

## Plant Biology (PLB) 161A. Plant Genetics and Biotechnology Laboratory (4) CRN# 86344 Fall 2003

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Lectures: Mon and Wed. 1:10-2:00 p.m., 109 Hutchison Hall  
Laboratory: Mon and Wed. 2:10-6:00 p.m., 109 Hutchison Hall  
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### Course Objective:

To provide experience in techniques used in genetic analysis at the molecular and organismal levels.

### Schedule of Lectures and Labs:

Wk.	D	No	Dt.	Topic
1	M	1	9/29	Lab safety, notebook, measurements, pipeting exercise.
	W	2	10/01	DNA restriction analysis, agarose gel electrophoresis (Dandekar).
2	M	3	10/06	Plasmid DNA isolation (Dandekar).
	W	4	10/08	Restriction mapping (Dandekar).
3	M	5	10/13	PCR, Cloning of DNA fragments and ligation (Dandekar).
	W	6	10/15	Transformation of E.coli and screening of transformants (Dandekar).
4	M	7	10/20	Basic bacterial culture techniques, growth, genetic selection, pure culture isolation and cryostorage. Inoculation broth and plates (Dandekar).
	W	8	10/22	Miniscreen of transformants and restriction analysis (Dandekar).
5	M	9	10/27	DNA sequencing, analysis of DNA sequences (Dandekar)
	W	9	10/29	Class number 10 Dandekar
6	M		11/03	No Class. Will be replaced by Thursday 11/13
	W	10a	11/05	Plant DNA isolation (Dubcovsky).
7	M	10b	11/10	Quantitative and qualitative analysis of DNA (Dubcovsky).
	W	11a	11/12	RFLPs: Electrophoresis of genomic DNA (Dubcovsky).
	T	11b	11/13	Southern Blots (Dubcovsky). Hours to be arranged
8	M	12a	11/17	RFLPs: Hybridization and autoradiography (Dubcovsky).
	W	12b	11/19	RFLPs: Autoradiography (Dubcovsky).
9	M	13	11/24	PCR markers. Microsatellites (Dubcovsky).
	W	14a	11/26	Genetic Maps. Concepts and Exercises (Dubcovsky).
10	M	14b	12/01	Construction of genetic maps using Mapmaker (Dubcovsky).
	W	14c	12/03	QTL Analysis and Mapmaker QTL (Dubcovsky).
11	M	--	12/08	Lab notebooks and take home exam.

**Laboratory Notebooks:**

All laboratory experiments should be filed neatly in a three ring binder. Start with a table of contents that includes the title of each experiment. Handouts for each experiment will be provided before each class. File each of these handouts and read them carefully before each class so that you can ask questions during the lecture period before the lab.

Format of each experiment: Each experiment must include the following sections; title, experimental objectives, materials and stocks, procedures, results, discussion/conclusions.

The first sections, i.e., title, experimental objectives, materials and stocks and procedures should be written up and included in your notebook before coming to class. All data, results, graphs and photographs should be taped directly in the notebook. Draw an outline for each experiment to reflect the way it was performed in class. The results, discussion, and conclusion should be written in a timely manner and giving time for thought and reflection. The discussion/conclusions is the most important: spend time reflecting on the problems encountered or anomalous data, giving explanations, making observations, and drawing conclusions. Please note that achieving the expected experimental results will not determine notebook grading. Rather, a thorough interpretation of your individual results will be the most important aspect of the notebooks. What do the results mean in the broader experimental context? Propose a hypothesis to explain your results (especially anomalous results). How could you test the hypothesis?

Laboratory notebooks and reports will constitute the major portion of your final grade in the class. Lab reports are due each Monday for the previous weeks labs.

**Grading:**

Final grades will be determined as follows:

Laboratory notebook	70%
Final Exam	30%

## Safety

Safety is an individual responsibility, and you must ensure that you are aware of the safety requirements before proceeding with the molecular biology and genetic experiments. The following is included merely as a guide:

The major hazards encountered in molecular biology research are:

- microorganisms
- radiochemicals
- organic solvents
- mutagens/carcinogens
- toxic chemicals
- ultraviolet radiation
- high voltage electricity

## Genetic manipulation

Genetic manipulation experiments must be carried out in accordance with guidelines laid down by National Institutes of Health, and our local Environmental Health and Safety on our campus. A copy of these documents will be available in the lab. You must understand and comply with these guidelines before starting any experiments involving recombinant DNA.

