

THE
UNIVERSITY OF
LETHBRIDGE

BIOLOGY 4200
TECHNIQUES IN MOLECULAR BIOLOGY

LABORATORY EXERCISES

2006

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Quintin Steynen

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Course Outline
BIOLOGY 4200/5200 - TECHNIQUES IN MOLECULAR BIOLOGY
Fall, 2006

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Office Hours: Monday & Tuesday 11:00 to 12:00
or by appointment

Lectures/ Laboratories: Wednesdays and Fridays 1:00 p.m. - 3:50 p.m. C740.

Lab Book: Available from the course website

Grading:

Assignments	**20%**
Outline for Grant Proposal – Due September 27, 2007 prior to 10:00 AM	**5%**
Laboratory Report 1-Due November 1, 2006 prior to 10:00 AM	**15%**
Grant Proposal – Due November 22, 2006 prior to 10:00AM	**30%**
Laboratory Report 2 – Due December 8, 2006 prior to 4:00 PM	**20%**
Participation*	**10%**

*The Participation mark will be based upon your being prepared for lab, making an effort to help out, not leaving the lab for extended periods of time, participating in class discussions, etc. Failure to participate in any out-of-lab setup will result in a deduction of this 10% from your overall grade.

- **Late written work (laboratory reports, assignments, grant proposal outlines and grant proposals) must still be completed to my satisfaction and handed in within 2 weeks of the scheduled due date but will receive a grade of 0. Failure to hand in any of the aforementioned components will result in a grade of F being assigned for the course.**
- **Late materials will not be accepted. Extensions will only be considered if a written application to the Instructor including work completed on the assignment to date is made a minimum of 2 days prior to the due date.**
- **Plagiarism will result in a grade of F in the course and a letter placed in the student's file in the Registrar's office. Please consult the University of Lethbridge Calendar (2006/2007) pages 70-71.**

Student Expectations:

- Students are expected to have completed all of the prerequisites. To this end, a prerequisite check is automatically performed at the beginning of the course. If you have achieved less than a grade of **C+** in the prerequisites, you should be prepared to invest additional time in learning the related material.

- Attendance at all laboratories is mandatory. If you miss a laboratory, it is your responsibility to make arrangements with the instructor to perform a make-up laboratory that may or may not be the same as the exercise missed. Note that this opportunity is dependent upon the instructor's schedule. If you fail to schedule and complete a make-up laboratory, you will lose 5% of your final grade for each lab missed. This is distinct from the laboratory participation grade.
- Unfortunately, absences of greater than 2 laboratories can not be accommodated; consequently, students who miss more than 2 laboratories will need to see a Student Advisor for options regarding deferring the course until the following Fall semester.
- For certain labs, students are expected to set up experiments outside of regularly scheduled lab time. Effort and participation in these out-of-lab activities as well as in laboratory discussions of results and papers, and in-class participation form the basis of the laboratory participation mark.
- Students are expected to have read the laboratories prior to coming to the labs themselves. In addition, most labs have associated background readings and/or appendices that the students should review and understand prior to coming to the lab. Such preparation is also considered to be part of the laboratory participation grade.

Concept List – As background preparation for the course you should ensure that you can define/explain each of the following concepts:

- Gene structure – what is a gene? What are parts of a prokaryotic gene?
- Transcription and translation in prokaryotes
- DNA replication in prokaryotes
- The lac operon
- Bacterial growth; nutritional requirements; techniques for isolation and cultivation of bacteria
- Recombination
- Transformation
- Type II Restriction endonucleases – mechanism of action, formation of sticky or blunt ends
- Plasmid
- Expression vectors
- SDS PAGE
- Agarose gel electrophoresis
- Cloning
- PCR

Tentative Schedule

Project I	
Sept. 6 (Wed)	Introduction and Project I Overview Lab 1: Solution preparation
Sept. 8 (Fri)	Lab 2: DNA isolation (start) Lab 3: Agarose gel preparation Grant proposals
Sept. 13 (Wed)	Lab 2: DNA isolation (complete)
Sept. 15 (Fri) Morning	Gel Electrophoresis of isolated DNA
Sept. 15 (Fri)	Lab 3: Extract DNA from gel and column purify Check DNA purity and quantify (start)
Sept. 20 (Wed)	Lab 3: Check DNA purity and quantify (complete) Lab 4: PCR setup Agarose gel preparation Lecture: Cloning of PCR products
Sept. 22 (Fri)	Lab 4: Gel Electrophoresis of PCR Products Lab 5: Extract/purify PCR products from gel
Sept. 27 (Wed)	Quantify purified PCR Product (gel and/or UV) Ligation of product into a vector **Grant Proposal Outline Due (10am)**
Sept. 28 (Thu)	Lab 6: Media preparation
Sept. 29 (Fri)	Lab 6: Competent cell preparation (DH5α) Pour media for transformation Lecture: Plasmids as cloning vectors
Oct. 4 (Wed)	Lab 6: Transformation of ligation products Spread plating of potential transformants
Oct. 5 (Thu)	Lab 7: Replica plating for Eckhardt gel electrophoresis
Oct. 6 (Fri)	Lab 7: Screen plasmids – Eckhardt gel electrophoresis Lecture: Expression vectors
Oct. 10 (Tue) Afternoon	Lab 8: Setup liquid o/n cultures
Oct. 11 (Wed)	Lab 8: Alkaline lysis prep of plasmids with column clean-up (start)
Oct. 13 (Fri)	Lab 8: Alkaline lysis prep of plasmids with column clean-up (complete) Check DNA purity and quantify (gel & spec) Note: DNA will be sent to U of C for sequencing Lecture: Sequence analysis
<u>Project II</u>	
Oct. 18 (Wed)	Project II Overview Primer design for *rnb* deletion mutations Lecture: Primer design
Oct. 20 (Fri)	Primer design (con't)
Oct. 25 (Wed)	Boiling lysis plasmid prep on p*rnb*::Teasy R.E. digest of *rnb* clone and expression vector
Oct. 27 (Fri) Morning	Gel Electrophoresis of digestion products

Oct. 27 (Fri)	Extract digestion products from agarose gel
Nov. 1 (Wed)	Quantify DNA (gel) Setup ligation reactions **Lab Report #1 Due (10am)**
Nov. 3 (Fri)	Competent cell prep (BL21) Transform DH5α with ligation products
Nov. 7 (Tue) Afternoon	Inoculate liquid cultures from transformants
Nov. 8 (Wed)	Plasmid preparation Setup digests for restriction analysis
Nov. 10 (Fri)	Gel electrophoresis of digests Transformation of BL21 with plasmids of interest
Nov. 15 (Wed) Morning	Inoculate liquid cultures for expression testing
Nov. 15 (Wed)	Small scale expression testing (SDS-PAGE)
Nov. 17 (Fri)	Setup ATW PCR using islolated plasmids
Nov. 22 (Wed) Morning	Gel electrophoresis of PCR products
Nov. 22 (Wed)	Agarose gel analysis Adjust PCR conditions and re-run (if needed) **Grant Proposal Due (10am)**
Nov. 24 (Fri)	Gel purify/extract PCR products (if req'd) Setup intramolecular ligation
Nov. 29 (Wed)	Transformation of BL21 with ligation mixture
Dec. 1 (Fri) Morning	Inoculate liquid cultures from transformants
Dec. 1 (Fri)	Induction of liquid cultures Preparation of cultures for expression testing
Dec. 6 (Wed)	Small scale expression testing (SDS-PAGE)
Dec. 8 (Fri)	**Lab Report #2 Due (4pm)**

LABORATORY 1

Objective: To prepare solutions, gain experience in use of the pH meter, and review handling of microorganisms.

Background Reading: Appendices 1, 2, 3, 5, and 6

Supplies and Equipment

2x balances	Weigh boats
2x pH meters	Scoops
Tris	Disodium salt of EDTA
NaOH pellets	5M NaCl solution
Nanopure H_2O in large carboy	5x Graduated cylinders
Labeling tape	Permanent markers
2x waste beakers for the pH meters	Glucose
Propipettors	16x 100 mL beakers
16x bottles (holding 50 mL)	Stir bars
Stirring plates	Kimwipes
Autoclave tape	Microbiology kits
CTAB	Concentrated HCl (in the fume hood)
Gloves	Safety goggles
Pasteur pipettes, bulbs	10 mL disposable pipettes
2x squeeze bottles containing d_2H_2O	

A PREPARATION OF SOLUTIONS
Please see Appendix 9 for recipes.

General Tips:

- Use a fresh weigh boat for each chemical. Don't throw them out! At the end of the lab, rinse out your weigh boats and prop up so that they dry.
- Rinse out and dry the scoops in between chemicals and reuse.
- Use Nanopure water for making up solutions. Use Optima-water for resuspending DNA. Prior to using the Optima-water, use aseptic technique (hint, Bunsen burners are involved) to aliquot out 1 mL portions of Optima-water into sterilised microfuge tubes).
- When adjusting pH, always add acid to water!
- For autoclaving anything, high pressure is involved so always leave your lids loose.

Procedure:
Note - Each group should make up solutions according to Table 1. There are only

supplies for each group to prepare one of each solution. If you make a mistake in preparation, the Instructor will supply you with solutions (as you may suspect, there is an associated participation mark penalty!).

Table 1. Types of solutions, concentrations, and volumes for each group to prepare.

Solutions to Prepare	**Volumes (in mL)**
500 mM Tris pH 8	50
500 mL EDTA pH 8	50
Lysis Buffer	50
Solution I	50
STET	50

For making up the EDTA solution:

- After calculating the appropriate amount of EDTA required, weigh out this amount and place it in a labeled beaker containing a stir bar.

Caution

*** Prior to working with solid NaOH, ensure that you are wearing a lab coat and gloves.**

- add half of the amount of Nanopure water called for (use the water in the carboy), and place the beaker onto a stirring plate. Use solid NaOH pellets to pH EDTA (note that the disodium salt of EDTA will not go into solution until the pH is at ~8.0). Please see **Appendix 6** for guidelines on using the pH meter.
- Pour the solution into a graduated cylinder and adjust the volume with Nanopure water.

