APPENDIX

APPENDIX I : Chemical and reagent preparation

1. Agarose gel loading buffer:

   - Bromophenolblue 0.25% (0.0625 g.)
   - Xylencyanol 0.24% (0.0625 g.)
   - glycerol 30% (7.5 ml.)

   made up to 25 ml with water

2. APS:

   - APS 1 g.
   - H_2O 10 ml.

3. ITPG (0.5 M):

   - 1.2 g ITPG
   - H_2O 10 ml.

4. LB (Luria-Bertaini) broth, (800 ml.):

   - 8.0 g. NaCl (ROTH)
   - 8.0 g. Pepton (ROTH)
   - 4.0 g. Hefer extract (ROTH)

- 480.0 µl. 1N NaOH (ROTH)

LB plate : add 12 g. Agar-agar (ROTH)

5. 1xTE buffer:

- 1 M. tris-HCl pH8.0 10 ml. (10 mMole)
- 0.5 M. EDTA pH8.0 2 ml. (1 mMole)

made up to volume 1,000 ml.

6. 50xTAE buffer:

- 242 g. Tris Base
- 57.1 ml. conc.Acetic acid
- 0.5 M. EDTA pH8.0 100 ml.

made up to volume 1,000 ml.

APPENDIX II : PRELAB NOTES (Bloom *et al.*, 1996)

1. Nutrient Agar

Almost any rich nutrient agar can be used for plating cells, although there are prefer LB (Luria-Bertani) agar. Presterilized, ready-to-pour agar is a great convenience. It only needs to be melted in a microwave oven or boiling water bath, cooled to approximately 60°C, and poured onto sterile culture plates. To prevent boiling over, the agar container should be no more than half full. Loosen the cap to prevent bottle from exploding.

2. Ampicillin

Plasmids having ampicillin resistance are most commonly used for cloning DNA sequences in *E. coli*. Ampicillin is very stable in agar plates, thresholds for selection are relatively broad, and contaminants are infrequent. Despite its stability, ampicillin, like most antibiotics, is inactivated by prolonged heating. Therefore, it is important to allow the agar solution to cool until the container can be held comfortably in the hand (about 60°C) before adding the antibiotics. Use sodium salt, which is very soluble in water, instead of free acid form, which is difficult to dissolve.

3. *Escherichia coli.*

E. coli has simple nutritional requirements and grows slowly on a minimal medium containing an energy source such as glucose, salts such as NaCl and $MgCl_2$, the vitamin biotin, and the nucleoside thymidine. *E. coli* synthesizes all necessary vitamins and amino acids from these precursors. It grows rapidly in a complete medium, such as LB, in which yeast extract and hydrolyzed milk protein (casein) provide a ready supply of vitamins and amino acids.

A liquid bacterial culture goes through a series of growth phases. For approximately 30 minutes following inoculation, there is a *lag phase* during which there is no cell growth. The bacteria begin dividing rapidly during *log phase*, when the number of cells double every 20-25 minutes. As nutrients in the media are depleted, cells stop dividing and enter the *stationary phase*, with

a concentration of approximately 10^9 cells/ml. During the *death phase*, waste products accumulate and the cells begin to die.

Optimum growth in liquid culture is achieved with continuous agitation, which aerates the cells, facilitates the exchange of nutrients, and flushes away waste products of metabolism. It can safely be assumed that a culture in complete medium has reached stationary phase, following overnight incubation with continuous shaking.

A culture in the stationary phase will look very cloudy and turbid. Discard any overnight culture where vigorous growth is not evident. Expect less growth in cultures incubated for several days *without continuous shaking*. To gauge growth, shake the tube to suspend the cells that have settled at the bottom of the tube.

4. Storing and Handling Restriction Enzymes

Restriction enzymes, like many enzymes, are most stable at cold temperatures, and lose activity if they are warmed for any length of time. Because maintaining these enzymes in good condition is critical to the success of the experiments in this course, follow the guidelines below for handling them.

