can be stored at 4 deg C for short periods prior to use.

B. Permanent Storage

For every culture used, in particular, for newly constructed strains or for cells containing plasmids, a permanent glycerol stock must be prepared as soon as the construct has been confirmed and **this stock must be placed in the laboratory stock collection with the appropriate documentation and location information. Failure to follow these procedures will result in serious penalties.** This procedure pertains not only to *E. coli* but to any organism for which a deep freeze stock can be prepared. Also, all plasmid constructs, including construction intermediates, must be maintained in cells, not as naked DNA stocks. For each construct, at least 2 stocks should be made. To prepare a glycerol stock for *E. coli* cells, combine 1.4 ml of a freshly grown overnight culture with 0.6 ml of sterile 50% glycerol. Mix well. Transfer to two freezer vials labeled with the strain name, the date and your initials (not an eppendorf tube). Immediately place into a dry ice/ethanol bath or into a box in the -80 deg C freezer. Note the location and enter data into the strain book.

CHAPTER 2: Instructions for Notebook Keeping

1. A notebook should be kept for laboratory experiments only using a scientific notebook book or other bound book. The notebook should be written in ink, and each page signed and dated. Mistakes are not to be erased but should be marked out with a single line. Try to keep your notebook with the idea that someone else must be able to read and understand what you have done. The notebook should always be up-to-date and can be collected at any time.

2. **INDEX:** An index containing the **title** of each experiment and the page number should be included at the beginning of the notebook.

3. **WHAT SHOULD BE INCLUDED IN THE NOTEBOOK?** Essentially everything you do in the laboratory should be in your notebook. The notebook should be organized by **experiment** only and **should not** be organized as a daily log. Start each new experiment on a new page. The top of the page should contain the **title of the experiment, the date, and the page number**. The page number is important for indexing, referring to previous experiments, and for labeling materials used in a given experiment. If an experiment spans more than one page, note the page on which the experiment continues if it's not on the next page. Each experiment should include the following:

4. **Title/Purpose:** Every experiment should have a title and it should be descriptive. An example would be "Large-scale plasmid preparation of plasmid pXGH-5 for transfection into mouse L cells". When starting a new project, it is a good idea to introduce the overall strategy prior to beginning the first experiment. This serves two purposes. First, it forces you to think about what you are doing and why and sometimes things look differently when written down than they do in your head. Second, ideas can be patented, and a thorough description of your hypothesis and experimental strategy with appropriate documentation can be helpful for any future intellectual property issues.

Many experiments should also describe the purpose of the experiment and include any information that is pertinent to the execution of the experiment or to the interpretation of the results. For example, if it is a repeat experiment, state what will be done differently to get the experiment to work. If it's a cloning experiment, include what the strategy is and how the recombinants will be screened. A simple drawing of the plasmid map can be helpful. **This is not like the introduction to a paper.** Include anything that will be helpful in carrying out the experiment and deciphering the experiment at a later date. For the most part, notebooks are not written for today but for the future.

5. **Background information:** This section should include any information that is pertinent to the execution of the experiment or to the interpretation of the results. For example, if it is a repeat experiment, state what will be done differently to get the experiment to work. If it's a cloning experiment, include what the strategy is and how the recombinants will be screened. A simple drawing of the plasmid map can be helpful. **This is not like the introduction to a paper.** Include anything that will be helpful in carrying out the experiment and deciphering the experiment at a later date. For the most part, notebooks are not written for today but for the future.

6. **Materials:** This section should include the key materials, i.e., solutions or equipment, that will be needed. It is not necessary to include every piece of lab equipment required, i.e. vortexer, pipetman, etc, but you should include any specialized equipment and the manufacturer, i.e, a phosphoimager or real-time PCR instrument. Composition of all buffers should be included unless they are standard or are referenced. Pre-packaged kits should be identified as to the name of the kit, the vendor, and the catalog number. Biological samples should be identified by genus and species, strain number, tissue type, and/or genotype with the source of the material identified. Enzymes should be identified by name, vendor, and concentration. DNA samples should be identified as to 1: type of DNA, i.e., chromosomal, plasmid, etc, 2: purity (miniprep, gel purified, PCR product) 3: concentration, if known, and 4: source, (include prior experiment number if the DNA was isolated in a previous experiment). Include all calculations made in preparing solutions. The sequence of all oligonucleotides must be

included or referenced. Agarose gels should be identified by percentage and buffer used. If any of these materials were used in previous experiments, include only the reference to that earlier experiment, do not repeat the information again.

7. Procedure: Write down exactly what you are going to do before you do it and make sure you understand each step before you do it. In general, Xerox copies alone of procedures are not acceptable for several reasons: 1. You should include everything you do including all volumes and amounts; many protocols are written for general use and must be adapted for a specific application. 2. Writing a procedure out helps you to remember and to understand what it is about. It will also help you to identify steps that may be unclear or that need special attention. 3. Some procedures can be several pages long and include more information than is necessary in a notebook. However, it is good laboratory practice to have a separate notebook containing methods that you use on a regular basis (this is not required for this course). If an experiment is a repeat of an earlier experiment, you do not have to write down each step but refer to the earlier experiment by page or experiment number. If you make any changes, note the changes and why. Flow charts are sometimes helpful for experiments that have many parts. Tables are also useful if an experiment includes a set of reactions with multiple variables. It is good practice to check off steps as they are completed or reagents as they are added to prevent you from losing your place or for forgetting to add something. **All procedures should be referenced.**

8. Results: This section should include **all** raw data, including gel photographs, printouts, colony counts, autoradiographs, etc. All lanes on gel photographs must be labeled and always identify the source and the amount of any standards. This section should also include your analyzed data, for example, transformation efficiencies, calculations of specific activities or enzyme activities.

9. Conclusions/Summary: This is one of the most important sections. You should summarize all of your results, even if they were stated elsewhere and state any conclusions you can make. If the experiment didn't work, what went wrong and what will you do the next time to try to trouble shoot?

CHAPTER 3: Vector NTI User's Guide

One of the most useful programs for basic DNA analysis and for laboratory maintenance of plasmid constructs and oligonucleotides is Vector NTI Advance 10.0, a computer program available from Invitrogen (<http://www.invitrogen.com>). The following is a short introduction to the program and the steps for completing some common tasks - it is not meant to be comprehensive - see the complete users manual at the vendors web site.

To **initiate** the program, go to the start menu and look for the program in the **Invitrogen** folder.

General Program Organization:

Once the program is started, the initial view is a tripartite screen into which existing or new DNA molecules can be loaded. To access database entries (samples are included with the program), click on the toolbar icon (shown in the left box). This opens the local **Database Explorer** which comprises the databases, organized by type of molecule, e.g., DNA, protein, oligonucleotide, or enzyme. To work with an individual sequence file, locate that file in the database, and double-click on it. You will see a three-part screen: a text pane, with annotation; a graphic pane with a visual map of the sequence; and a sequence pane, with the complete nucleic acid or protein sequence shown.

Useful icons to know:

A. First Row (only selected icons are described – others are intuitive or nonessential) :

- **Save** – files can be saved in one of two formats- as **database** files or as **molecule** files. By default, all sequences are saved into the Explorer **database**. However, to view files at another location or to save changes made to a file after graphic editing, they must be saved as a molecule file. Under the save tab, chose save as file and the file will be given a “.gb” extension and you will be prompted for a location. Molecule files can be saved to personal directories or drives and they can be viewed on computers not running Vector NTI using the free Vector NTI viewer program available at the following website: http://register.informaxinc.com/solutions/vectornti/molecular_viewer.html

Note: Many modifications made to database files will not be saved unless you save a display profile and after opening database files, change the display setup to this profile.

- **Camera** – this is Vector NTI's method of copying images, etc, to the Windows clipboard. The camera will only copy the information in the active window (i.e., text, graphic or sequence)
- **Local Database** – this will open the database explorer
- **Add fragment to goal list** – used to construct molecules (described in Task #4)
- **Add to oligo list** – This icon is activated if sequence in the sequence pane is highlighted. Hence, oligonucleotides can be copied directly from DNA sequence to a working “oligo list” – a temporary place to work with oligos until they are to be saved in the database. To do this, after highlighting the sequence in the sequence pane, click on “**Add to oligo list**”. Give the oligo a name, analyze, if desired, and save to database if you wish to make a permanent file. By default, the top strand will be saved. To create oligos to the bottom strand (reverse complement), check the reverse complement box in the oligo tab.
- **Open Goal list ; Open oligo list** – this button opens the Vector NTI lists that includes the **goal** list, if you are making constructions; the **oligo** list, which is a temporary list for collecting oligo sequences until they are saved permanently into the database; and a few others. If you click on one of these buttons and the list doesn't appear, it may be because it is already open but hidden either at the top or at the right of the main Vector NTI screen. To access it, drag the mouse to a double-headed arrow to maximize the list.