Caution

*** Prior to working with concentrated HCl, ensure that you are wearing a lab coat, goggles and gloves.**

For making up the Tris solutions:

- Again, calculate amount required per solution, add only half of the water required (just enough to dissolve the compound), and read the pH. After adjusting to the appropriate pH, stir and pour the solution into a clean graduated cylinder and adjust the volume using Nanopure water.

When you are finished:

- Ensure that solutions are labeled as completely as possible. Include name of solution, pH, date, and your initials or group designation. Place your labeled bottles in a metal tray on the side for autoclaving.

At some point during the lab, there will be a demo of the autoclave. To prepare for this, ensure that all lids to bottles are barely on the bottles (you want to allow for pressure

changes during the autoclave cycle!!)

B HANDLING OF MICROORGANISMS

Objective: To practice streaking for single colonies using aseptic technique

Background Reading: Appendices 1, 2

Additional Supplies and Equipment:

Liquid cultures of *Escherichia coli* strain DH5α	Microbiology kits
Racks for test tubes	Tape
LB plates – 1 per person	Inoculating loops

Procedure: The following should be performed using aseptic technique (Appendix 1).

1) Clean off your bench surface using the dettol/ethanol mixture provided.

2) Obtain 1 LB plate and a tube of bacterial culture.

3) Following the instructions found in Appendix 2, set up a streak plate for single colonies using the broth culture of bacteria and a fresh LB plate. Ensure that you label your plate completely, then place your plate into the 37 ^{o}C incubator in C741. Typically, *E. coli* is incubated for 16 – 20 hours to allow for single colony formation. In the presence of antibiotics other than ampicillin, incubation time may be increased up to 24 hours.

C PREPARATION OF AGAROSE FOR GEL ELECTROPHORESIS (if time permits – if not, this will be performed during Day 2 of Laboratory 2)

Rather than weighing out agarose and preparing a single gel each time you require one, it makes more sense to create a larger volume of molten agarose, let solidify, then re-melt and pour gels when required.

Each group should prepare 200 mL of 0.8% agarose in 1x TAE

Additional Supplies and Equipment

Agarose	250 mL bottles
1x TAE	Graduated cylinders
Stir bars	

1) Weigh out an appropriate amount of agarose to create a 0.8% mixture (check with the instructor if you are unsure).

* **% refers to g/100 mL of solution**

Place agarose into a 250 mL bottle. Add a stir bar.

2) Use a graduated cylinder to add 200 mL of 1x TAE. Heat on high power in the microwave for 1 minute intervals stopping frequently to ensure that the agarose mixture does not boil over.

3) Swirl the flask each time you remove it. Hold it up to the light to check to see if any agarose is not in solution (you should not see any floating particles).

4) Once the agarose is in solution, let it cool, label the bottle clearly with your group designation, and store for use throughout the semester. Don't remove the stir bar.

Thought Questions:

- What is the genotype of DH5α? (Often catalogues from suppliers of molecular biology cells and reagents have this information either in paper versions or on-line)
- Why might *E. coli* genotype be important in molecular biology experiments? Speculate using specific examples from DH5α..

LABORATORY 2

ISOLATION OF DNA FROM COMPOST

Objective: To extract total DNA from a sample of compost.

Background Reading: Amann et. al., 1995, Cole et. al., 2003, Gabor, et. al., 2003; Pace, 1997, DeLong and Pace, 2001, Woese et. al. 1990, http://pacelab.colorado.edu/index.html; the Ribosomal Database Project: http://rdp.cme.msu.edu/index.jsp

The classical approach to isolation and identification of microbes in a complex environment such as soil or compost requires placing the material into a medium containing all of the necessary nutrients for growth, and then incubating the medium under appropriate conditions. Solid or liquid media may be used and each has particular advantages. Unfortunately, these techniques greatly underestimate the number of microbes present in a particular sample (Atlas and Bartha, 1993). For instance, counts obtained using epifluoresence microscopy are usually two orders of magnitude higher than those obtained by classical culturing techniques (Atlas and Bartha, 1993). Other studies estimate culturability of bacteria from soil at 0.3% (as cited in Roose-Amsaleg et. al., 2001). In order to obtain more meaningful information on diversity of microbial communities in complex environments, isolation and analysis of DNA from environments such as soil and compost may be performed; thereby circumventing the difficulties associated with attempting to culture all microbes present. Once DNA is isolated, it may be studied via construction of BAC (Bacterial Artificial Chromosome) libraries (for an example, see Rondon, et al., 2000). More simply, an appreciation of diversity may be obtained by using universal primers for PCR amplification of rDNA genes from the Bacterial domain on a preparation of total DNA from an environment such as compost. The resulting pool of nucleotide fragments (approximately 1500 bp long) may then be cloned, unique clones sequenced, and the resulting sequences analysed in order to characterise microbes present.

Prelab Preparation:

- Ensure waterbaths are set at 37 °C and 65 °C

Supplies and Equipment:

2x vortex mixers	Lysis Buffer prepared in Lab 1
Compost	Balance
Weigh boats	Scoops
Sterile 2 mL microfuge tubes	Lysozyme
Pronase from *Streptomyces griseus*	Waterbath at 65 °C

Waterbath at 37 °C	Floating racks
10% SDS	Micropipettors and sterile tips

Procedure:

Work individually. Use 2 mL microfuge tubes.

1) Weigh out 500 mg of compost avoiding any large pieces.

2) Add 750 μL of lysis buffer. Vortex for 5 minutes.

3) Use a sterile yellow pipette tip to remove a few crumbs of lysozyme from the stock bottle.

***Caution Lysozyme is a very fine powder and will blow off very easily.**

Add the lysozyme to your compost mixture.

4) Repeat step 3 with a fresh tip and with Pronase. Cap and mix by shaking.

5) Incubate your sample for 30 minutes at 37 °C.

6) Add 400 μL of 10% SDS to your sample. Mix by vigorously shaking by hand. Place sample into the 65 °C waterbath for the remainder of the lab. After gel preparation and the lecture, transfer your tube back into the 37 °C waterbath where it will incubate overnight.

Also during today's laboratory: Please complete Part A of Laboratory 3 – Purification of Compost DNA

The next laboratory period:

Supplies and Equipment

Microfuges	Lysis buffer prepared in Lab 1
Waterbath at 65 °C	Chloroform (in the fume hood)
Weigh boats	Scoops
Sterile 2 mL microfuge tubes	Optima-water
Micropipettors	Organic waste disposal (in the fume hood)
Vortex mixer	Floating racks
Isopropanol	Sterile tips
70% ethanol	Gloves
Waste beaker for tips and tubes in the fume hood	4x small beakers for liquid waste
Vortex	

Procedure:

Work individually to complete extraction of compost DNA.

1) Place tubes containing compost/buffer mixture into the microfuge.

- **Caution - Ensure that your tubes are in a balanced configuration. Get into the habit of placing your tubes with the hinges facing outward. Your pellets will then always be located under the hinge – a handy thing to note particularly when the pellets may not be very large!**

2) Spin at 8000 rpm for 10 minutes. Use a pipette with a sterile tip to transfer the supernatant to a labeled sterile tube.

3) Add 500 μL of Lysis buffer to the pellet. Vortex vigorously for a few seconds, then incubate the tube at 65 °C for 10 minutes.

4) Repeat the spin step outlined in Steps 1 and 2 above. Transfer this supernatant to the same tube used in Step 2.

5) Repeat the extraction and incubation in Lysis buffer one additional time. Repeat the centrifugation step and pool the supernatants. Discard the tube containing the pellet.

6) Use the gradations on the microfuge tube to estimate the volume of supernatant you have. If it exceeds 1 mL, split the supernatant evenly between 2 tubes. Put on gloves and move to the fume hood.

7) To each tube of supernatant, add an approximately 50% volume of chloroform (for instance, if you have 500 μL of supernatant, add 250 μL of chloroform). Shake the tube for 1 minute.

* Addition of an organic solvent, mixing, centrifuging and subsequent transfer of the aqueous mixture is often termed extraction. Extraction with organic solvents such as phenol or chloroform (or a mixture of the two) is a standard way of removing proteins from nucleic acid solutions. Deproteinisation is more efficient when two different organic solvents are used instead of one (Sambrook et. al., 1989; Sambrook and Russell, 2001)

8) Place your tube in the centrifuge in a balanced configuration with tubes from other students. Spin at 10000 rpm for 3 minutes.

9) The aqueous phase (upper layer) contains your DNA. Remove the supernatant to a fresh tube (in the fume hood). Discard the liquid chloroform waste into the container labeled “organic waste”, and discard the tube in the beaker in the fume hood.

10) To precipitate DNA, add an equal volume of isopropanol to each tube. Mix by hand until two phases are no longer distinguishable. Store the tube at room temperature for 10 minutes.

*In solution, nucleic acids are surrounded by water molecules attracted by hydrogen bonds and dipole attractions. Ethanol (alcohol) depletes some of these molecules of water, most likely by displacement. Consequently, the negatively charged phosphate groups on the nucleic acids are exposed. When cations such as Na^+ are added, they associate with the charged phosphate groups (an ionic attraction). This shielding of the negative charges allows the nucleotide chains to associate closely with each other (DNA associates with itself rather than with water) resulting in precipitation of the nucleic acids. (Sambrook and Russell, 2001)

11) Centrifuge in a balanced configuration for 10 minutes at 13000 rpm. Decant the supernatant into a waste beaker (pour away from the pellet).

12) Add 500 μL of 70% ethanol and flick the tube with your finger to dislodge the pellet. Spin at 13000 rpm for 3 minutes and decant the ethanol. Pulse the tube (again, balance your tubes, put the lid on, and hold down the button on the front panel of the microfuge until the microfuge is up to speed, then release the button) and draw off any remaining ethanol with a micropipettor.