## Microorganisms

Most molecular biology experiments are carried out with attenuated strains of *E. coli* that are unable to survive outside of the test-tube. Nevertheless, precautions to avoid contamination, as described in a later section of this handout, should be observed whenever bacteria are being handled.

## Radiochemicals

Although  $^{32}\text{P}$  and  $^{35}\text{S}$  are not the most dangerous radionuclides in biological research they are used in large amounts in molecular biology experiments. We will be using  $^{33}\text{P}$ , a safer alternative to  $^{32}\text{P}$ . Our national (NRC) and local (EH&S) regulations for handling and disposal are to be followed at all times. Some molecular biology techniques that involve radiochemicals are messy. Before running a polyacrylamide gel with labeled DNA check that you can dispose of the (highly radioactive) contents of the lower reservoir without spillage. Before you carry out your first hybridization experiment make sure you can seal and open plastic bags without causing a mess.

## Organic solvents

The most dangerous solvent used in molecular biology is phenol. Always wear gloves when you are carrying out phenol extractions and make sure the gloves do actually provide a barrier to phenol: some lightweight disposable gloves do not

## **Mutagens/carcinogens**

Most chemicals that bind to DNA in the test-tube are mutagenic and/or carcinogenic. An example is ethidium bromide, when it is used in large amounts to detect DNA in agarose gels. Some manuals recommend adding ethidium bromide to the running buffer, but in general terms this should be avoided. It does not provide any major advantage over post-staining (unless you want to follow the electrophoresis) and creates a major health hazard. Our local regulations do not allow ethidium bromide solutions to be disposed of down a sink. We have to follow a decontamination procedure. Remember that gels stained with ethidium bromide are dangerous and should not be disposed with the general lab waste. Always wear gloves when handling ethidium bromide stained gels.

## **Toxic chemicals**

Several toxic chemicals are used in molecular biology. The most dangerous is probably acrylamide because of the possibility of creating aerial dust contamination when handling the powder. avoid weighing out acrylamide if possible and handle the powder in a fume hood. We will be using pre-made solutions.

## **Ultraviolet radiation**

The most dangerous piece of equipment used by molecular biologists is the UV transilluminator. Standard laboratory models have recommended exposure levels of 4.5 sec or less at 12 inches. Always use a suitable screen when observing agarose gels and wear face and eye protection when the screen is removed for photography. Be particularly careful that your wrists and neck are protected when you are excising bands from gel.

## **High-voltage electricity**

The voltages used in electrophoresis can kill. Commercial gel apparatus is designed so that lids cannot be removed before the power supply is disconnected. Homemade equipment should incorporate similar safeguards. Broken gel tanks should not be used. Remember that liquids can conduct electricity, and that leaky apparatus is potentially lethal.

## **Basic microbiological techniques**

Recombinant DNA technology depends on the manipulation of particular strains of bacteria and bacteriophages (phages). Expertise in isolating, checking, growing, and analyzing these strains is crucial for the success of genetic engineering. Many experiments including those that we will conduct during this lab course are now permitted under conditions of 'good microbiological technique' and anybody embarking on research in this area should check exactly what is meant by this phrase. Essentially, all of the basic techniques depend on culturing a particular microbe in the absence of other organisms (sterile or aseptic technique), the isolation of a genetically-pure culture or clone derived from a single cell (single colony or single plaque isolation) and the characterization of the known genetic features of the strain. From a safety point of view, experiments are done in such a way that recombinant microbes do not escape into the environment or infect the experimenter or other people. The basis of sterile technique is the efficient sterilization of all equipment and growth media followed by the protection of the media from contamination during manipulations. If contamination of a culture is suspected then colony morphology, smell of the culture or staining of the cells followed by microscopic examination can be useful. Growth of the strains in liquid or solid media allows larger numbers of cells to be cultured and it is important to understand the various phases of the growth curve as different types of cells are required for different experiments. Plating techniques such

as dilution and streak plates can give rise to single colonies or plaques derived from single-cell or phage particles. Selective techniques are often used to isolate particular strains or to detect rare events, for example in transformation, and such techniques are very sensitive to low levels of contamination by unrelated strains which happen to have the same characteristics as the desired strain. This may be resistance to antibiotics or nutritional independence.