B. Row 2:

This row will change depending on which pane is active. The first three buttons allow you to toggle between the text, graphic and sequence pane. Alternatively, clicking in the respective window activates that window. The active pane will determine what information will be sent to the printer or the camera as well as changing the icons available.

1. **Display Setup** – this icon is always available and allows you to make changes in:
 - the restriction enzyme set-up, i.e., which enzymes are shown on a given molecule;
 - the feature setup, i.e., which features are displayed;
 - the sequence setup, i.e. spacing of sequence in sequence pane; 3 or 1 letter amino acid codes, single-strand vs. double-strand display, and fonts
 - motifs setup, i.e., allows you to search for motifs or oligonucleotide sequences within a sequence file and display them on the sequence.
2. **Text Pane Icons:**
 - **Link Panes** – when this button is selected, the only features that will show up in the graphic pane are those features whose folders are open in the text pane (the features haven't disappeared from the file, they are just not shown)
3. **Graphic Pane Icons:**
 - **Add a feature** – add annotation to graphic
 - **Find a sequence**
 - **Edit picture** – this enables individual parts of the graphic pane to be edited to customize the display. The file must be saved in the molecule format (as a *.gb) file in order to save these changes. The version of the file saved in the database will not have these changes.
4. **Sequence specific icons**
 - **Translate direct or complementary strand** icon is available if sequence in the sequence pane is highlighted
 - **Erase translations** removes the translation

Some standard manipulations in VectorNTI:

Task 1: Create a new sequence file (DNA or protein) from scratch.

Options – in Database Explorer, select DNA/RNA Molecules if not already visible using the drop-down menu on the top left, and navigate through **Table, New, Molecule (Using Sequence Editor)**; in main Vector NTI page, navigate through **File, Create new sequence, Using Sequence Editor**. The initial screen asks for the name of the molecule to be created; then tab over to enter the information, including the sequence that you wish to include in this file. You may paste sequence from the Windows clipboard if desired. **If the sequence to be included is present in Genbank or Swissprot, do not make a file this way because you will lose all accompanying annotation.**

Task 2: Create a new sequence file (DNA or protein) from a Genbank file

From either the database screen or the main screen, navigate through Tools, Open, Retrieve (choose appropriate originating location). To open a file from Genbank, use the number followed by the "GI:", don't use the accession number. Note that creating files by this method preserves some of the annotations that should be found in the text screen.

Task 3: Create a new oligo file

Open the Database Explorer oligo database (use the drop-down menu) and navigate through **Table, New, Oligo**.

Task 4: Create recombinant molecule from pre-existing sequence files using standard restriction digestions

- Open all of the files containing the component parts.
- Change "display setup", if necessary, to ensure that all restriction enzymes to be used in the cloning are displayed.

- Starting with the insert, open the file containing the insert sequence. In the graphic pane, click on the restriction enzyme site that is located at the left end of the fragment to be cloned. Holding the Shift key, click on the restriction enzyme site that is located at the right end of the fragment to be cloned.
- Using the menu at the top of the screen, click on **Add fragment to Goal List**. (If this was done correctly, you should just have to click on **Next** until the **Add to List** is displayed).
- Open the file containing the vector sequence; in the graphic pane, click on the restriction site that is compatible to the last enzyme chosen in step #3. Holding the Shift key, click on the other restriction enzyme site. Make sure that the correct piece of the vector is chosen in the display.
- Click on **Add fragment to Goal List**.
- If additional fragments are to be included in this construct, add them using the same procedure.
- Using the icon at the top, **Open Goal list**. The fragments that you added should be in the list. If more than two fragments are included in the construction, make sure that they appear in the order in which you want them to ligate to each other. The order can be changed at this time.
- Click on **Run**. You will be prompted for a name for this molecule and, if the fragments were entered correctly, the construct should be successfully created and will be shown in a new file.
- If you wish to change the starting coordinates of the newly created file, go to **Molecule, Operations, Advanced DNA/RNA, Change starting coordinates**.

Task 5: Overlay oligonucleotide sequences onto DNA sequence files

- Open the DNA sequence file. In the Sequence pane, **Display Setup**, check **Motifs Setup**. If the oligo sequence is not already in the database or in the oligo list, you can add it here. If it is in either the database or in the oligo list, find the name of the oligo file in the list and click on it to add it to the window. If you wish to include oligos that are not completely identical to the DNA sequence, file, you can change the similarity parameters on this screen. Click **OK**.
- Open all of the files containing the component parts.
- Change “display setup”, if necessary, to ensure that all restriction enzymes to be used in the cloning are displayed.
- Starting with the insert, open the file containing the insert sequence. In the graphic pane, click on the restriction enzyme site that is located at the left end of the fragment to be cloned. Holding the Shift key, click on the restriction enzyme site that is located at the right end of the fragment to be cloned.
- Using the menu at the top of the screen, click on **Add fragment to Goal List**. (If this was done correctly, you should just have to click on **Next** until the **Add to List** is displayed).
- Open the file containing the vector sequence; in the graphic pane, click on the restriction site that is compatible to the last enzyme chosen in step #3. Holding the Shift key, click on the other restriction enzyme site. Make sure that the correct piece of the vector is chosen in the display.
- Click on **Add fragment to Goal List**.
- If additional fragments are to be included in this construct, add them using the same procedure.
- Using the icon at the top, **Open Goal list**. The fragments that you added should be in the list. If more than two fragments are included in the construction, make sure that they appear in the order in which you want them to ligate to each other. The order can be changed at this time.

- Click on **Run**. You will be prompted for a name for this molecule and, if the fragments were entered correctly, the construct should be successfully created and will be shown in a new file.
- If you wish to change the starting coordinates of the newly created file, go to **Molecule, Operations, Advanced DNA/RNA, Change starting coordinates**.

Task 6: Checking primers for PCR

1. Open the DNA sequence file that will be used as the template.
2. Highlight the region to be amplified and click **Analysis, Primer Design, Find PCR Primers**.
3. Click on **More** to see additional information about the primers. Generally, there will be oligo sequences in this window that need to be deleted (they are left over from the previous oligo design). If the oligos aren't in the database, they may be entered at this time. Also, any parameters that you wish to change can be changed at this time.
4. If the primers are already in the database, click on the box with the three dots next to the sense and find the sense primer in the database. Repeat with the antisense. Cut any non-template derived sequence from the 5' end of each oligo and paste it into the box **Attach to 5' terminus**.
5. Click **Apply**.
6. If the primers you designed are consistent with the chosen parameters and if they are appropriate for the template, the primers will be shown in the text pane with information about the PCR product size, the optimal annealing temperature, and other information about the primers including T_m, %GC, and so on. The primers can be checked for dimers, hairpins, and predicted duplex formation either in this screen, in the PCR design screen, or directly from the oligonucleotide database. **Print this text screen because this information will not be saved in the database file.** To save this information, the sequence file must be saved as a **molecule** file .
7. The PCR product that is predicted to result from the primers shown can be saved in the database by clicking on the product description with the right mouse button and selecting **Save to database**. This is particularly useful for constructing recombinant molecules using this PCR product as the cloned insert.