13) Leave the tube open on the bench and let the pellet air dry for 10 minutes. Resuspend the pellet in 25 μL of Optima-water. Note that the pellet will be brown in colour at this point.

DNA extraction protocol modified from: Gabor, E. M., deVries, E. J., and Janssen, D. B. 2003. Efficient recovery of environmental DNA for expression cloning by indirect extraction methods. FEMS Microbiol Ecol. 44: 153-163.

LABORATORY 3

PURIFICATION OF COMPOST DNA

Objective: To use two techniques to purify total DNA isolated from compost in order that the polymerase chain reaction may be carried out.

Background Reading: Instructions from Qiagen DNEasy Kit, Appendix 7

A PREPARATION OF LOW MELTING POINT AGAROSE GELS (PLEASE REVIEW APPENDIX 7) (Performed during Laboratory 2)

Supplies and Equipment:

Low melting point agarose	Scissors
2 small gel trays	2x 6 well combs
Weigh boats	Scoops
Balance	2x 500 mL graduated cylinders
1x 100 mL graduated cylinder	Microwave
2x 250 mL flasks	Oven mitts
Rubbermaid container for finished gels	Electrophoresis tape
1x TAE	Stir bars

Procedure:

Two gels will be prepared for the entire class.

1) Each gel tray holds approximately 40 mL of agarose. You want to prepare a 0.5% agarose gel in 1x TAE buffer. Use this information to calculate how much agarose to weigh out.

2) Weigh out the appropriate amount of agarose (check with the instructor if you are unsure). Place into a flask and add 40 mL of 1x TAE. Heat on high power in the microwave for 1 minute

intervals stopping frequently to ensure that the agarose mixture does not boil over.

3) Swirl the flask each time you remove it. Hold it up to the light to check to see if any agarose is not in solution (you should not see any floating particles).

4) Once the agarose is in solution, you will need to let it cool by stirring it gently so as to avoid introducing bubbles. Once you can hold the flask comfortably in your hand, you can pour the gel.

5) While the agarose is cooling, prepare the gel tray. Use electrophoresis tape to cover both ends of the tray. Ensure that the ends are completely sealed so that agarose does not leak out. Place the comb into the slots approximately 1 cm from one end of the gel tray.

6) Pour the agarose gently into the prepared tray. Leave the gel at least 10-15 minutes. When it doesn't move when touched, transfer the whole tray to a fridge and chill.

Caution – these gels will be very fragile!

7) At the end of the lab period, remove the gel from the fridge, remove the tape and put the gel plus comb into a gel tray. Cover the gel with 1x TAE, then remove the comb. The gel can remain in buffer for several days prior to using.

Stop Point – these gels will be run the morning prior to the next laboratory

Loading the gel:

Supplies and Equipment

Power supplies	Lambda DNA digested with *Hind*III
Loading dye	Waterbath set at 65 °C
Parafilm	Micropipettors and sterile tips
Tubes of DNA	Ethidium bromide bath

***Place tubes of λ DNA digested with *Hind*III into 65 °C water bath for at least 10 minutes prior to loading. Why?**

9) Obtain tubes of DNA. Set a micropipettor to 3 μL and add Loading Dye to your sample.

Loading dye is considered to be 10x concentration; you should add to your sample to create a concentration that is approximately 1x.

*Loading Dye - 1) increases density of sample ensuring that it drops evenly into well; 2) adds colour to sample to simplify loading; and 3) contain dyes that in an electric field move toward the anode at predictible rates

Another Option:

In situations where you do not want to load your entire DNA sample, you can cut off a small square of parafilm and dot out the loading dye in a grid pattern. Remove the desired volume from your tube of DNA and mix gently on the parafilm, then proceed with loading.

10) Load the gel as follows:

-Starting with the lane farthest to the right side of the gel, load 7.5 μL of λ DNA digested with *Hind*III resuspended in loading dye. Lambda DNA is at a final concentration of 0.05 μg/μL. Place tip into top part of well (white paper underneath gel tray may help in seeing the wells) and **gently** release digest mixture into well, only pipetting to the first stop. At this point, decide on the order (from right to left) in which you will load your samples.

*Lambda DNA digested with *Hind*III serves as a reference as all of the λ band sizes are known and can be used to compare with digested sample bands (Appendix 7).

-Using a fresh tip for each tube, pipette up and down VERY GENTLY to mix DNA and dye - if you introduce bubbles, there is nothing you can do to get rid of them!!! Draw up all of the DNA/loading dye mixture ensuring that no air is left at the end of the tip - bubbles in the well will cause the sample to float out!

-Place tip into top part of well (white paper underneath gel tray may help in seeing the wells) and **gently** release digest mixture into well.

11) Load remainder of DNA samples in the same way.

12) Connect leads to power supply (a good rule to remember is **Run to Red**) and turn on. Turn the voltage up to 70 V, and run the gel for 1.5 hours. The time and voltage used depends upon the type and concentration of agarose as well as on the size of DNA to be separated.

13) After run is complete, turn off power and disconnect leads. Wearing gloves, remove gel tray and gently slide gel into staining tray avoiding splashing the ethidium bromide.

***Ethidium bromide is a mutagen and a suspected carcinogen. At very dilute concentrations, and with responsible handling, this risk is minimised**

14) Stain gel for 10 minutes. View using transilluminator and UV light.

***Ultraviolet light is damaging to naked eyes and exposed skin. Always view through filters or safety glasses that absorb harmful wavelengths.**

The gels will be photographed and pictures posted on the Biology 4200 web site or emailed out. You will be extracting DNA from these gels and purifying it.

B PURIFICATION OF DNA BY EXTRACTION FROM LOW MELTING POINT AGAROSE

Prelab Preparation:

- Two student volunteers are needed to come in the morning prior to the laboratory in order to load and run the gels.
- At the beginning of the lab, ensure a waterbath is set at 68 °C.

Supplies and Equipment

Micropipettors	Waste beakers
Sterile tips	Optima-water (in kits)
Ethidium bromide bath	UV transilluminator
Gloves	Spatulas
Microfuge tubes	Eye protection or face shields
68 °C waterbath	Buffer saturated phenol (in the fume hood)
Organic waste disposal	Phenol:Chloroform (1:1) (in the fume hood)
Microfuges	3 M NaOAc pH 5.2
Isopropanol	70% ethanol
Goggles for working with organic solvents	95% ethanol

Procedure:

Every student should extract his/her own DNA. Once the DNA is extracted from the agarose, volumes will be pooled prior to Qiagen column purification.

Suggestion:
Use the 2 mL microfuge tubes initially or use the tubes labeled for phenol use only (the other tubes may leak).

1) Have labeled microfuge tubes handy with the lids open. While wearing gloves and face

protection, examine the gels under UV light. Cut out all of the DNA visible (try and avoid taking excess agarose) and place the agarose plus DNA into a labeled microfuge tube. If you are the last person to remove your DNA, please dispose of the gel in a biohazard bag and wipe up the transilluminator.

2) Melt the agarose at 68 °C for 10 minutes or until you no longer see any pieces of agarose.

3) Estimate the volume. If it is greater than 500 μL, separate the liquid into two equal volumes in two separate tubes. While wearing gloves and goggles, move to the fume hood and add 0.5 volumes of buffer saturated phenol. **Note:** the phenol itself is the bottom layer of liquid in the bottle (the top layer of liquid is aqueous buffer!).

- **Caution - phenol is extremely corrosive.**

4) Mix by inversion for 1 full minute.

5) Spin in a balanced configuration at 13000 rpm for 3 minutes.

6) Back in the fume hood, remove just the supernatant to a new tube (avoid removing any of the white interface). Pour out the phenol/agarose mixture into the organic waste disposal in the fume hood and discard the tube and tips in the beaker provided.

7) Extract one more time with phenol, mixing for another minute, spinning, and transferring the supernatant as above.

8) Extract a final time with phenol:chloroform (1:1).

9) Add 1/10 volume (again, estimate) of 3M NaOAc pH 5.2 to the tube as well as 2.5 volumes of 95% ethanol. Mix well and place in the freezer for 20 minutes.

10) Spin your tubes for 10 minutes at 13000 rpm. Discard the ethanol by pouring away from the pellet into the waste beaker.

11) Wash the pellet using 500 μL of 70% ethanol, flicking the tube to dislodge the pellet.

12) Spin for 3 minutes at 13000 rpm. Remove as much of the ethanol as possible as you have done in Laboratory 2.

13) Let the pellet air dry for 10 minutes and resuspend in 50 μL of Optima water. Pool your volume of DNA with that from another student (you should have a total of 100 μL).

C PURIFICATION OF DNA USING THE DNEASY KIT FROM QIAGEN

Prelab preparation:

- **Ensure that a waterbath is set at 70 ºC**

Additional Supplies and Equipment

Qiagen DNEasy Kit 99% ethanol

70 ºC waterbath

Procedure:

Please work in pairs to clean up your DNA.

Notes:

- On page 29 of the protocol booklet under the heading Protocol for isolation of genomic DNA from crude lysates, start at Step #3. DO NOT VORTEX AT ANY POINT! Vortexing will shear the high molecular weight DNA. After Step #4, there is no need to check the pH of the mixture; proceed to Step #4 on page 17.
- Elute using 100 μL of Buffer AE
- Place second elution into a fresh tube (do not mix elutions). Label clearly.
  Store your DNA at –20 ºC (again, ensure your tubes are very clearly labeled so that you can find them again).

You will quantitate your DNA either using agarose gel electrophoresis or UV spectrophotometry.

D QUANTIFICATION OF DNA USING UV SPECTROPHOTOMETRY (May or may not be performed at this point during the term)

Background Reading: Appendix 8 Quantification of DNA

Procedure:

1) Prepare a 1/5 dilution of your compost DNA from the first elution. Note that in order for the sample to be read, you should prepare it in a final volume of 100 μL of the same solvent used to resuspend your DNA (this could be water or elution buffer).

2) Small groups of students will use the UV spectrophotometer in Brent Selinger's research laboratory. Please do not all pile into his lab at once!