## Basic techniques of microbial genetics

Genetic purity of strains, plasmids or bacteriophages is critical and it should be clearly understood that no strains can ever be completely stable. Growth can lead to rearrangements of DNA, mutation of essential genes, or infection by foreign DNA. Strains should therefore be carefully checked to make sure that they are of the correct genotype before starting crucial experiments. Chemicals and enzymes are not self-replicating and they are therefore usually purchased from suppliers. In contrast strains of bacteria and phages are frequently subcultured many times and passed from one research group to another. Quality control and frequent checking is necessary or significant alternations in the genome of the organism may occur. It is particularly important to make a careful note of any deliberate changes or improvements in particular strains. This means recording the pedigree of the strains and using a unique numbering system and detailing the full genotype. The dangers here are that essential gene mutations such as *recA* (deficient for the major recombination system) may have reverted to wild-type and transposons may have caused rearrangements. An empirical approach can be adopted such that if the technique works the strain must be correct, but this can delay the problem until a later stage. Unless you are absolutely sure of the origin of the strain it is much safer to obtain the correct culture from a reputable supplier. When the correct strain has been successfully characterized it is essential that it is preserved efficiently. Any growth can lead to mutation and any desiccation or contamination can lead to death of the culture. The basis of preservation is to stop growth by lyophilization or freezing in the presence of cryoprotectants such as glycerol. Duplicate cultures should be kept in different locations to avoid dangers of apparatus failure.

## Safety in the molecular biology laboratory

Although *Escherichia coli* K12 is not considered to be pathogenic, there are other strains of *E. coli* which are dangerous and can cause septicaemia or kidney infections. The development of disease symptoms depends on the type and number of organisms which gain access to the body as well as on the efficiency of the immune system of the laboratory worker. Most healthy people can cope with small numbers of microbes but depression of the immune system caused by illness or infection or the use of immunosuppressive drugs can markedly increase the risk of a serious infection. It is safer to assume that all microbes are potential pathogens and to treat them with respect. Some basic precautions are given below.

- (a) Laboratory overalls, which should ideally be side- or back-fastening, must be worn when using radioactive chemicals.
- (b) Avoid all hand-to-mouth operations such as licking pencils or labels.
- (c) Make a habit of washing your hands before leaving the laboratory.
- (d) Before starting to work, protect all cuts with adequate waterproof dressings.
- (e) Culture split on the bench, floor, apparatus or on yourself or others, should be treated with disinfectant. Material used to wipe up should be discarded for incineration or sterilization.
- (f) Do not eat, drink, smoke, or apply cosmetics in the laboratory.
- (g) All contaminated apparatus should be sterilized before washing or disposal.
- (h) All contaminated glassware such as pipettes and tubes should be discarded into disinfectant prior to sterilization and washing.
- (i) All contaminated disposable plasticware should be discarded into strong autoclavable bags for sterilization or incineration.

- (j) All apparatus for autoclaving or incineration should be carried in leak-proof containers.
- (k) All apparatus used for the culture of microbes should be clearly labeled before inoculation. Apparatus to be left in communal areas should show your name, the organism, and the date.
- (l) All contaminated syringe needles should be discarded into special 'sharps' containers for special sterilization and disposal.
- (m) Every effort should be made to avoid the production of aerosols. These are produced, for example, by blenders, centrifugation, ultrasonication, and movement of liquids against surfaces. If the microbes present a hazard then the equipment used should be placed in a suitable safety cabinet.
- (n) Records should be kept of the storage and transport of all microbes.

Essentially, exposure to microbes by inhalation, ingestion, absorption through the skin or mucous membranes should be treated seriously and precautions taken to limit the access of microbes to the environment, laboratory workers, and the public. Safety is not the blind following of regulations but an awareness of the hazards and the methods that can be used to minimize them.

## **Sterilization and disinfection**

It is important to distinguish between these two processes. The aim of sterilization is to eliminate all microbes from laboratory equipment or materials. Disinfection aims to eliminate organisms which may cause infection. Space does not permit a theoretical treatment of this subject but the important practical principles will be given.