1. Always store enzymes in a *non-frost-free* freezer that maintains a constant temperature of - 10° to - 20°C. *Non-frost-free* freezers typically develop a layer of frost around the chamber, which acts as an efficient insulator and helps maintain a constant temperature. Frost-free freezers, on the other hand, go through freeze-thaw cycles that would subject enzymes to repeated warming

and subsequent loss of enzymatic activity. If a frost-free freezer must be used, store the enzymes in their Styrofoam shipping container within the freezer. The container will help to maintain a constant temperature during the thaw cycle.

2. Remove restriction enzymes from the freezer directly onto crushed or cracked ice in an insulated ice bucket or cooler. Make certain that the tubes are pushed down into the ice and not just sitting on top of it. Keep the enzymes on ice at all times during handling, and return them to the freezer immediately after use.

3. When a large shipment of an enzyme is received, split it into several smaller aliquots of 50-100 µl in 1.5-ml tubes. Use a permanent marker on tape to clearly identify a aliquots by enzyme type, concentration in units/µl, and date received. Use up one aliquot before starting another.

4. Keep aliquot of enzymes, buffer, and DNA in a cooler filled with ice before lab and also for dispensing; unused aliquots will remain fresh.

5. Although it is good technique to set up restriction digests on ice, it is much simpler to set up reaction in a test tube rack at room temperature. Little loss of enzyme activity will occur during the brief time needed to set up the reaction.

6. Storing DNA and Restriction Buffer

Purified DNA is generally stored in the refrigerator (at approximately 4°C). DNA can be kept in the freezer (at approximately - 20°C) for longterm storage of several months or longer. However, it is not advisable to store DNA in the freezer during times of active use: Ice crystals formed during repeated

freezing will nick and shear DNA over time. Freeze damage is especially relevant to plasmid DNA used for transformations; nicked or linearized plasmid does not transform as well as the supercoiled form. Restriction buffer is best stored frozen and is not affected by freeze-thaw cycles.

7. Buffers

Many types of buffers are used in this course: restriction buffer, ligation buffer, PCR buffer, electrophoresis buffer, hybridization buffer, and various wash buffers. Each has a different chemical composition and use. Always double-check to ensure that you are using the proper buffer.

Tris-Borate-EDTA (TBE) electrophoresis buffer can be reused several times. Collect the used buffer, and store it in a large carboy. If different gels will be run over a period of several days, store the buffer in an electrophoresis chamber with the cover in place to retard evaporation. Before reusing buffer that has been stored in an electrophoresis chamber, rock the chamber back and forth to mix the buffer at either end. This reequilibrates ions that accumulate at either end during electrophoresis.

Groups of restriction enzymes operate under various salt and pH conditions. For optimal activity, several different buffers would be needed for the enzymes used in this course. Wherever possible, we use a "compromise" restriction buffer-a universal buffer that is a compromise betweem the conditions preferred by various enzymes.

All buffers are used at a final working concentration of 1×. Rely on the standard $C_1 V_1 = C_2 V_2$ formular to determine how much buffer to add to obtain a 1 × solution:

(vol. Buffer)	**(conc. of buffer)=(total vol. Of reaction)**	**(IX buffer)**
(1 µl)	(10 × buffer) = 10 µl	(1×)
(5 µl)	(2× buffer) = 10 µl	(1×)

For convenience, we use 2× restriction buffer whenever possible in 10 µl reactions: It saves a pipetting step to add water to bring the reaction up to 10 µl total volume. It is also easier and more accurate to pipet 5 µl than to pipet 1 µl. Compare a typical restriction reaction using 2× versus 10× restriction buffer:

	2×Buffer	**10×Buffer**
DNA	4 µl	4 µl
Enzyme	1 µl	1 µl
Buffer	5 µl	1 µl
Water	-	4 µl
Total solution	10 µl	10 µl

7.1 Suggested agarose concentrations. (SeaKem^{R} LE agarose., 1998)

Size Range (base pairs)	Final agarose concentration (%)	
	1x TAE buffer	1x TBE buffer
1,000-23,000	0.60	0.50
800-10,000	0.80	0.70
400-8,000	1.00	0.85
300-7,000	1.20	1.00
200-4,000	1.50	1.25
100-3,000	2.00	1.75

8. Diluting DNA

DNA for near-term use can be diluted with distilled or deionized water. However, it should be diluted with Tris-EDTA (TE) buffer for long-term storage. EDTA in the buffer binds divalent cations, such as Mg^{++}, that are necessary cofactors for DNA-degrading nucleases. Always dilute DNA to the concentration specified by the protocol.