3) Turn on the spectrophotometer and choose the mode for Nucleic Acids.

4) Enter the pathlength for the cell used (we are using the microcells and the pathlength is 10 mm).

5) Select units (probably ng/μL).

6) Do not alter the background correction option.

7) Enter the dilution factor.

8) Pipette 100 μL of Optima-water or elution buffer into a cell. Wipe off using a Kimwipe and place into the machine so that the red dot is closest to you. Press the green run key. This is your reference.

Note: If you are using the larger cuvettes, two sides are smooth to allow light to pass through, while the other two sides are irregular resulting in readings that don't make sense.

9) Pipette out the water. Add your sample to the cell, insert and press the run key. Record all of the values.

10) Pipette out your sample. Pipette in some sterile water, remove and repeat to rinse the cell out for the next pair.

Thought Question:

Evaluate your absorbance values with respect to concentration and purity.

LABORATORY 4
THE POLYMERASE CHAIN REACTION

Objective: To use universal primers in the polymerase chain reaction (PCR) to amplify DNA from the Eubacterial domain.

Background Reading: Same as for Laboratory 2, Atlas and Bej, 1994.

Prelab Preparation:

- Working in groups of 4, decide what reactions you need to set up (note that you need to demonstrate that what you amplify is actually bacterial DNA). If you need any additional materials, please let the instructor know.

Supplies and Equipment

Micropipettors
Sterile tubes for PCR
PCR machines
Compost DNA
2x Ice buckets
Taq (NEB)
5x vials of 1492R (20 pmol/μL)
5x vials of dNTP mix
Sterile tips
Coloured dots
Optima-water
Microfuges
Biohazard bags
5x vials of 10X Buffer (NEB)
5x vials of FP1 (20 pmol/μL)

Procedure:

Work in groups of four.

Use Aseptic Technique (Appendix 1)
For preparation of your reaction mixtures:

If you are using the 250 μL tubes, label on the point of the non-hinged part of the lid. Do not label on the sides or on the rounded part of the lid – these markings will come off.

1) Using the information provided in Table 4.1, calculate the final volumes of each component of your PCR mixes and fill these out in the chart handed out. Decide on codes for each tube so that you know what tubes contain what mixtures.

Table 4.1 Components and concentrations or volumes for set-up of PCRs.

Component	**Final Concentration or volume**
Optima-Water	Calculated to make up the final volume to 50 μL
10x PCR buffer	1x
dNTP mix	5 μL
FP1 (sequence: AGAGTTYGATYCTGGCT)*	50 pmol per reaction
RP1492 (sequence: TACGGYTACCTTGTTACGACT)*	50 pmol per reaction
Template DNA	10 μL of each of 4 different dilutions: Undiluted, 1/10, 1/100, 1/1000
Taq DNA polymerase	0.5 μL

Y = C or T

2) It is worthwhile to set up a "Master Mix" containing 1x PCR buffer (final concentration), primers, water and Taq. To do this, calculate how many reactions you will be performing and add 1. Then, calculate how much of each of the components listed are be required for all of the reactions. When you are ready to set up your reactions, mix all of these components well in a tube, and leave on ice until needed. This ensures that you don't need to pipette 0.5 μL volumes, for instance.

3) After preparation of Master Mix, add the appropriate volume of template to each tube, then add Master Mix last (after all of the groups are at approximately the same stage)

GENTLY tap tubes to mix. When everyone is ready, the instructor will then show you how to operate the PCR machines. If you are using the Perkin Elmer machine, remember which number slot you placed your tube into – that way, if your label rubs off, you can still locate your mixture.

4) The parameters you are using for the PCR are:

- 5 minutes at 95 °C

30 cycles of:

- 20 seconds at 94 °C
- 30 seconds at 50 °C
- 3 minutes at 72 °C

A final elongation of:

- 7 minutes at 72 °C

Questions for Discussion:

- What are the purposes of the primers in PCR?
- What happens at each temperature?
- How is annealing temperature determined?
- What is meant by stringency? How can you ensure high stringency?

- If you left out the forward primer, would you expect to see a band resulting on the gel? If you did, explain what this would mean.
- Is it possible to design PCRs given only an isolatable protein? Why or why not? What are some of the problems associated with such an experiment? How might you adapt the reaction conditions to optimise yield of desired product?

The protocol for PCR was modified from:

Whitford, M. F., Forster, R. J., Beard, C. E., Gong, J., and Teather, R. M. 1998. Phylogenetic analysis of rumen bacteria by comparative sequence analysis of cloned 16S rRNA genes. Anaerobe. **4**: 153-163.

Also during today's laboratory:

Pouring an Agarose Gel

Additional Supplies and Equipment

Bottle of agarose	1x TAE
4 small gel trays	4x 6 well combs
Oven Mitts	Microwave
Tape	Scissors

Work in groups of 4

In order to view your PCR results, you will need to run the DNA out on an 0.8% agarose gel. Gel trays hold approximately 40 mL of agarose. One or two group members should prepare the gel tray as per Page 14. The remaining group members should take care of melting and pouring the gel.

1) Loosen the top of your bottle of agarose, then microwave in 1 minute bursts to liquefy the agarose. Swirl the bottle each time you remove it, and check the progress by holding it up to the light .

2) Once all of the agarose is melted, cool the bottle either by placing on a stir plate and stirring until you can comfortably handle the bottle, or by swirling under running cold water and checking the temperature periodically.

3) Pour your gel as per instructions on page 16.

Stop Point – these gels will be run the morning prior to the next laboratory

The morning prior to the next laboratory, representatives from each group will need to come in and load their samples on the gel they have prepared.

- For each sample, 25 μL of DNA will be loaded. Follow the instructions using the option involving mixing the sample on parafilm found in Laboratory 3. Remember that you will need to alter the volume of loading dye.
- Don't forget heat-treated Lambda *Hind*III!
- Run this gel at 70V for 1 – 1.5 hours
- Gel may be left in ethidium bromide until lab time.

LABORATORY 5

EXTRACTION OF DNA FROM AGAROSE; SUBCLONING USING THE T VECTOR SYSTEM FROM PROMEGA

Objectives: To isolate, purify and subclone populations of bacterial rDNA amplified from compost

A EXTRACTION OF DNA FROM AGAROSE

Background Reading: MinElute Handbook; Qiagen web site http://www1.qiagen.com/

Prelab Preparations:

- Set a water bath to 50 ^{o}C

Supplies and Equipment:

Optima-water	Micropipettors
Gel from Laboratory 4	MinElute Gel Extraction Kit
Isopropanol	Permanent marker
Microfuges	Transilluminator
Eye protection	Gloves
Balance	Spatulas
Floating racks	Waterbath
Hand held UV lamp	Gel tray or other work surface

Procedure:

Work in pairs.

1) Ensure that you are wearing gloves and eye protection. View your gel using the hand-held UV lamp using the Long Wave setting (our transillulminator has only one setting for UV light intensity. This setting results in DNA degradation by the UV light rendering ligation less efficient). Use a spatula to excise the agarose containing just the fragment of interest (note, this fragment should correspond to approximately 1500 bp in size). Cut the band into two approximately equal fragments and place each band into a clean, labeled microfuge tube. Save the gel and the gels will be photographed.

Each pair will purify the DNA from the agarose fragment.

2) Check to ensure that 99% ethanol has been added to Buffer PE before getting started. Follow the guidelines on page 16-17 of the MinElute Handbook to purify your DNA. Use Buffer EB for elution.

B LIGATION OF ISOLATED DNA FRAGMENT INTO THE pGEM-T VECTOR (perform the same laboratory period)

Background Reading: Promega Technical Manual for pGEM-T and pGEM-T-Easy Vector Systems http://www.promega.com/vectors/t_vectors.htm (or in the binder); Hengen, P. N., 1995.

T-vectors are plasmid cloning vectors that have been digested with *Eco*RV (this enzyme produces blunt ends), and had single 3' T overhangs added at this digest point. Certain DNA polymerases such as Taq will preferentially add a single deoxyadenosine triphosphate to the 3' end of the newly synthesised DNA (as reviewed in Sambrook and Russell, 2001). Consequently, some annealing between the T (on the vector) and A (on the PCR product) overhangs could occur. Such annealing would stabilise the hybrid molecule so that ligation is more likely. According to Hengen (1995), pGEM-T vectors produced by Promega are the most reliable of the T vectors that are commercially available; therefore, this vector is the one that we will be making use of in cloning 16S rDNA amplified from compost DNA.

Supplies and Equipment:

Optima-water
Extracted DNA
Microfuges
2x Ice buckets with ice
Micropipettors
5x aliquots of pGEM -T vector
Permanent marker

Procedure:

Each pair should set up one ligation.

- You will be setting up a ligation in a final volume of 10 μL exactly according to the instructions found on page 7 of the Promega manual.
- We will be leaving the ligations overnight at 4 ^{o}C.
- One group will be setting up a ligation using vector only (Background control).
- One group will be setting up a ligation using control insert provided.

Thought Questions:

- Diagram the reaction that is taking place (catalysed by DNA ligase)
- What is the purpose of the Background Control? If you see colonies resulting from your transformation, what do they mean?

LABORATORY 6
PREPARATION OF COMPETENT CELLS AND TRANSFORMATION

A PREPARATION OF COMPETENT CELLS

Objective:

To prepare competent *E. coli* cells (strain DH5α).

Transformation:

Background Reading: Mandel and Higa, 1970; Cohen, et. al., 1972.

Definition: One of 3 mechanisms by which DNA is transferred from a donor cell to a recipient cell (Atlas, 1995). Specifically, transformation is the uptake of naked DNA in organisms such as *E. coli*, *Bacillus* and *Streptococcus*; in a natural system, this may occur when the donor cell lyses although this process is very inefficient (Atlas, 1995).