Sterilization can be achieved by heat, chemicals, radiation, or by filtration. Nichrome loops are sterilized by flaming in a bunsen burner, and disposable plastics can be sterilized by incineration. Glassware can be sterilized either by autoclaving or by dry heat. Autoclaving which uses wet heat is much more efficient than dry heat as hydrated microbes are killed more easily. Autoclaves vary from domestic pressure cookers to large, industrial-size motorclaves. It is very important that you follow the operating instructions and in particular do not overload the autoclave, as the central region may not reach the necessary temperature. It is important to remove all of the air from the autoclave because the presence of air depresses the final temperature reached. Autoclave tape which changes color after the correct time and temperature is a useful check. Loosen the caps of all bottles and do not autoclave completely sealed bags in small autoclaves. Always make sure that there is sufficient water in pressure cookers and check that the correct procedure for autoclaving and recovery of materials is followed. Autoclavable plastic tubes such as pipette tips and microfuge tubes should be wrapped in autoclavable nylon bags. Dry-heat sterilization is normally used for flasks and glass pipettes, which should be left on a 6-12 h cycle at 160°C. Sterilization with chemicals and radiation are not practical methods in the average laboratory. Sterile plastic ware has normally been produced by gamma irradiation.

Disinfection procedures will vary from laboratory to laboratory but a general-purpose disinfectant is 2% (v/v) Hycolin which turns green to blue when no longer effective. Chlorine-based disinfectants such as Chlorox are more effective against spores and viruses but have the disadvantage that they need to be checked more frequently. Any contaminated glassware or unwanted culture should be immersed in disinfectant before autoclaving and washing. Contaminated disposal plastic ware should be placed in autoclavable bags or bowls, which must be leak-proof. This material should be disposed of by incineration.

## Basic principles of aseptic technique

There are two basic principles of aseptic technique:

- protection of yourself and of others.
- protection of cultures and apparatus from contamination by unwanted microbes.

In normal laboratory areas, microbes are everywhere in the air, in dust, on your fingers. It is in fact very difficult to produce an environment completely free from microbes, and special equipment such as laminar-flow cabinets and sterile areas are required. In a clean laboratory, with reasonable precautions, it is not necessary to use inoculating cabinets for the preparation of media and the manipulation of cultures.

Unless laboratory air has been efficiently filtered, it will contain many suspended bacterial cells and spores, fungal spores, and, in some laboratories, air-stable phage particles. This population of particles is added to by air movements which resuspend dust particles from bench surfaces. These airborne particles will settle on to any exposed surface and this is a major source of contamination, therefore anything which is to be kept sterile must be exposed to the air for a minimum period of time. Dust and aerosol particles tend to settle rather than drift sideways unless there is a draught of air. Consequently, containers and Petri dishes should not be left open, surface upwards, although tubes opened at an angle are less at risk. All apparatus which cannot be flamed in a bunsen burner immediately prior to use should be left in the wrappers or containers in which they have been sterilized, until actually needed. No sterile equipment should be allowed to come into contact with unsterile surface. Plugs and caps from sterile tubes and bottles should not be placed on the bench, although they can be placed on a tile swabbed with a disinfectant.

The commonest source of contamination in the laboratory is the access of unsterile air to the apparatus. This is increased by draughts and general movement of air and it follows that every effort should be made to work in still-air conditions. Windows and doors should be closed and all rapid movements in the laboratory should be eliminated. It is obvious that the laboratory should be free from dust which could be resuspended by air movement but it should be remembered that cleaning techniques such as sweeping and dusting can be a serious source of aerial contamination. The major advantage of inoculating cabinets is that they give protection from these air movements and allow a small volume of air to be sterilized by UV radiation or by a 70% (v/v) alcohol spray. The principles of spraying the air in a cabinet or over a bench is that it settles dust particles onto the bench, from where they can be removed with a paper towel. Fungal spores are adapted to aerial transmission and particular care should be taken when handling fungi or disposing of apparatus contaminated by fungi. Fungal spores released into the environment can take up to 7 h to settle and therefore can be a source of contamination for a considerable period.

Skin, hair, breath, and clothing are all sources of microbes and it is particularly important that you do not touch sterile surfaces such as the tips of pipettes and the inside of containers. Do not bend over your equipment such that skin scales or dust from your hair might fall into your cultures. Problems have arisen from contamination with yeasts traced to home baking of bread. Where strict asepsis is required (as in operating theatres) sterile caps, gloves, and gowns are worn.