CHAPTER 4: Molecular Biology Methods

Preparation of Genomic DNA from Bacteria- using Phase Lock Gel™

(Modified from Experimental Techniques in Bacterial Genetics, Jones and Bartlet, 1990)

Materials: see [Solutions](#) for Recipes

- TE buffer
 - 10% (w/v) sodium dodecyl sulfate ([SDS](#))
 - 20 mg/ml proteinase K
 - phenol\chloroform (50:50)
 - isopropanol
 - 70% ethanol
 - 3M [sodium acetate](#) pH 5.2
 - Phase Lock Gel™ (Eppendorf-Brinkmann)
1. Grow *E. coli* culture overnight in rich broth.
 2. Transfer 2 ml to a 2-ml micro centrifuge tube and spin 2 min.
 3. Decant the supernatant.
 4. Drain well onto a Kimwipe.
 5. Resuspend the pellet in 467 µl TE buffer by repeated pipetting.
 6. Add 30 µl of 10% SDS and 3 µl of 20 mg/ml proteinase K, mix , and incubate 1 hr at 37 ° C.
 7. Add an equal volume of phenol/chloroform and mix well but very gently to avoid shearing the DNA by inverting the tube until the phases are completely mixed. CAUTION: PHENOL CAUSES SEVERE BURNS, WEAR GLOVES GOGGLES, AND LAB COAT AND KEEP TUBES CAPPED TIGHTLY.
 8. Carefully transfer the DNA/phenol mixture into a Phase Lock Gel™ tube (green) and spin at 12,000 RPM for 10 min.
 9. Transfer the upper aqueous phase to a new tube and add an equal volume of phenol/chloroform.
 10. Again mix well and transfer to a new Phase Lock Gel™ tube and spin 10 min.
 11. Transfer the upper aqueous phase to a new tube.
 12. Add 1/10 volume of sodium acetate. Mix.
 13. Add 0.6 volumes of isopropanol and mix gently until the DNA precipitates.
 14. Spool DNA onto a glass rod (or Pasteur pipet with a heat-sealed end).
 15. Wash DNA by dipping end of rod into 1 ml of 70% ethanol for 30 sec.
 16. Resuspend DNA in at least 200 µl TE buffer. Complete resuspension may take several days. Store DNA at 4 ° C short term, -20 or -80 ° C long term.
 17. After DNA has dissolved, determining the concentration by measuring the absorbance at 260 nm.

PCR Amplification of DNA

Materials:

- sterile water
- 10X amplification buffer with 15mM MgCl₂
- 10 mM dNTP
- 50 µM oligonucleotide primer 1
- 50 µM oligonucleotide primer 2
- 5 unit/µl Taq Polymerase
- template DNA (1 µg genomic DNA, 0.1-1 ng plasmid DNA) in 10 µl mineral oil (for thermocyclers without a heated lid)

- Combine the following for each reaction (on ice) in a 0.2 or 0.5 ml tube:

• 10X PCR buffer	• 10 μ l
• Primer 1	• 1 μ l
• Primer 2	• 1 μ l
• dNTP	• 2 μ l
• template DNA and water	• 85.5 μ l
• Taq Polymerase	• 0.5 μ l

- Prepare a control reaction with no template DNA and an additional 10 μ l of sterile water.
- If the thermocycler does not have a heated lid, add 70-100 μ l mineral oil (or 2 drops of silicone oil) to each reaction.
- Place tubes in a thermal cycler preheated to 94 degrees C.
- 5. Run the following program:
 - 94 degrees C 1 min
 - 55 degrees C 1 min or annealing temperature appropriate for particular primer pair
 - 72 degrees C 1 min (if product is <500 bp), 3 min (if product is >500 bp) for 30 cycles.
 - Program a final extension at 72 degrees C for 7 min.

Restriction Enzyme Digestion of DNA

Materials:

- 10X restriction enzyme buffer (see manufacturer's recommendation)
 - DNA
 - sterile water
 - restriction enzyme
 - phenol:chloroform (1:1) (optional)
1. Add the following to a microfuge tube:
 - 2 μ l of appropriate 10X restriction enzyme buffer
 - 0.1 to 5 μ g DNA
 - sterile water to a final volume of 19 μ l (Note: These volumes are for analytical digests only. Larger volumes may be necessary for preparative digests or for chromosomal DNA digests.)
 2. Add 1 to 2 μ l (3 to 20 units) enzyme and mix gently. Spin for a few seconds in microfuge. Incubate at the appropriate temperature (usually 37 degrees C) for 1 to 2 hours. Run a small aliquot on a gel to check for digestion.
 3. If the DNA is to be used for another manipulation, heat inactivate the enzyme (if it is heat labile) at 70 degrees C for 15 min, phenol/chloroform extract and ethanol precipitate, or purify on DNA purification column.

Phenol/chloroform Extraction of DNA

Materials:

- phenol:chloroform (1:1)
 - chloroform
1. Add an equal volume of buffer-saturated phenol:chloroform (1:1) to the DNA solution.
 2. Mix well. Most DNA solutions can be vortexed for 10 sec except for high molecular weight DNA which should be gently rocked. (If using Phase-Lock Gel, follow procedure [M.1](#))
 3. Spin in a microfuge for 3 min.

4. Carefully remove the aqueous layer to a new tube, being careful to avoid the interface. (Steps 1-4 can be repeated until an interface is no longer visible).
5. To remove traces of phenol, add an equal volume of chloroform to the aqueous layer.
6. Spin in a microfuge for 3 min.
7. Remove aqueous layer to new tube.
8. Ethanol precipitate the DNA(see [ethanol precipitation](#))

Ethanol Precipitation of DNA

Materials: see [Solutions](#) for Recipes

- 3 M [sodium acetate](#) pH 5.2 or 5 M ammonium acetate
 - DNA
 - 100% ethanol
1. Measure the volume of the DNA sample. **Adjust the salt concentration** by adding 1/10 volume of sodium acetate, pH 5.2, (final concentration of 0.3 M) or an equal volume of 5 M ammonium acetate (final concentration of 2.0-2.5 M). These amounts assume that the DNA is in TE only; if DNA is in a solution containing salt, adjust salt accordingly to achieve the correct final concentration. Mix well. Add 2 to 2.5 volumes of cold 100% ethanol (calculated after salt addition). Mix well.
 2. Place on ice or at -20 degrees C for ≥ 20 minutes.
 3. Spin a maximum speed in a microfuge 10-15 min. Carefully decant supernatant. Add 1 ml 70% ethanol. Mix. Spin briefly. Carefully decant supernatant. Air dry or briefly vacuum dry pellet.
 4. Resuspend pellet in the appropriate volume of TE or water.

Agarose Gel Electrophoresis

Materials:

- agarose solution in [TBE](#), [TAE](#) or [SB](#) (generally 0.7-1%)
 - 1X TBE, TAE, or SB (same buffer as in agarose)
 - gel loading dye
 - 10 mg/ml ethidium bromide
1. To prepare 100 ml of a 0.7% agarose solution, measure 0.7 g agarose into a glass beaker or flask and add 100 ml 1X buffer. Microwave or stir on a hot plate until agarose is dissolved and solution is clear.
 2. Allow solution to cool to about 55 degrees C before pouring. (Ethidium bromide can be added at this point to a concentration of 0.5 $\mu\text{g}/\text{ml}$)
 3. Prepare gel tray by sealing ends with tape or other custom-made dam. Place comb in gel tray about 1 inch from one end of the tray and position the comb vertically such that the teeth are about 1-2 mm above the surface of the tray
 4. Pour 50 degree C gel solution into tray to a depth of about 5 mm. Allow gel to solidify about 20 minutes at room temperature.
 5. To run, gently remove the comb, place tray in electrophoresis chamber, and cover (just until wells are submerged) with electrophoresis buffer (the same buffer used to prepare the agarose). Excess agarose can be stored at room temperature and remelted in a microwave. To prepare samples for electrophoresis, add 1 μl of 6x gel loading dye for every 5 μl of DNA solution. Mix well. Load 5-12 μl of DNA per well (for minigel). Electrophorese at 50-150 volts until dye markers have migrated an appropriate distance, depending on the size of DNA to be visualized.
 6. If the gel was not stained with ethidium during the run, stain the gel in 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide until the DNA has taken up the dye and is visible under short wave UV light, if the DNA will not be used further, or with a hand-held long-wave UV light if the DNA is to be cut out and purified.

Transformation of *E. coli* by Electroporation (electroporation procedure from Cell-Porator™ Voltage Booster, Life Technologies, Cat. Series 1612)

Background:

There are two methods to transform *E. coli* cells with plasmid DNA - chemical transformation and electroporation. For chemical transformation, cells are grown to mid-log phase, harvested and treated with divalent cations such as CaCl₂. Cells treated in such a way are said to be competent. To chemically transform cells, competent cells are mixed with the DNA, on ice, followed by a brief heat shock. Then, cells are incubated with rich medium and allowed to express the antibiotic resistant gene for 30-60 minutes prior to plating. For electroporation, cells are also grown to mid-log phase but are then washed extensively with water to eliminate all salts. Usually, glycerol is added to the water to a final concentration of 10% so that the cells can be stored frozen and saved for future experiments. To electroporate DNA into cells, washed *E. coli* are mixed with the DNA to be transformed and then pipetted into a plastic cuvette containing electrodes. A short electric pulse, about 2400 volts/cm, is applied to the cells causing small holes in the membrane through which the DNA enters. The cells are then incubated with broth as above before plating.