In the laboratory, to maximise this process, the recipient cells must be **competent**. In other words, the cells must contain sites for binding the donor DNA at the cell surface and their plasma membranes must be in a state so that free DNA can pass across them (Atlas, 1995). Experiments performed by Mandel and Higa (1970) demonstrated that *E. coli* cells could be made competent by treating them with ice-cold $CaCl_2$, and that the efficiency of this process may be improved by exposing strains of *E. coli* to combinations of divalent cations (Maniatis *et al.*, 1989). Although it is unknown by what mechanism cells become competent, it is hypothesised that uptake of DNA is normally prohibited due to electrostatic repulsion between negatively charged phospholipids in the cell membrane and negatively charged DNA molecules (Micklos & Freyer, 1988). Exposure to cold results in the crystalisation of the fluid cell membrane that then stabilises the distribution of the charged phosphate groups (Micklos & Freyer, 1988). Cations such as Ca^{++} or Mg^{++} can then complex with exposed phosphate groups shielding the negative charges. DNA molecules are then able to move through the adhesion pores in the cell membrane (Micklos & Freyer, 1988).

You will be preparing competent cells of *E. coli* strain DH5α. Use one of the catalogues (for example GIBCO, or Promega) to locate the genotype of DH5α and make note of it. What do all of the gene symbols mean?

Prelab Preparations:

- The day before the laboratory exercise, an overnight suspension culture of *E. coli* strain DH5α was prepared according to the directions in Appendix 2.
- Approximately 2 1/2 hours before the laboratory, a sub culture of mid-log DH5α cells was prepared according to the directions in Appendix 2. This ensures that the culture will be in mid-log phase prior to starting the procedure (having an OD_{550} of 0.35 - 0.50).

Supplies and Equipment:

Ice bucket, ice
100mM $CaCl_2$ (sterile)
80 mM $MgCl_2$ 20 mM $CaCl_2$ (sterile)
Glycerol (sterile)
Mid-log phase *E. coli* DH5α cells (50 mL per group of 4)
Waste containers
Racks for centrifuge tubes
Micropipettors
Sterile tips
1.5 mL microfuge tubes
40 mL sterile centrifuge tubes
Balance/small beaker
Sterile d_2H_2O for balancing, beaker

Procedure:
Note - Keep cells on ice as much as possible during this procedure. Perform all procedures using aseptic technique (Appendix 1) and sterile equipment.

Work in groups of 4 to prepare cells.

1) Place all solutions on ice to chill.

2) Pour roughly 40 mL of mid-log phase cells into 2 sterile centrifuge tubes (fill the centrifuge tube to the base of the neck). Balance your tubes to within 0.1 g.

- place 1 tube in a beaker on the balance pan and zero the balance
- remove the first tube and place the second in the beaker. Note the mass.
- adjust the volume in the tubes with sterile d^2H_2O such that the balance reads the same for both tubes.

Place your tubes into the centrifuge in a balanced configuration; ie, the 2 tubes your group balanced against each other should be across from each other.

3) Centrifuge the cells at 2,700 g for 10 minutes. This spin will throw the cells into a pellet at the bottom of the tube.

4) Remove tubes from the centrifuge. Pour off the supernatant into the original flask being careful not to disturb the pellet. Tap the tube gently over paper towel to remove excess supernatant and let the tube stand inverted on a piece of paper towel for 1 min.

5) Add 30 mL of ice cold 80 mM $MgCl_2$ 20 mM $CaCl_2$ to each tube using aseptic technique. Resuspend the pellet of cells by pipetting the mixture up and down. Ensure that cells are completely resuspended before proceeding.

6) Balance the tubes as needed and centrifuge in the same manner as above to pellet the cells. Pour off the supernatant, being careful not to disturb the pellet.

7) Resuspend each pellet in 2 mL of ice cold 100 mM $CaCl_2$ by swirling gently.

8) Pool the contents of your tubes so that you have a final volume of 4 mL..

9) Add 1.2 mL of sterile 100% glycerol and swirl gently until thoroughly mixed.

10) Transfer 100μL aliquots of the competent cells into 1.5 mL microcentrifuge tubes on ice. Cells will be frozen at -80 ^{o}C to be used the next laboratory and later in the course.

B POURING OF LB PLATES CONTAINING AMPICILLIN (to be performed during the same laboratory)

Objective:

To gain experience in preparing and pouring solid bacteriological media.

Note: Preparation and autoclaving media will be performed out of lab. Pouring media will be performed during the same lab period as preparation of competent cells.

Supplies and Equipment for Media Prep (available in the Biology Storeroom):

Agar	Balance
Spatulas	Weigh boats
Tryptone	Yeast Extract
NaCl	250 mL Flasks
Tape	Permanent markers
Stir bars	d^2H_2O
Graduated cylinders	Stir plate
Aluminum foil	Autoclave tape

In Lab Supplies and Equipment

5x syringe filters	5x 10 mL syringes
5x sterile 15 mL Falcon tubes	Ampicillin
Gloves	Balance
5x 50 mL beakers	Scoops
Weigh boats	

Procedure: Each pair should prepare and pour 250 mL of LB + Amp medium.

1) Arrange a time the day prior to Laboratory 6 Part B. In terms of timing, weighing out the ingredients and mixing them will take about 20 minutes. Autoclaving will be carried out by your instructor or the storeroom supervisor. After autoclaving. the medium will be stored in a water bath until the next day.

2) Obtain a flask or bottle that holds 2x the volume of medium you are making. Label the flask

using tape and a permanent marker and add a stir bar. Use the recipe for LB found in Appendix 9 and weigh out the ingredients required. Use a fresh weigh boat for each different component. Rinse the spatula with distilled water and dry with a Kimwipe in between each component. Rinse out the weigh boats when finished and save them.

3) Use a graduated cylinder to add the required amount of d^2H_2O (use the carboy containing Nanopure or d^2H_2O) to the flask, and place the flask on a stir plate to mix the ingredients. Note that unlike making a solution, you do not dissolve the ingredients first in a small volume of liquid then bring up the volume. For media for *E. coli*, this is not necessary (although it may be necessary for more fastidious organisms)

4) Wrap foil over the top of the flask or place the top loosely onto the bottle (do not tighten the lid), and place a piece of autoclave tape onto the flask.

5) Autoclave the medium on liquid cycle.

The Following Laboratory Period:

5) Media will be stored overnight in a water bath. The next day, complete preparation of any antibiotic solutions required (Appendix 4), then remove the medium and place on a hot plate and stir gently to speed up the cooling process.

*Preparation of antibiotic solutions. Stock concentrations are provided for you in Appendix 4. Each group of 4 should prepare and filter sterilise 10 mL of the appropriate concentration of ampicillin. Wear gloves when you weigh out the antibiotic. The tubes should be labeled thoroughly including date of preparation, concentration of antibiotic, group designation.

6) Calculate the amount of stock solution to add to your medium preparation. Double check your calculations. Obtain a sleeve of Petri dishes. Label the agar side of each dish with a code indicating what antibiotic is present.

7) When the flask containing the medium can be held, add the antibiotic (flame the mouth of the flask!). Place back on the stir plate for 30 s to mix.

8) Pour the plates ensuring that you flame the mouth of the flask periodically. Allow plates to dry for at least 24 hours at room temperature prior to using. After drying, place into a sleeve and store the plates at 4 ^oC. Note, for plates containing tetracycline, store in the dark at 4 ^oC.

Questions for Discussion

- Why is it necessary to add an antibiotic when culturing bacteria containing a plasmid?

- Why is it necessary to select single colonies when creating new subcultures?
- What is an autoclave? What is the purpose of autoclaving? What is a typical liquid cycle for autoclaving?

C TRANSFORMATION OF LIGATION MIXTURES (the next laboratory)

Objective:

To transform the competent *E. coli* DH5α cells prepared in a previous laboratory with a DNA construct containing 16S rDNA.

Supplies and Equipment

Competent *E. coli* DH5α cells – on ice	Sterilised spreaders
LBAmp plates (prepared in previous lab)	Micropipettors
Waterbath set at 42 ^{o}C	Ice bucket/ice
Waterbath set at 37 ^{o}C	Sterile tips
Racks for microfuge tubes	Sterile Optima water
X-gal solution (2% in dimethylformamide)	DNA from ligation
Gloves	Microfuges
Floating racks	Beaker for used spreaders

Procedure:

Work in pairs – each member of the pair should be responsible for one transformation mixture.

1) Obtain enough LBAmp plates for all of your transformation mixtures. Do not add X-gal to plates you aren't going to use today! Working with one plate at a time, pipette 40 μL of 2% X-gal onto the surface. Obtain a sterile spreader and use to spread the solution carefully and evenly over the surface of the plate. Place the used spreader into the marked container for re-autoclaving. Repeat for the remaining plates. Let these plates dry while you are carrying out the rest of the laboratory.

* X-gal or 5-bromo-4-chloro-3-indolyl-β-D-galactoside is coverted by β-galactosidase into an insoluble blue compound.

* X-gal is prepared in dimethylformamide (DMF). To minimise risk, wear gloves when handling the stock solution.

2) Obtain 2 tubes of competent cells. Thaw cells **on ice** as they are very fragile. While thawing, label 1 as **ligation mix**, and one as **control** (no DNA will be added to this tube). For groups carrying out additional controls, you will require more tubes of competent cells.

3) To the tube labeled ligation mix, add the entire contents of your ligation reaction. To the control, add the same volume of sterile water.

4) Incubate tubes on ice for 35 minutes.

* It is hypothesised that this incubation allows the DNA to approach the outer cell surface.

5) Bring your ice bucket over to the 42 °C water bath. Place your transformation mixtures into the water bath for exactly 1 minutes.

* Heat shocking may allow for the formation of a temperature gradient between the inside and outside of the cell, facilitating passage of DNA into the cell.

6) Return your tubes immediately to the ice bucket. Aseptically add 890 μL of LB to each transformation mixture. Place the tubes into the 37 °C water bath and incubate for 40 min.

*This incubation allows expression of the antibiotic resistance genes. It also allows the *E. coli* to go through at least 1 generation time.