1. Determine the total volume of DNA required by multiplying the number of experiments times the total volume of DNA per experiment, including overage.

(10 experiments) (20 µl DNA) = 200 µl DNA

2. Plug this number into the $C_1 V_1 = C_2 V_2$ formula, along with the desired final DNA concentration and the concentration of the stock DNA. Solve for V_1, the volume of stock DNA needed in the dilution.

(C_1 stock DNA) (V_1) = (C_2 final DNA) (V_2 total volume)

(0.5 µg/µl) (V_1) = (0.1 µg/µl) (200 µl)

$$(V_1) = \frac{(0.1\,\mu g / \mu l)(200\,\mu l)}{(0.5\,\mu g / \mu l)} = 40\ \mu l \text{ stock DNA}$$

3. Add water or TE to make the total volume of final solution. 40 µl stock DNA + 160 µl H_2O or TE = 200 µl final solution

9. Pooling Reagents

Reagent aliquots often become spread in a film around the sides or caps of 1.5-ml tubes, during aliquoting and moving to and from the freezer or refrigerator and ice bucket. Use one of the following methods to pool reagent droplets to make them easier to find in the tube.

1. Spin the tubes briefly in a microfuge.
2. Spin the tubes briefly in a preparatory centrifuge, using adapter collars for 1.5-ml tubes. Alternatively, spin the tubes within a 15-ml tube, and remove them carefully.
3. Tap the tubes sharply on the bench top.

10. Electrophoresing

Hydrogen gas bubbling off of the negative electrode and oxygen gas rising from the positive electrode (products of electrolysis of water) are the first signs that current is flowing through the electrophoresis system. Shortly after, bands of loading dye should be seen moving into the gel and migrating toward the positive pole of the apparatus. The loading dye band quickly resolves into two bands of color: The faster-moving, purplish band is bromophenol blue, and the slower-moving, aqua band is xylene cyanol. Bromophenol blue, and the slower-moving, aqua band is xylene cyanol. Bromophenol blue migrates through a 0.8% gel at the same rate as a DNA fragment of approximately 300 base pairs (bp). Xylene cyanol migrates at a rate approximately equivalent to 9,000 base pairs. The best separation for analysis of λ and plasmid DNA is achieved when the bromophenol blue migrates 40-70 mm from the origin.

The migration of DNA through an agarose gel depends upon voltage: The higher the voltage, the faster the rate of migration. However, higher voltages accentuate imperfections in the gel—such as differences in density and thickness from one part of the gel to another. Common effects include slanted and U-shaped bands ("smiles"). Slanted effects most typically occur at the edges of the gel, where the liquid gel adheres to the casting tray to form a miniscus, making the edges of the solidified gel thicker than the center. Resistance is decreased in the thicker edges of the outermost lanes, thus allowing DNA molecules toward the outer edge of the outermost lanes, thus allowing DNA molecules toward the outer edge of the band to migrate faster

than like-sized molecules toward the inner edge of the band. Also, heat generated at high voltages can begin to melt the gel and change its sieving properties. For these reasons, avoid using more than 125 volts in a minigel system.

APPENDIX III : Caution

1. EtBr (Ethidium Bromide)

EtBr is mutagen by the Ames microsome assay and a suspected carcinogen. Because mixing the 5 mg./ml. Solution from the concentrated powder poses the greatest hazard, there are recommend obtaining ready-mixed 5 mmg./ml. solution from a supplier. If you choose to mix ethidium bromide solution, handle the powder carefully to avoid creating dust. Wear rubber gloves and a mask that covers your nose and mouth.

2. Phenol

Phenol is corrosive and can causes serve burns. Be sure to handle phenol under a chemical hood, wear protective clothing, gloves, and safety glasses. Rinse with a large volume of water any area of skin that comes into contact with phenol. Wash with soap and water. Do not use ethanol.

3. Ultraviolet light

Ultraviolet light can damage the retina of the eye. Never look directly at an unshieldded UV-light source without eye protection. View only though a filter or safety glasses that absorb the harmful wavelengths.

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