For chemical transformation, there is no need to pre-treat the DNA. For electroporation, the DNA must be free of all salts so the ligations are first precipitated with alcohol before they are used.

Experimental Design:

To determine the efficiency of transformation, a positive control transformation should be done using 1 ng of uncut plasmid DNA, e.g. pUC19. The efficiency of transformation is calculated as the number of transformants/μg of input DNA. A negative control should also be included that contains cells with no added DNA.

A negative control with cells only (no added DNA) should also be included.

Host Cells

For most cloning applications, we use DH5α host cells. These cells are compatible with *lacZ* blue/white selection procedures, are easily transformed, and good quality plasmid DNA can be recovered from transformants. One notable exception is when transforming with plasmid constructs containing recombinant genes under control of the T7 polymerase. These constructs are typically transformed into DH5α for the cloning phase, but need to be transformed into a different bacterial strain, BL21(DE3) for expression of the recombinant protein (BL21 strains carry the gene for expression of the T7 polymerase).

Electroporation of *E. coli*:

Materials:

- Sterile centrifuge bottles – 250 ml for GSA rotor
- SOB medium
- *E. coli* host strain such as DH5α
- WB (10% redistilled glycerol, 90% distilled water, v/v) chilled to 4°C– need 500 ml of WB for each 250 ml of culture
- tRNA (5-10 μg/ml – used as a mass carrier to increase the efficiency of precipitation)
- 5 M ammonium acetate
- 100% ethanol
- 70% ethanol
- 0.5X TE or EB (10 mM Tris, pH 8.3)
- SOC medium
- transformation plates

I. Preparation of *E. coli* cells for electroporation.

1. Use a fresh colony of DH5 α (or other appropriate host strain) to inoculate 5 ml of SOB (without magnesium) medium in a 50 ml sterile conical tube. Grow cells with vigorous aeration overnight at 37°C.
2. Dilute 2.5 ml of cells into 250 ml of SOB (without magnesium) in a 1 liter flask. Grow for 2 to 3 hours with vigorous aeration at 37°C until the cells reach an OD₅₅₀ = 0.8.
3. Harvest cells by centrifugation at 5000 RPM in a GSA rotor for 10 min in sterile centrifuge bottles. (Make sure you use autoclaved bottles!).
4. Wash the cell pellet in 250 ml of ice-cold WB as follows. First, add a small amount of WB to cell pellet; pipet up and down or gently vortex until cells are resuspended. Then fill centrifuge bottle with ice cold WB and gently mix. NOTE- the absolute volume of WB added at this point is not important.
5. Centrifuge the cell suspension at 5,000 RPM for 15 min and carefully pour off the supernatant as soon as the rotor stops. Cells washed in WB do not pellet well. If the supernatant is turbid, increase the centrifugation time.
6. Wash the cell pellet a second time by resuspending in 250 ml of sterile ice-cold WB using the same technique described above. Centrifuge the cell suspension at 5000 RPM for 15 min.
7. Gently pour off the supernatant leaving a small amount of WB in the bottom of the bottle. Resuspend the cell pellet in the WB - no additional WB needs to be added – and the final volume should be about 1 ml. Cells can be used immediately or can be frozen in 0.2 ml aliquots in freezer vials using a dry ice-ethanol bath. Store frozen cells at -70°C.

II. Preparing DNA for Electroporation

DNA for electroporation must have a very low ionic strength and a high resistance. The DNA may be purified by either dilution, precipitation or dialysis.

- For transformation of purified plasmid DNA, dilute DNA in 10 mM Tris pH 8-8.3 to about 1-50 ng/ μ l (**do not use TE**). Use 1 μ l for transformation.
- For ligation reactions, use the following procedure.

Purifying DNA by Precipitation:

1. Add 5 to 10 μ g of tRNA to a 20 μ l ligation reaction in a 1.5 ml tube. Add 22 μ l 5M ammonium acetate (or an equal volume of ligation reaction with added tRNA). Mix well.
2. Add 100 μ l absolute ethanol (or 2.5 volumes of ligation reaction, tRNA and salt). Ice 15 min.
3. Centrifuge at >12,000 x g for 15 min at 4°C. Carefully decant the supernatant.
4. Wash the pellet with 1 ml of 70% ethanol. Centrifuge at >12,000 x g for 15 min at room temperature. Remove the supernate.
5. Air dry the pellet (speed vac okay but don't overdry).
6. Resuspend the DNA in EB buffer (10 mM Tris-HCl, pH 8.3) or 0.5X TE buffer [5 mM Tris-HCl, 0.5 mM EDTA (pH 7.5)] to a concentration of 10 ng/ μ l of DNA. For ligation reactions, it is convenient to resuspend in 10 μ l. Use 1 μ l per transformation of 20 μ l of cell suspension.

III. Electroporation.

1. Mark the required number of micro centrifuge tubes. Place the required number of Micro-electroporation Chambers on ice. Fill the temperature control compartment of the Chamber Safe with ~250 ml of ice-water slurry and place the Chamber Rack in the Chamber Safe.
2. Thaw an aliquot of cells that have prepared as in Section I and aliquot 20 μ l of cells to the required number of microfuge tubes on ice. Add 1 μ l of the DNA (or ligation reaction) prepared as in Section II.
3. Using a micro pipette, pipette 20 μ l of the cell-DNA mixture between the bosses in a Micro-Electroporation Chamber. Do not leave an air bubble in the droplet of cells; the pressure of a bubble may cause arcing and loss of the sample. Place the chamber in a slot in the Chamber Rack and note its position. Repeat the process if more than one sample is to be pulsed. Up to 4 samples can be placed in the Chamber Rack at one time. Handle the chambers gently to avoid accidentally displacing the sample from between the bosses.
4. Close the lid of the Chamber safe and secure it with the draw latch.

5. Plug the pulse cable into the right side of the Chamber safe.
6. Turn the chamber selection knob on top of the Chamber Safe to direct the electrical pulse to the desired Micro-Electroporation Chamber.
7. Set the resistance on the Voltage Booster to 4 k Ω ; set the Pulse Control unit to LOW and 330 μ F; double check connections.
8. Charge the Pulse Control unit by setting the CHARGE ARM switch on the Pulse Control unit to CHARGE and then pressing the UP voltage control button until the voltage reading is 5 to 10 volts higher than the desired discharge voltage. For *E. coli*, the standard conditions are 2.4 kv, which means setting the Pulse Control unit to 405 volts (400 volts is the desired discharge voltage + 5). The voltage booster amplifies the volts by ~6-fold such that the total discharge voltage is 2400 volts, or 2.4 kv. The actual peak voltage delivered to the sample will be shown on the Voltage Booster meter **after** the pulse is delivered.
9. Set the CHARGE/ARM switch to the ARM position. The green light indicates that the unit is ready to deliver a DC pulse. Depress the pulse discharge TRIGGER button and hold for 1 second.
NOTE: The DC voltage display on the Pulse Control unit should read <10 volts after a pulse has been delivered. If not, discharge the capacitor using the DOWN button.
10. For additional samples, turn the chamber selection knob to the next desired position and repeat steps 8 and 9 until all samples are pulsed.
11. For ampicillin selection, inoculate the samples into 2 ml of SOC medium and shake for 30 minutes (for amp), 60 minutes (for Kan) to allow expression of the antibiotic gene. Plate cells on LB medium with appropriate antibiotic or screening reagent (e.g. 100 μ g/ml ampicillin, and/or 40 μ l of 20 mg/ml X-Gal, XP, and 40 μ l of 100 mM IPTG).

Preparative DNA Fragment Isolation from an Agarose Gel

Background:

DNA can be easily isolated and purified after size selection on an agarose gel. The fragment of interest is simply cut out of the gel with a razor blade and purified by a number of different methods. The easiest is to use a method that involves first dissolving the agarose slice in a solution at 50°C, then binding the DNA from the melted agarose to a silica-gel membrane.

1. Prepare an agarose gel in TAE buffer using the four-well combs. (Preparative agarose gels should be run using 1X TAE electrophoresis and gel buffer as the borate in TBE interferes with some purification resin). Load the DNA. To visualize the DNA after staining, **do not expose the DNA to shortwave UV light as this will introduce nicks**. Visualize the bands with a hand-held long wave UV light and cut out the band with a clean razor blade (Note: place gel on a glass slide to avoid cutting the surface of the transilluminator).
2. After cutting out the band, follow the procedure for DNA fragment purification using Qiagen QIAquick or Qiaex II purification systems following the manufacturer's procedure (<http://www.qiagen.com>). Estimate the approximate concentration of the DNA obtained by running 10% of the eluate on an agarose gel against a DNA mass ladder.