7) On one plate, spread 100 μL of ligation mixture/competent cells. Label the plate appropriately. For the remaining 2 plates, place both transformation mixtures (the remaining DNA/cell mixture plus the control lacking DNA) in a balanced configuration in a microfuge and spin at 13 000 rpm for 3 minutes to pellet the cells. Pour off all but approximately 100 μL of liquid from each tube into the liquid waste container. Resuspend the cell pellets at the bottom of the tube in the remaining liquid and plate. Ensure that your plates are clearly labeled.

8) Place your plates into the 37 °C incubator overnight (12-16 hours). Tomorrow, the instructor will remove the plates and store them at 4 °C. This will allow for maximum blue colour development.

Questions for Discussion:

- Why when using ampicillin as the selection agent is it necessary to remove the plates from the incubator at no later than 16 hours? What are the additional colonies that develop called? Do they contain the plasmid of interest? Why or why not?
- What is blue-white selection?
- Is blue-white selection possible using any strain of *E. coli*? Why or why not?
- If you obtain blue colonies from your Background Control, what does this mean? Should you be concerned?

LABORATORY 7
ECKHARDT GEL ELECROPHORESIS OF PUTATIVE SUBCLONES CONTAINING BACTERIAL 16s rDNA

Objective: To become familiar with screening colonies using Eckhardt gel electrophoresis

Background Reading: Eckhardt, 1978.

Prelab Preparation:

The day prior to the laboratory, representatives from groups with white (or pale blue) colonies should replica-plate these colonies onto 3-4 plates of LB Amp medium as the procedure works best with fresh cultures. Each group should also pick 1-2 blue colonies to run as well. When you replica-plate, don't just dot the colony onto the fresh medium. Instead, put little scribbles on the new plate so that you have little more culture to work with. Is it necessary to include X-gal? Why or why not? Incubate plates overnight (12 – 16 hours) at 37 °C.

Supplies and Equipment:

Micropipettors	Parafilm
Electrophoresis kits	Tape
10% SDS	10x TBE stock
4x graduated cylinders	4x large beakers
4x 250 mL flasks	Power supply capable of running at 5-10 volts
Agarose	Balance
Scoops	Weigh boats
Microwave	Fresh plate cultures
Solution I (GTE)	10 mL pipettes
Propipettors	Lysozyme powder
10x Loading dye	Aliquots of λ *Hin*dIII DNA
RNAse	

Procedure:

Work in groups. Note, each group should run an Eckhardt gel on all of the possible subclones even if the group did not obtain any!

Screening a large number of clones for the desired construct can often be time consuming as well as wasteful. In Dr. Brent Selinger's and Dr. Michael Hynes' laboratories, they routinely use modified Eckhardt gels (Eckhardt, 1978; Hynes et al., 1985) to examine potential *Escherichia coli* clones. The procedure is simple and can be performed on the original transformant colonies (no

subcultures required!).

1) Cast an 0.8% agarose gel (for Eckhardts, it is best to use 1x TBE for casting and for running the gel. Use of TAE results in overheating) containing 1% SDS. This is accomplished with a minimal amount of bubbles by adding the 10% SDS stock solution (SDS may or may not be prepared in TBE) **after** the agarose is melted in 0.9 volumes of TBE. To minimise bubbles further, trickle the SDS down the side of the flask into the agarose and stir **very gently** to mix.

2) Aseptically, pipette 1 mL of Solution 1 into a microfuge tube. Use a sterile yellow tip to add a scoop of lysozyme powder. Add 20 μL of RNAse to the tube. Close the tube and mix well. On a piece of parafilm place 15 μL droplets of Solution I containing lysozyme and RNAse.

3) For each colony, place a sterile tip on a suitable pipettor and pick up a bit of the colony with the end of the tip. Pipet the cells up and down in the droplet of solution until cells are dispersed. Try not to introduce any bubbles. Load the suspension into a well of the agarose SDS gels.

4) Load 7.5 μL of lambda DNA digested with *Hin*dIII as a marker.

5) Once all of the samples are loaded, turn power supply on to 5 - 10 V and run until cells are lysed (i.e., the suspensions in the wells clear). When *E. coli* colonies are used this usually takes less than 0.5 h. Following lysis, increase power supply voltage to 70 V for 2 hours to resolve plasmids. Upon completion of run, stain gel in ethidium bromide and photograph.

6) Select clones containing the plasmid of expected size.

Thought Questions:

- How many bands do you expect to see for uncut plasmid DNA? What does each band represent? Hint: **http://bio.classes.ucsc.edu/bio20L/animate/anim2/topo.htm** has excellent animations and explanations)
- What is the expected size of a plasmid containing the desired insert? How do you know?
- Are there any additional bands? Hypothesise as to what these bands may represent.

References

Eckhardt, T. 1978. A rapid method for the identification of plasmid deoxyribonucleic acid in bacteria. Plasmid 1: 584-588.

Hynes, M.F., R. Simon, and A. Puhler. 1985. The development of plasmid-free strains of *Agrobacterium tumefaciens* by using incompatibility with a *Rhizobium meliloti* plasmid to eliminate pAtC58. Plasmid 13:99-105.

Selinger, L. B. Personal Communication.

LABORATORY 8
MIDI-PREPARATION AND COLUMN CLEAN-UP OF SUBCLONES OF INTEREST

Background Reading: Appendix 8 Quantification of DNA; Sanger et. al, 1977; Hunkapiller et. al., 1991, GenElute handbook (SIGMA)

Objective:

To prepare plasmid DNA for sequencing.

Prelab Preparations:

- The day before the laboratory, each pair needs to prepare one overnight suspension culture of *E. coli* DH5α containing the putative clone of bacterial 16s rDNA according to the instructions found in Appendix 2. Ensure that you add the correct amount of ampicillin to your tube of culture (assuming a final volume of 5 mL).
- Prior to starting the lab, ensure that a waterbath is set to 70 ^{o}C

Supplies and Equipment:

Micropipettors	5 mL of fresh overnight culture
Microfuges	Floating racks
Permanent markers	Ice bucket, ice
Waste beakers for bacterial waste	Solution I (GTE) (ice cold)
Solution III (KOAc) (ice cold)	2 M NaOH
10% SDS	Sterile d_2H_2O
Isopropanol (ice cold)	75% ethanol
Sterile tips	Sterile tubes
SIGMA GenElute Plasmid Miniprep kit	Lysozyme
2 bottles of Phenol:Chlorofom (1:1)	Gloves
Goggles	Waste beakers labeled for phenol waste

Procedure: Work individually - 1 tube per person. When completed, resuspend all pellets from 5 mL of culture in 200 μL of water total, then proceed to using the SIGMA kit to clean up 100 μL of your DNA (Save the other tube). Use aseptic technique (Appendix 1).

Note: Flame your tubes of bacterial culture while transferring to the microfuge tube. This way, if there is a problem, the culture can be recovered with less worry about contamination.

1) Obtain 2 microfuge tubes and label accordingly. Aseptically transfer 1.5 mL of culture into

each tube and centrifuge in a balanced configuration at 13 000 rpm for 1 minute to pellet the cells.

2) Pour off the supernatant into a designated container (NOT the biohazard bag – the storeroom staff will hunt you down). To the same tubes, aseptically add another 1 mL of culture and spin as in the previous step. Again, pour off the supernatant. Repeat until all of the 5 mL culture has been pelleted into the two microfuge tubes (2.5 mL per tube).

3) Remove as much of the supernatant as possible. Aseptically transfer 1 mL of Solution I to a fresh microfuge tube. Use a sterile yellow micropipettor tip to add a few crumbs of lysozyme to the Solution I. Resuspend each pellet in 200 μL of Solution I (GTE) by pipetting up and down.

*Glucose-Tris-EDTA - The Tris buffers the cells at pH 7.9. EDTA weakens the cell envelope by binding divalent cations in the lipid bilayer.

4) Prepare 1 mL of Solution II (0.2M NaOH/1% SDS). Calculate the amounts of NaOH (2 M stock solution) and SDS (10% stock solution). Determine the amount of water required to make the volume up to 1 mL and add the water to a tube **first**. Then, add NaOH and SDS, using a fresh tip for each. Mix well. Add 300 μL of freshly prepared Solution II to each tube. Mix the contents of the tube by inversion 6-8x only. Then, incubate the tubes on ice for 5 minutes.

*SDS-sodium hydroxide - SDS is a detergent which solublises both the lipid components of the cell membrane and the cell proteins, thus lysing the cells. Sodium hydroxide denatures the DNA, both chromosomal and plasmid, although the circular plasmid DNA strands remain intertwined.

*Potassium acetate-acetic acid - Potassium acetate precipitates the SDS from the suspension taking with it the proteins and lipids which are associated with the SDS. Acetic acid neutralises the pH of the solution and causes the renaturation of the plasmid DNA, but only partial renaturation of the chromosomal DNA. This chromosomal DNA tangle becomes trapped in the SDS mixture, and as a result, is precipitated out. Plasmid DNA and RNA (which are both smaller) remain in solution.

5) Neutralise the solution by adding 300 μL of **cold** Solution III (KOAc), mix by inverting the tube, and incubate on ice for 5 minutes.

6) Transfer your mixture to a 2 mL tube . Put on gloves and goggles, move to the fume hood and extract using an equal volume of phenol:chloroform (1:1).

7) Remove the aqueous phase to a new tube. Extract the contents with an equal volume of chloroform.

8) Remove the aqueous phase to a new tube. Add 2.5 volumes of 95% ethanol (you may have to split up the contents into two tubes). Place the tube in the freezer for 10 minutes.

*95% Ethanol vs Isopropanol – Isopropanol is more efficient – an equal volume is added to a

solution of DNA to preferentially precipitate nucleic acids; however, after time it will begin to precipitate proteins. 95% ethanol – a larger volume (2.5x) is required, but precipitation is cleaner

STOP POINT: DNA may be stored in ethanol at -20^{o}C until ready to continue.

8) Spin the tubes in a balanced configuration in a microfuge for 10 minutes at maximum. Discard the supernatant into the labeled waste beaker. Wash the DNA pellet with 500 μL of 70-75 % ethanol, remove as much ethanol as possible, then pulse in the microfuge and draw off the last few drops of ethanol. Allow the tube to sit open on your lab bench for 10 minutes to let the remaining ethanol evaporate off.

*Ethanol - removes salt and any remaining SDS from the solution.

9) Dissolve **all** DNA pellets resulting from 5 mL of culture in 200 μL of Optima-H_2O.

10) Treat 100 μL of DNA **exactly** like a pellet of cells and follow the protocol (steps 1 through 8) found in the GenElute manual. After step 2 – neutralise RIGHT AWAY – there is no need to wait for cells to lyse!

* Do you need the optional wash? Why or why not? Check the genotype of DH5α cells.

11) Elute the DNA in 100 μL Optima-water. Check 5 μL of your DNA on an agarose gel. DNA will also be quantified (using 260/280 ratios) and the four samples having the highest concentration and purity will be sent off to the University of Calgary for DNA sequencing.

The previous protocol is modified from the following references:

Birnboim, H. C. and Doly, J. 1979. A rapid alkaline extraction method for screening recombinant plasmid DNA. Nucleic Acid Res. **7**: 1513.

Micklos, D. A., and Freyer, G. A. 1990. DNA Science A First Course in Recombinant DNA Technology. Cold Spring Harbor Laboratory Press.

Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning A Laboratory Manual. Cold Spring Harbor Laboratory Press.

GUIDELINES FOR SAFETY PROCEDURES

EMERGENCY NUMBERS

City Emergency	9-911
Campus Emergency	2345
Campus Security	2603
Student Health Centre	2484 (Emergency - 2483)

THE LABORATORY INSTRUCTOR MUST BE NOTIFIED AS SOON AS POSSIBLE AFTER THE INCIDENT IF NOT PRESENT AT THE TIME IT OCCURRED

EMERGENCY EQUIPMENT:

Know the location of the following equipment which will be indicated to you at the beginning of the first lab:

1) Closest emergency exit
2) Closest emergency telephone and emergency phone #'s
3) Closest fire alarm
4) Fire extinguisher and explanation of use
5) Safety showers and explanation of operation
6) Eyewash facilities and explanation of operation.
7) First aid kit

GENERAL SAFETY REGULATIONS

1) Eating and drinking are prohibited in the laboratory.
2) Always wash your hands prior to leaving the laboratory.
3) Laboratory coats are required for all laboratories.
4) Report equipment problems to instructor immediately.
5) Report all spills to the instructor immediately.
6) Long hair must be kept restrained to keep from being caught in equipment, Bunsen burners, chemicals, etc.

SPILLS

Spill of ACID/BASE/TOXIN: Contact instructor immediately!

BACTERIA/VIRUS SPILLS: If necessary, remove any contaminated clothing. Prevent anyone from going near the spill. Cover the spill with dilute bleach and leave for 10 minutes before wiping up.

DISPOSAL

Upright Cardboard Boxes:

CLEAN LAB GLASSWEAR - broken glass, Pasteur pipets; NO CHEMICAL, BIOLOGICAL, OR

RADIOACTIVE MATERIALS

Biohazard Bags:

Petri plates, microfuge tubes, tips. All of this material will be autoclaved prior to disposal.

BACTERIAL OR VIRAL LIQUID: Tubes and flasks containing liquid cultures are placed in marked trays for autoclaving.

LIQUID CHEMICALS: Place in labeled bottle

THE UNIVERSITY OF LETHBRIDGE
Policies and Procedures
Occupational Health and Safety

SUBJECT: CHEMICAL RELEASE PROCEDURE

Precaution must be taken when approaching any chemical release.

1. **Unknown/Known Release**

- Clear the area
- Call Security **2345**
- Do not let anyone enter the area
- Call Utilities at 2600 and request the air be turned off at the release site
- Security will immediately notify:

Chemical Release Officer: **331.5201**

Occupational Health and Safety: **394.8937**
394.8716

EMERGENCY CALL LIST 0800 – 1600

2345	**SECURITY**
331-5201	**CHEMICAL RELEASE OFFICER**
2301	**ADMIN. ASSISTANT**
394.8937 **394.8716**	**OCCUPATIONAL HEALTH AND SAFETY**

EMERGENCY CALL LIST 1600 -0800

2345	**SECURITY**
331-5201	**CHEMICAL RELEASE OFFICER**
394-8937 **394-8716**	**OCCUPATIONAL HEALTH AND SAFETY**

IF THE CHEMICAL RELEASE OFFICER CANNOT BE LOCATED CALL:
328-4833 DBS

If the area must be evacuated all employees will be evacuated to the North Parking Lot.

APPENDIX 1 - ASEPTIC TECHNIQUE

Purposes: 1) To prevent the contamination of the environment and people working in the laboratory from the cultures used in the exercises.

2) To prevent accidental contamination of cultures of microorganisms and of solutions and equipment used in the laboratory

Correct methods of handling cultures and apparatus will be demonstrated. These methods should be followed. Consider carefully and remember the following points:

-Prior to starting any work in the laboratory, wash hands with soap, and wash down bench area using 10% bleach. This procedure should be repeated after the lab is complete.

-Avoid working on your lab book or lab notes.

-Clean laboratory coats must be worn. If you have long hair, tie it back before working in the laboratory environment.

-Eating or drinking are not permitted in the laboratory. Do not place pencils, fingers or anything else in your mouth.

-Clean air contains many bacteria and fungal spores carried on dust particles or in water droplets. Any surface exposed to air quickly becomes contaminated. If material is to be kept sterile, it should be exposed only as much as is absolutely necessary for manipulation.

-**Plugs and caps of tubes, tops of Petri dishes and bottles of solutions, (even water!!) must not be laid on the bench nor must sterile containers and cultures be left open and exposed to the air.**

INOCULATION OF CULTURE TUBES

Again, the important thing to remember is that exposure of sterile liquids or bacterial cultures to air **must** be minimised.

-Ensure that you have the tubes, plate of inoculum, inoculating loop and a sterile tube of medium available within easy reach.
-Flame the inoculating loop until red hot. When removing inoculum from a tube, remove the cap from the tube by grasping the cap between the last finger and the hand which is also holding the inoculating needle (Figure 1). **Do not place the cap on the bench!!**

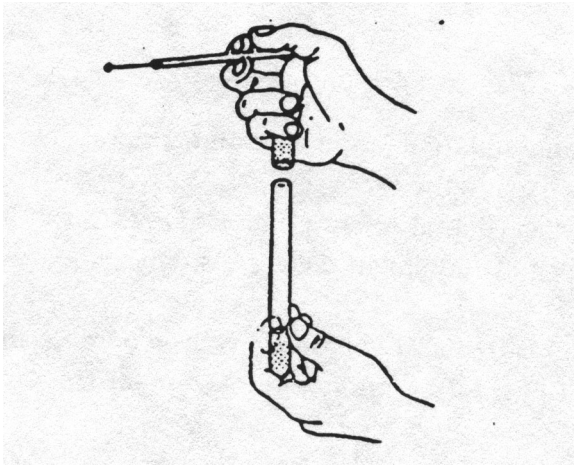


Figure 1.1 Technique for manipulating test tubes aseptically.

-Flame the mouth of the tube by passing it rapidly through the Bunsen burner 2-3 times. This sterilises the air in and immediately around the mouth of the tube.
-Cool the loop on the inside of the tube, remove the inoculum.
-Reflame the mouth of the tube and replace the cap
-Flame the inoculating loop before replacing
-Note, when removing inoculum from a plate, cool the loop in the agar before picking up the bacteria

STREAKING FOR SINGLE COLONIES

-A loop of liquid culture or a small amount of bacterial growth from a plate culture is transferred aseptically to a sterile plate in the area shown by Diagram 1. One of 2 different methods may be followed to produce single colonies.
-Once the first set of streaks have been made, the inoculating loop is reflamed until red hot. **DO NOT REINTRODUCE THE LOOP INTO THE ORIGINAL CULTURE!!!**
-Cool the loop in the agar of the streak plate, and make a second set of streaks as shown in Diagram 2, only crossing over the initial set of streaks once.
-Flame the loop again, cool in the agar, and repeat for 3 more sets (Diagram 3). Note, try not to gouge the agar while streaking the plate.

Diagram 1

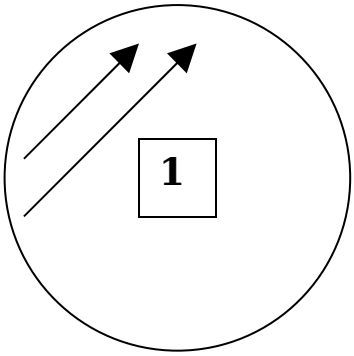


Diagram 2

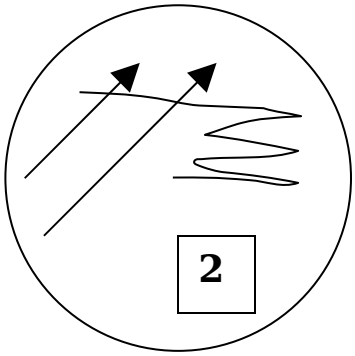
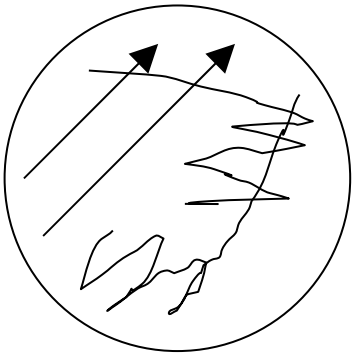


Diagram 3



APPENDIX 2 - ASEPTIC PREPARATION OF LIQUID CULTURES OF BACTERIA ; CULTURE CONDITIONS FOR *Escherichia coli*.