Ligations of plasmid DNA to insert DNA

A typical ligation reaction consists of about 20-200 ng of a vector and a 1-3 fold molar excess of insert DNA. A typical ligation reaction consists of the following:

- 20-100 ng vector
- 3-fold MOLAR excess (not mass) of insert DNA
- 4 μ l 5X ligase buffer or 2 μ l 10X ligase buffer (note: ligase buffer contains ATP and is unstable when repeatedly frozen and thawed. Prepare small aliquots of buffer and discard aliquot after use)
- water to 19 μ l
- 1 μ l T4 DNA ligase

Incubate at room temperature for 2-24 hours. For transformation by electroporation, ethanol precipitate as described in the transformation procedure.

Procedure for Transfection of Mammalian Cells

Materials:

- Lipofectamine (Invitrogen)
- IMDM containing 10% fetal bovine serum, 1% glutamine, 1% aa
- IMDM containing 1% glutamine
- IMDM containing 20% fetal bovine serum, 1% glutamine, 1% aa

1. In a six-well or 35 mm tissue culture plate, seed $\sim 2 \times 10^5$ cells per well in 2 ml IMDM containing 10% FBS and nonessential amino acids.
2. Incubate the cells at 37°C in a CO₂ incubator until the cells are 70-80% confluent. This will usually take 18-24 h.
3. Prepare the following solutions in 12 x 75 mm sterile tubes:

Solution A: For each transfection, dilute 2 µg DNA (plasmid) in 375 µl serum-free IMDM (containing nonessential amino acids).

Solution B: For each transfection, dilute 12 µl LIPOFECTAMINE Reagent in 375 µl serum-free IMDM.

4. Combine the two solutions, mix gently, and incubate at room temperature for 15-45 min. The solution may appear cloudy, however this will not impede the transfection. Wash the cells once with 2 ml serum-free IMDM.
5. For each transfection, add 750 µl serum-free IMDM to each tube containing the lipid-DNA complexes. Do not add antibacterial agents to media during transfection. Mix gently and overlay the diluted complex solution onto the washed cells.
6. Incubate the cells for 5 h at 37°C in a CO₂ incubator.
7. Add 1.5 ml IMDM with 20% FBS without removing the transfection mixture. If toxicity is a problem, remove the transfection mixture and replace with normal growth medium. Replace medium at 18-24 h following start of transfection.
8. Assay cell extracts for gene activity 24-72 h after the start of transfection, depending on cell type and promoter activity.

Southern Blotting

1. Electrophoresis of DNA is carried out in a neutral agarose gel system. Prepare a 0.8-1% agarose gel containing 1x TAE buffer. Ethidium bromide can be added to a final concentration of 0.2 µg/ml.
2. Apply the samples to the gel.
3. Run the gel in 1x TAE buffer at 4V/cm until the bromophenol blue indicates that the sample has run for a sufficient distance.
4. Following electrophoresis, visualize the gel under UV transillumination and photograph with a ruler.
5. i) Depurination, 10 minutes at room temperature with gentle agitation (optional). This step is necessary if target sequences are greater than 10 Kb in size
ii) Denaturation, 25 minutes at room temperature with gentle agitation.
iii) Neutralization, 30 minutes at room temperature with gentle agitation. When using nitrocellulose membranes, the neutralization time should be extended to 45 minutes. Include a rinse in distilled water between each step
6. Assemble the capillary blotting apparatus using 10X SSC as the transfer buffer. Allow the DNA to transfer overnight onto Hybond N+.
7. The following day, disassemble the apparatus, mark the membrane appropriately and fix the DNA to the membrane by UV crosslinking or baking (2 hours at 80°C). For nitrocellulose membranes, bake for 2 hrs. at 80°C in a vacuum oven.

Solutions:

Hybridization buffer

5x SSC

1 in 20 dilution Liquid Block (Amersham) or other blocking reagent

0.1%(w/v) SDS

5%(w/v) Dextran sulphate

EDTA stock

0.5M EDTA pH8.0

SDS stock

10% or 20% (w/v) SDS

Depurination solution

250mM HCl

Denaturation solution

1.5M NaCl

0.5M NaOH

Neutralization solution

1.5M NaCl

0.5M Tris-HCl

pH adjusted to 7.5

20x SSC

0.3M Na(3) citrate

3M NaCl

Western Blot Analysis of Epitoped-tagged Proteins Using The Chemifluorescent Detection Method - for alkaline phosphatase conjugated antibodies

1. Cut PVDF membrane to the appropriate size, activate with absolute methanol for 5 sec, and incubate in distilled water for 5 min.
2. For electroblotting, equilibrate in transfer buffer and follow the standard blotting procedure to transfer the proteins to the membrane. For dot blotting, keep membrane wet until ready to use.
3. After protein has been transferred to the membrane, wash again in absolute methanol for a few seconds and allow to dry at room temperature for 30 min. or more.
4. Block in 30 ml of 1X Western buffer (containing 0.1% Tween-20 and 0.2% I-Block), gently rocking, 1 hr, room temperature.
5. Add appropriate dilution of primary antibody (typically 1:5000 or 1:10,000) prepared in 1X Western buffer (containing 0.1% Tween-20 and 0.2% I-Block), incubate 30 min, room temperature, gently rocking.
6. Wash three times in 20 ml 1X Western buffer (containing 0.1% Tween-20 and 0.2% I-Block) for 5 min each.

Add appropriate dilution of secondary antibody conjugated to alkaline phosphatase prepared in 1X Western buffer (containing 0.1% Tween-20 and 0.2% I-Block), gently rocking, 30 min, room temperature.

7. Wash as in step #6.
8. Then, wash twice with 1X Western buffer **without I-block**.
9. At the end of the second final wash, leave some buffer in the container to keep the membrane moist. With the membrane facing protein-side up, add 0.5 ml of substrate solution directly into the remaining liquid, mix well, and pipet (with a p1000) the solution over the membrane to ensure the entire surface comes into contact with the substrate. Gently agitate for a few minutes, remove membrane to a paper towel and let dry completely. The substrate solution can be reused immediately for additional membranes.
10. Scan membrane using the Molecular Dynamics Storm or other suitable instrument.

Western Blotting Solutions:

- **1X Transfer buffer:** 25 mM Tris, 192 mM Glycine, pH 8.3. Mix 3.03 g Tris and 14.4 g glycine; add water to 1 liter - do not add acid or base to pH - it should be >8.0. Use 0.5X for transfer in 20% methanol.
- **10X Western Buffer:** 200 mM Tris pH = 7.5; 1.5 M NaCl (containing 0.1% Tween-20 and 0.2% I-Block). To prepare 1X Western Buffer, dilute 10X buffer to 1X, adding Tween-20 to 0.1%. Remove 50 ml and set aside for the last two washes. To the remainder, add I-Block to 0.2% (Cat #T2015, Applied Biosystems - formerly Tropix). To dissolve I-Block, heat solution in a beaker briefly in a microwave to about 60°C, then stir until dissolved (solution will be cloudy). Bring to room temperature before using.
- **Primary antibody:** For his tagged proteins - Anti-His monoclonal antibody - BD Bioscience #631212
- **Secondary antibody:** Goat anti-mouse alkaline phosphatase conjugated - Biorad #170-6520.
- **Substrate:** ECF chemifluorescent substrate - Amersham #RPN5785. Mix substrate with accompanying buffer as per manufacturer's recommended instructions, prepare 1 ml aliquots and store at -20°C.

Recommended Cycle Sequencing Protocols For ABI 3100

Template Quantity

Template	Quantity
PCR product:	
100-200bp	1-3 ng
200-500bp	3-10 ng
500-1000bp	5-20 ng
1000-2000bp	10-40 ng
>2000bp	20-50 ng
Single-stranded	25-50 ng
Double-stranded	150-300 ng
Cosmid, BAC	0.5-1.0 mg
Bacterial genomic DNA	2-3 mg

Reaction Mixtures			
Reagent	Full Reaction	Half Reaction	Half Reaction(10 µl)
Big Dye Premix	8 µl	4 µl	2 µl
Big Dye Seq. Buffer	-	2 µl	1 µl
Template	See Table above	See Table above	See Table above
Primer (10 µM)	1 µl	1 µl	1 µl
Water	q.s.	q.s.	q.s.
Total	20 µl	20 µl	10 µl

Primer Quantity

Primer needed = 3.2 - 10 pmoles

PCR Cycle Sequencing Settings for Big Dye V3.1

Initial denaturing	96°C	1min
25 cycles of	96°C	10sec
	50°C	5sec
	60°C	4min
Hold at	4°C	

Ethanol/EDTA Precipitation to clean up reactions

- Add 5uL of 125 mM EDTA. *Make sure the EDTA reaches the bottom of the tube.*
- Add 60uL of 100% ethanol to each tube.

- Finger vortex and incubate at room temperature for 15 min.
- Spin samples in a microcentrifuge at max speed in 4 ° C for 20min.
- Carefully aspirate off the supernatant.
- Add 60 µl of 70% Ethanol.
- Spin samples in a microcentrifuge at maximum speed at 4 ° C for 15 minutes.
- Aspirate off the supernatant.
- Dry sample for 15 minutes in a Speed-Vac (longer if air-drying). *Protect samples from light while they are drying.*

Colony PCR

1. Prepare a master mix containing the following:

1X reaction:

- 2.5 µl 2 mM dNTPs
- 2.5 µl 10 µM primer 1
- 10 µl 2.5X [cresol red loading dye](#)
- 2.5 µl 10X PCR buffer
- 5.0 µl sterile water
- 0.5-1 µl Taq polymerase

Note: If using PuReTaq Ready-To-Go PCR Beads (Amersham Biosciences), use 10 µl of water instead of dNTP's, buffer and enzyme]

To prepare the master mix, multiply the volumes above by the number of colonies to be screened + 1.

2. Aliquot to 0.2 ml labeled PCR tubes and keep on ice.
3. Prepare one selection plate, e.g., LB + ampicillin, for every 20 colonies screened.
4. Label the plate with a grid so that each colony can be associated with a number that matches the number on the PCR tube and can be retrieved once PCR results are known.
5. With a toothpick or sterile loop, pick colonies from transformation plate, patch onto the selection plate and then place remainder in PCR with the same identifier.
6. PCR cycle using the same conditions for the original PCR with those same primers with one modification: include a 5 min 94°C denaturation at the beginning of the cycling reaction.
7. Analyze PCR results by running reactions directly onto an agarose gel (no additional loading dye is required)

One-Step Gene Assembly

(Reference: Gang Wu, Julie B. Wolf, Ameer F. Ibrahim, Stephanie Vadasz, Muditha Gunasinghe and Stephen J. Freeland, Simplified gene synthesis: A one-step approach to PCR-based gene construction, Journal of Biotechnology, 124(3):496-503)

1. Design overlapping oligonucleotides, generally 40 bases in length, that encompass the sense strand of the gene of interest. Design antisense oligonucleotides that stagger the sense oligos by 20 bases.
2. Design outside PCR amplification primers to incorporate the appropriate restriction enzyme recognition sites, if desired, and to overlap the assembled gene sequence by at least 15 nucleotides. (Order oligonucleotides from IDT or Invitrogen using their standard desalting purification).
3. Reconstitute oligonucleotides to 100 µM in 10 mM Tris pH=8.5 (same as Qiagen buffer EB). Vortex well to reconstitute and store at -20°C.
4. Prepare a gene assembly mix by combining 5 µl of each of the gene assembly oligos. Dilute this mix so that each oligo is at a final concentration of 1 µM. (This will be referred to as 1X). Then dilute this mix 1:2 (0.5X), 1:5 (0.2X) and 1:10 (0.1X) in Tris buffer. This step is to optimize the assembly/amplification of the required product which varies from one gene to the next – so best to set up 4 different reactions, one for each dilution of the mix, so determine which gives you the best yield and the least background.

5. Prepare 10 μM dilutions of outside amplification primers.
6. Perform one step gene assembly/amplifications using the following:

Reaction conditions:

Template	5 μl gene assembly mix (1X, 0.5X, 0.2X, and 0.1X)
Primers (0.4 μM final)	2 μl of 10 μM outside amplification primer #1 (OP-5') 2 μl of 10 μM outside amplification primer #2 (OP-3')
dNTP (0.2 mM final)	5 μl of 2 mM each (provided with KOD enzymes)
10X PCR buffer	5 μl (provided with KOD enzymes)
25 mM MgCl_2	2 μl for KOD HiFi; none needed for XL
Sterile water	28 μl for KOD HiFi; 30 μl for XL
KOD HiFi enzyme for highest accuracy or KOD XL for TA cloning	0.4 μl KOD HiFi (Novagen) or 1 μl for KOD XL (Novagen)
Total	50 μl

Cycling conditions:

	KOD XL– up to 2 kb		KOD HiFi – up to 2 kb	
25 cycles	94°C	30 sec	98°C	15 sec
	52°C	5 sec	52°C	2 sec
	72°C	30-60 sec/kb	72°C	20 sec
1 cycle	74°C	10 min		

- Analyze 5 μl on agarose gel.
- Purify remainder using Qiagen PCR purification columns, digest overnight, then gel purify.
Ligate to vector of choice.

CHAPTER 5: Tissue Culture Methods

I. TYPES OF CELLS GROWN IN CULTURE

Tissue culture is often a generic term that refers to both organ culture and cell culture and the terms are often used interchangeably. Cell cultures are derived from either primary tissue explants or cell suspensions. Primary cell cultures typically will have a finite life span in culture whereas continuous cell lines are, by definition, abnormal and are often transformed cell lines.

II. WORK AREA AND EQUIPMENT

A. Laminar flow hoods. There are two types of laminar flow hoods, vertical and horizontal. The vertical hood, also known as a biology safety cabinet, is best for working with hazardous organisms since the aerosols that are generated in the hood are filtered out before they are released into the surrounding environment. Horizontal hoods are designed such that the air flows directly at the operator hence they are not useful for working with hazardous organisms but are the best protection for your cultures. Both types of hoods have continuous displacement of air that passes through a HEPA (high efficiency particle) filter that removes particulates from the air. In a vertical hood, the filtered air blows down from the top of the cabinet; in a horizontal hood, the filtered air blows out at the operator in a horizontal fashion. NOTE: these are not fume hoods and should not be used for volatile or explosive chemicals. They should also never be used for bacterial or fungal work. The hoods are equipped with a short-wave UV light that can be turned on for a few minutes to sterilize the surfaces of the hood, but be aware that only exposed surfaces will be accessible to the UV light. Do not put your hands or face near the hood when the UV light is on as the short wave light can cause skin and eye damage. The hoods should be turned on about 10-20 minutes before being used. Wipe down all surfaces with ethanol before and after each use. Keep the hood as free of clutter as possible because this will interfere with the laminar flow air pattern.

B. CO₂ Incubators. The cells are grown in an atmosphere of 5-10% CO₂ because the medium used is buffered with sodium bicarbonate/carbonic acid and the pH must be strictly maintained. Culture flasks should have loosened caps to allow for sufficient gas exchange. Cells should be left out of the incubator for as little time as possible and the incubator doors should not be opened for very long. The humidity must also be maintained for those cells growing in tissue culture dishes so a pan of water is kept filled at all times.

C. Microscopes. Inverted phase contrast microscopes are used for visualizing the cells. Microscopes should be kept covered and the lights turned down when not in use. Before using the microscope or whenever an objective is changed, check that the phase rings are aligned.

D. Preservation. Cells are stored in liquid nitrogen (see Section III- Preservation and storage).

E. Vessels. Anchorage dependent cells require a nontoxic, biologically inert, and optically transparent surface that will allow cells to attach and allow movement for growth. The most convenient vessels are specially-treated polystyrene plastic that are supplied sterile and are disposable. These include petri dishes, multi-well plates, microtiter plates, roller bottles, and screwcap flasks - T-25, T-75, T-150 (cm² of surface area). Suspension cells are either shaken, stirred, or grown in vessels identical to those used for anchorage-dependent cells.

III. PRESERVATION AND STORAGE. Liquid N₂ is used to preserve tissue culture cells, either in the liquid phase (-196°C) or in the vapor phase (-156°C). Freezing can be lethal to cells due to the effects of damage by ice crystals, alterations in the concentration of electrolytes, dehydration, and changes in pH. To minimize the effects of freezing, several precautions are taken. First, a cryoprotective agent which lowers the freezing point, such as glycerol or DMSO, is added. A typical freezing medium is

90% serum, 10% DMSO. In addition, it is best to use healthy cells that are growing in log phase and to replace the medium 24 hours before freezing. Also, the cells are slowly cooled from room temperature to -80°C to allow the water to move out of the cells before it freezes. The optimal rate of cooling is $1^{\circ}\text{--}3^{\circ}\text{C}$ per minute. Some labs have fancy freezing chambers to regulate the freezing at the optimal rate by periodically pulsing in liquid nitrogen. We use a low tech device called a Mr. Frosty (C#1562 -Nalgene, available from Sigma). The Mr. Frosty is filled with 200 ml of isopropanol at room temperature and the freezing vials containing the cells are placed in the container and the container is placed in the -80°C freezer. The effect of the isopropanol is to allow the tubes to come to the temperature of the freezer slowly, at about 1°C per minute. Once the container has reached -80°C (about 4 hours or, more conveniently, overnight) the vials are removed from the Mr. Frosty and immediately placed in the liquid nitrogen storage tank. Cells are stored at liquid nitrogen temperatures because the growth of ice crystals is retarded below -130°C . To maximize recovery of the cells when thawing, the cells are warmed very quickly by placing the tube directly from the liquid nitrogen container into a 37°C water bath with moderate shaking. As soon as the last ice crystal is melted, the cells are immediately diluted into prewarmed medium.

IV. MAINTENANCE

Cultures should be examined daily, observing the morphology, the color of the medium and the density of the cells. A tissue culture log should be maintained that is separate from your regular laboratory notebook. The log should contain: the name of the cell line, the medium components and any alterations to the standard medium, the dates on which the cells were split and/or fed, a calculation of the doubling time of the culture (this should be done at least once during the semester), and any observations relative to the morphology, etc.

A. Growth pattern. Cells will initially go through a quiescent or lag phase that depends on the cell type, the seeding density, the media components, and previous handling. The cells will then go into exponential growth where they have the highest metabolic activity. The cells will then enter into stationary phase where the number of cells is constant, this is characteristic of a confluent population (where all growth surfaces are covered).

B. Harvesting. Cells are harvested when the cells have reached a population density which suppresses growth. Ideally, cells are harvested when they are in a semi-confluent state and are still in log phase. Cells that are not passaged and are allowed to grow to a confluent state can sometime lag for a long period of time and some may never recover. It is also essential to keep your cells as happy as possible to maximize the efficiency of transformation. Most cells are passaged (or at least fed) three times a week.

1. Suspension culture. Suspension cultures are fed by dilution into fresh medium.

2. Adherent cultures. Adherent cultures that do not need to be divided can simply be fed by removing the old medium and replacing it with fresh medium.

When the cells become semi-confluent, several methods are used to remove the cells from the growing surface so that they can be diluted:

- Mechanical - A rubber spatula can be used to physically remove the cells from the growth surface. This method is quick and easy but is also disruptive to the cells and may result in significant cell death. This method is best when harvesting many different samples of cells for preparing extracts, i.e., when viability is not important.
- Proteolytic enzymes - Trypsin, collagenase, or pronase, usually in combination with EDTA, causes cells to detach from the growth surface. This method is fast and reliable but can damage the cell surface by digesting exposed cell surface proteins. The proteolysis reaction can be quickly terminated by the addition of complete medium containing serum
- EDTA - EDTA alone can also be used to detach cells and seems to be gentler on the cells than trypsin. The standard procedure for detaching adherent cells is as follows:

1. Visually inspect daily
2. Release cells from monolayer surface
 - a. wash once with a buffer solution
 - b. treat with dissociating agent
 - c. observe cells under the microscope. Incubate until cells become rounded and loosen when flask is gently tapped with the side of the hand.
 - d. Transfer cells to a culture tube and dilute with medium containing serum.
 - e. Spin down cells, remove supernatant and replace with fresh medium.
 - f. Count the cells in a hemacytometer, and dilute as appropriate into fresh medium.

C. Media and growth requirements

1. Physiological parameters
 - A. temperature - 37C for cells from homeother
 - B. pH - 7.2-7.5 and osmolality of medium must be maintained
 - C. humidity is required
 - D. gas phase - bicarbonate conc. and CO₂ tension in equilibrium
 - E. visible light - can have an adverse effect on cells; light induced production of toxic compounds can occur in some media; cells should be cultured in the dark and exposed to room light as little as possible;
2. Medium requirements: (often empirical)
 - A. Bulk ions - Na, K, Ca, Mg, Cl, P, Bicarb or CO₂
 - B. Trace elements - iron, zinc, selenium
 - C. sugars - glucose is the most common
 - D. amino acids - 13 essential
 - E. vitamins - B, etc.
 - F. choline, inositol
 - G. serum - contains a large number of growth promoting activities such as buffering toxic nutrients by binding them, neutralizes trypsin and other proteases, has undefined effects on the interaction between cells and substrate, and contains peptide hormones or hormone-like growth factors that promote healthy growth.
 - H. antibiotics - although not required for cell growth, antibiotics are often used to control the growth of bacterial and fungal contaminants.
3. Feeding - 2-3 times/week.
4. Measurement of growth and viability. The viability of cells can be observed visually using an inverted phase contrast microscope. Live cells are phase bright; suspension cells are typically rounded and somewhat symmetrical; adherent cells will form projections when they attach to the growth surface. Viability can also be assessed using the vital dye, trypan blue, which is excluded by live cells but accumulates in dead cells. Cell numbers are determined using a hemocytometer.

V. SAFETY CONSIDERATIONS

Assume all cultures are hazardous since they may harbor latent viruses or other organisms that are uncharacterized. The following safety precautions should also be observed:

- pipetting: use pipette aids to prevent ingestion and keep aerosols down to a minimum
- no eating, drinking, or smoking
- wash hands after handling cultures and before leaving the lab
- decontaminate work surfaces with disinfectant (before and after)
- autoclave all waste
- use biological safety cabinet (laminar flow hood) when working with hazardous organisms. The cabinet protects worker by preventing airborne cells and viruses released during experimental activity from escaping the cabinet; there is an air barrier at the front opening and exhaust air is filtered with a HEPA filter make sure cabinet is not overloaded and leave

- exhaust grills in the front and the back clear (helps to maintain a uniform airflow)
- use aseptic technique
- dispose of all liquid waste after each experiment and treat with bleach

REFERENCES:

R. Ian Freshney, *Culture of Animal cells: A manual of basic techniques*, Wiley-Liss, 1987.

VI. TISSUE CULTURE PROCEDURES

Each student should maintain his own cells throughout the course of the experiment. These cells should be monitored daily for morphology and growth characteristics, fed every 2 to 3 days, and subcultured when necessary. A minimum of two 25 cm² flasks should be carried for each cell line; these cells should be expanded as necessary for the transfection experiments. Each time the cells are subcultured, a viable cell count should be done, the subculture dilutions should be noted, and, after several passages, a doubling time determined. As soon as you have enough cells, several vials should be frozen away and stored in liquid N₂. One vial from each freeze down should be thawed 1-2 weeks after freezing to check for viability. These frozen stocks will prove to be vital if any of your cultures become contaminated.

Procedures:

1. Media preparation. Each student will be responsible for maintaining his own stock of cell culture media; the particular type of media, the sera type and concentration, and other supplements will depend on the cell line. Do not share media with you partner (or anyone else) because if a culture or a bottle of media gets contaminated, you have no back-up. Most of the media components will be purchased prepared and sterile. In general, all you need to do is sterily combine several sterile solutions. To test for sterility after adding all components, pipet several mls from each media bottle into a small sterile petri dish or culture tube and incubate at 37EC for several days. Use only media that has been sterility tested. For this reason, you must anticipate your culture needs in advance so you can prepare the reagents necessary. But, please try not to waste media. Anticipate your needs but don't make more than you need. Tissue culture reagents are very expensive; for example, bovine fetal calf serum cost ~ \$200/500 ml. Some cell culture additives will be provided in a powdered form. These should be reconstituted to the appropriate concentration with double-distilled water (or medium, as appropriate) and filtered (in a sterile hood) through a 0-22 µm filter. All media preparation and other cell culture work must be performed in a laminar flow hood. Before beginning your work, turn on blower for several minutes, wipe down all surfaces with 70% ethanol, and ethanol wash your clean hands. Use only sterile pipets, disposable test tubes and autoclaved pipet tips for cell culture. All culture vessels, test tubes, pipet tip boxes, stocks of sterile eppendorfs, etc. should be opened only in the laminar flow hood. If something is opened elsewhere in the lab by accident, you can probably assume its contaminated. If something does become contaminated, immediately discard the contaminated materials into the biohazard container and notify the instructor.
2. Growth and morphology. Visually inspect cells frequently. Cell culture is sometimes more an art than a science. Get to know what makes your cells happy. Frequent feeding is important for maintaining the pH balance of the medium and for eliminating waste products. Cells do not typically like to be too confluent so they should be subcultured when they are in a semi-confluent state. In general, mammalian cells should be handled gently. They should not be vortexed, vigorously pipetted or centrifuged at greater than 1500 g.
3. Cell feeding. Use prewarmed media and have cells out of the incubator for as little time as possible. Use 10-15 ml for T-25's, 25-35 ml for T-75's and 50-60 ml for T-150's. a. Suspension cultures. Feeding and subculturing suspension cultures are done simultaneously. About every 2-3 days, dilute the cells into fresh media. The dilution you use will depend on the density of the cells and how quickly they divide, which only you can determine. Typically 1:4 to 1:20 dilutions are appropriate for

most cell lines. b. Adherent cells. About every 2-3 days, pour off old media from culture flasks and replace with fresh media. Subculture cells as described below before confluency is reached.

4. Subculturing adherent cells. When adherent cells become semi-confluent, subculture using 2 mM EDTA or trypsin/EDTA.

Trypsin-EDTA :

a. Remove medium from culture dish and wash cells in a balanced salt solution without Ca⁺⁺ or Mg⁺⁺. Remove the wash solution.

b. Add enough trypsin-EDTA solution to cover the bottom of the culture vessel and then pour off the excess.

c. Place culture in the 37°C incubator for 2 minutes.

d. Monitor cells under microscope. Cells are beginning to detach when they appear rounded.

e. As soon as cells are in suspension, immediately add culture medium containing serum. Wash cells once with serum containing medium and dilute as appropriate (generally 4-20 fold).

EDTA alone:

a. Prepare a 2 mM EDTA solution in a balanced salt solution (i.e., PBS without Ca⁺⁺ or Mg⁺⁺).

b. Remove medium from culture vessel by aspiration and wash the monolayer to remove all traces of serum. Remove salt solution by aspiration.

c. Dispense enough EDTA solution into culture vessels to completely cover the monolayer of cells.

d. The coated cells are allowed to incubate until cells detach from the surface. Progress can be checked by examination with an inverted microscope. Cells can be gently nudged by banging the side of the flask against the palm of the hand.

e. Dilute cells with fresh medium and transfer to a sterile centrifuge tube.

f. Spin cells down, remove supernatant, and resuspend in culture medium (or freezing medium if cells are to be frozen). Dilute as appropriate into culture flasks.

5. Thawing frozen cells.

a. Remove cells from frozen storage and quickly thaw in a 37°C waterbath by gently agitating vial.

b. As soon as the ice crystals melt, pipet gently into a culture flask containing prewarmed growth medium.

c. Log out cells in the "Liquid Nitrogen Freezer Log" Book.

6. Freezing cells.

a. Harvest cells as usual and wash once with complete medium.

b. Resuspend cells in complete medium and determine cell count/viability.

c. Centrifuge and resuspend in ice-cold freezing medium: 90% calf serum/10% DMSO, at 10⁶ - 10⁷ cells/ml. Keep cells on ice.

d. Transfer 1 ml aliquots to freezer vials on ice.

e. Place in a Mr. Frosty container that is at room temperature and that has sufficient isopropanol.

f. Place the Mr. Frosty in the -70°C freezer overnight. Note: Cells should be exposed to freezing medium for as little time as possible prior to freezing

g Next day, transfer to liquid nitrogen (DON'T FORGET) and log in the "Liquid Nitrogen Freezer Log" Book.

7. Viable cell counts. **USING A HEMOCYTOMETER TO DETERMINE TOTAL CELL COUNTS AND VIABLE CELL NUMBERS** (Reference: Sigma catalogue) Trypan blue is one of several stains recommended for use in dye exclusion procedures for viable cell counting. This method is based on the principle that live cells do not take up certain dyes, whereas dead cells do.

1. Prepare a cell suspension, either directly from a cell culture or from a concentrated or diluted suspension (depending on the cell density) and combine 20 µl of cells with 20 µl of trypan blue suspension (0.4%). Mix thoroughly and allow to stand for 5-15 minutes.

2. With the cover slip in place, transfer a small amount of trypan blue-cell suspension to both chambers of the hemocytometer by carefully touching the edge of the cover slip with the pipette tip and allowing each chamber to fill by capillary action. Do not overfill or underfill the chambers.3. Starting with 1 chamber of the hemocytometer, count all the cells in the 1 mm center square and four 1 mm corner square. Keep a separate count of viable and non-viable cells.4. If there are too many or too few cells to count, repeat the procedure either concentrating or diluting the original suspension as appropriate.5. The circle indicates the approximate area covered at 100X microscope magnification (10X ocular and 10X objective). Include cells on top and left touching middle line. Do not count cells touching middle line at bottom and right. Count 4 corner squares and middle square in both chambers and calculate the average.6. Each large square of the hemocytometer, with cover-slip in place, represents a total volume of 0.1 mm^3 or 10^{-4} cm^3 . Since 1 cm^3 is equivalent to approximately 1 ml, the total number of cells per ml will be determined using the following calculations: Cells/ml = average cell count per square x dilution factor x 10^4 ;
Total cells = cells/ml x the original volume of fluid from which the cell sample was removed; % Cell viability = total viable cells (unstained)/total cells x 100.

Common Stock Solutions

Prepare a 5x stock solution in 1 liter of H₂O:

54 g of Tris base

27.5 g of boric acid

20 ml of 0.5 M EDTA (pH 8.0)

The pH of the concentrated stock buffer should be approx. 8.3.. Some investigators prefer to use more concentrated stock solutions of TBE (10x as opposed to 5x). However, 5x stock solution is more stable because the solutes do not precipitate during storage. Passing the 5x or 10x buffer stocks through a 0.22 μm filter can prevent or delay formation of precipitates.

TE buffer:

10 mM Tris-Cl (pH, usually 7.6 or 8.0)

1 mM EDTA (pH 8.0)

Use concentrated stock solutions to prepare. If sterile water and sterile stocks are used, there is no need to autoclave. Otherwise, sterilize solutions by autoclaving for 20 minutes. Store the buffer at room temperature.

1 M Tris-Cl – used at various pHs

Using Tris base : To make 1 liter, dissolve 121 g Tris Base in 800 ml of water. Adjust pH to the desired value by adding approximately the following:

pH = 7.4 about 70 ml of concentrated HCl

pH = 7.6 about 60 ml of concentrated HCl

pH = 8.0 about 42 ml of concentrated HCl

Make sure solution is at room temperature before making final pH adjustments. Bring final volume to 1 liter. Sterilize by autoclaving.

Using Trizma tables: an alternate procedure for preparing Tris solutions is to combine the proper amount of Tris Base and Tris Hydrochloride to achieve the desired value using Sigma's Tris tables.

WB (10% redistilled glycerol, 90% distilled water, v/v)

In a 1-liter graduated cylinder, add 100 ml of glycerol and 900 ml of distilled water. Cover with parafilm and mix thoroughly. Sterilized by autoclaving, and chill to 4°C.

Western Blotting Solutions:

1X Transfer buffer 1: 25 mM Tris, 192 mM Glycine, pH 8.3

Mix 3.03 g Tris, 14.4 g glycine; add dd water to 1 liter – do not adjust pH.

1X Transfer buffer 2: 25 mM Tris, 192 mM Glycine, pH 8.3, 20 % methanol

Mix 3.03 g Tris, 14.4 g glycine; add 200 ml methanol; add dd water to 1 liter – do not adjust pH.

(NOTE: methanol is not needed for PVDF membranes)

10X Western Buffer: 200 mM Tris pH = 7.5; 1.5 M NaCl

To prepare 1X Western Buffer, dilute 10X buffer to 1X, adding Tween-20 to 0.1%. Remove 50 ml and set aside for the last two washes. To the remainder, add I-Block to 0.2%, heating gently with constant stirring until dissolved. Bring to room temperature before using.

X-gal 5-bromo-4-chloro-3-indolyl-b-D-galactoside (same recipe for **X-phosphate**)

Make a 2% (w/v) stock solution by dissolving X-gal in dimethylformamide at a concentration of 20 mg/ml solution. Use a glass or polypropylene tube. Wrap the tube containing the solution in aluminum foil to prevent damage by light and store at -20 ° C. It is not necessary to sterilize X-gal solutions.