A USING A PLATE CULTURE

Obtain an agar plate containing single colonies of the desired strain of bacteria. Working from single colonies ensures that the resulting culture arose from a single bacterial cell, and therefore consists of a pure culture of only the organism of interest.

Flame an inoculating loop until red-hot. Lift lid of Petri dish at an angle. *Do not put lid down on benchtop - continue to hold it until colony transfer is complete*. Cool loop in agar away from any colonies.

Pick up a colony well separated from any surrounding colonies with the edge of the loop. Close lid of Petri dish.

Have a tube of liquid medium ready. Using little finger, remove cap of tube. *While continuing to hold lid of tube*, flame opening of tube.

Place end of loop containing colony into the broth and swish gently in the medium to dislodge cells. Remove loop and flame top of culture tube again and replace the cap.

Flame the loop until red-hot to kill any remaining bacterial cells.

Incubate 8-24 hours at 37 ^{o}C for *E. coli*; 8-48 hours at 28 ^{o}C for *Rhizobium leguminosarum*.

B SUBCULTURING FROM LIQUID BROTH

Obtain a liquid culture of the bacteria of interest. A fresh overnight culture is best but older cultures may be used.

Obtain the appropriate micropipettor and a sterile tip to add approximately 1/50th the amount of culture to medium and have ready.

Using little finger, remove cap of tube. *While continuing to hold lid of tube*, flame opening of tube.

Tilt tube so that culture moves toward the entrance of the tube. Place tip of micropipettor into culture and carefully draw up the required amount of bacteria. This is done as the barrel of the micropipettor is most likely contaminated with other microorganisms so we want to avoid putting the barrel down into the test tube.

Flame the top of the culture tube and replace the cap.

Remove the top of the new flask of medium and flame the top *without setting down the micropipettor containing the inoculant.* Introduce the inoculant into the flask of fresh medium. Flame the flask opening and replace the top.

Eject tip into the appropriate waste container.

Incubate 8-24 hours at 37 ^{o}C for *E. coli*;.

APPENDIX 3 - DILUTIONS

When asked to prepare a solution of a certain molarity by diluting a more concentrated solution, the formula to use is:

$$C_1V_1 = C_2V_2$$

Where C_1 = initial concentration
V_1 = initial volume (ie, the volume to be used in the preparation of the final concentration)
C_2 = final concentration (the concentration of solution you are asked to prepare)
V_2 = final volume

For example: You have a 2M solution of NaOH and you are asked to prepare 40 mL of a 500 mM solution. How do you go about this?

C_1 = 2 mol/L
V_1 = unknown, so let's say 'x'
C_2 = 500 mmol/L which is equivalent to 0.5 mol/L
V_2 = 40 mL which is equivalent to 0.04 L

Substituting these into the formula, we get:

$$(2 \text{ mol/L})(x \text{ L}) = (0.5 \text{ mol/L})(0.04 \text{ L})$$

Solving for 'x' results in:

$$x \text{ L} = \frac{(0.5 \text{ mol/L})(0.04 \text{ L})}{2 \text{ mol/L}}$$

x = 0.01 L which = 10 mL
Remember, final volume = 40 mL, so 10 mL of your concentrated solution of NaOH should be added to (40 mL - 10 mL) = 30 mL of water (or whatever you are asked to dilute with)

APPENDIX 4 - FINAL CONCENTRATIONS OF ANTIBIOTICS FOR CULTURING *E. coli*

Whenever you are culturing cells containing plasmids or cosmids, it is necessary to maintain what is known as **selection pressure** - ie. a way of ensuring that cells continue to pass on the plasmid or cosmid DNA upon cell division. Expression of plasmid-encoded genes is costly to a cell, so if there is no use for the genes encoded on the plasmid, the plasmid may be lost when the cell divides.

How to Maintain Selection Pressure? Plasmid vectors contain genes coding for resistance to certain antibiotics which are useful in, for example, transformation experiments; they provide a way for a researcher to "track" the presence or absence of that particular piece of DNA. In culturing a strain of bacteria containing a particular plasmid, it is thus necessary to include an amount of the certain antibiotic to which the plasmid encodes resistance. This way, certain genes on the plasmid are necessary for cell survival, and the plasmid is not lost when the cell divides.

When setting up liquid cultures of plasmid-containing strains of *E. coli*, use the following chart as a guideline as to the final concentrations of antibiotic that each organism can tolerate.

Antibiotic	**Stock Conc. (mg/mL)**	**Final Conc. for *E. coli* (μg/mL)**
Ampicillin (Amp)	10	100
Kanamycin (Km)	10	50
Neomycin (Nm)	10	n/a
Streptomycin (Sm)	50	600
*Tetracyline (Tc)	5	10
Gentamycin (Gm)	10	15
*Chloramphenicol (Cm)	10	100

* Dissolved in 100% ethanol. May or may not be filter sterilised.
The rest of the antibiotics are dissolved in d_2H_2O and filter sterilised.

APPENDIX 5
HANDLING OF MICROPIPETTORS

Things NOT to Do!!:

*Do not rotate the volume adjustor beyond the upper or lower range of the pipet

*Do not use the micropipettors without tips in place - this could ruin the precision piston that measures the volume of fluid

*Do not lay down pipettor with filled tip (also, always hold with the filled tip facing down) - fluid could run back into the piston

*Do not let the plunger snap back after withdrawing or ejecting fluid - this could damage the piston

*Do not immerse the barrel of pipettor in fluid

*Do not ever flame a micropipettor tip

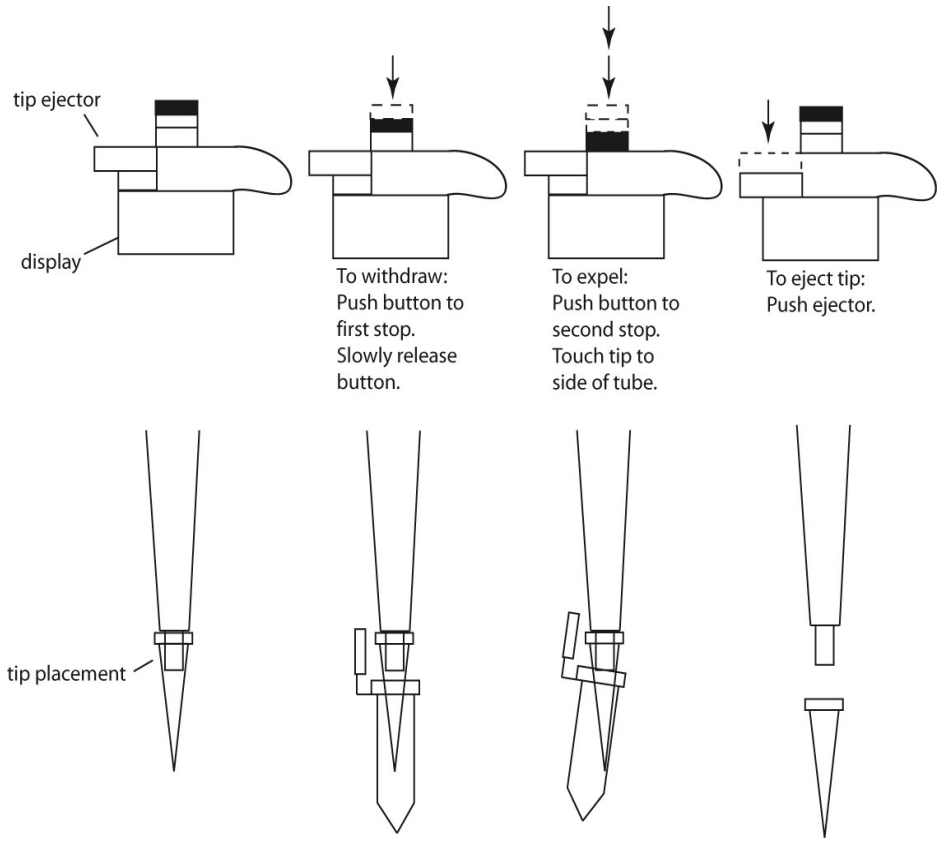


Figure 1.1. Use of Eppendorf Series 2100 Micropipettor. Figure prepared by Katrina White.

I SMALL VOLUME MICROPIPETTOR EXERCISE

Note: Each partner should try out one set of tubes

1) Obtain 2 1.5 mL microfuge tubes. Label as A and B

2) Following the chart below, add the appropriate solutions to each of the tubes

Tube	SolI.(μL)	SolII.(μL)	SolIII.(μL)	SolIV.(μL)
A	2	3	1	4
B	10	5	7	3

*For each solution, use a fresh tip!!

3) Pool and mix the reagents by placing in a microfuge in a balanced configuration (Figure 6.2). Ensure that each volume is balanced with a tube containing an equal volume so balance with tubes from other groups. If tubes are not balanced, this will damage the microfuge motor. Apply a short several-second pulse to the tubes.

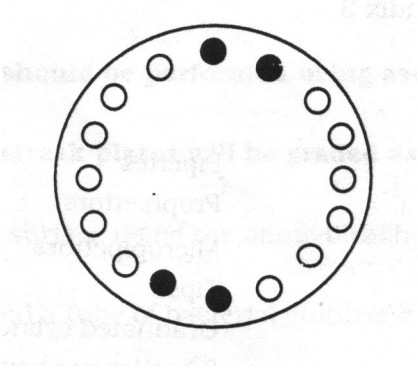


Figure 6.2. Balancing samples in the microfuge rotor.

4) 10 μL and 25 μL were added to tubes A and B respectively. Set your micropipettor(s) to each of these volumes and withdraw the solution from each tube. Evaluate your pipetting technique - is the tip just filled? or is a small volume of fluid left in the tube? or is there an air space left in the tip of the tube?

*While you are doing this exercise, try to note what each volume should look like when in a microfuge tube. Sometimes being able to eyeball a volume is a good indication if your volumes are correct, and can save a great deal of problems in experiments such as PCR where errors in pipetting small volumes can result in the reaction not occurring.

II LARGE-VOLUME MICROPIPETTOR EXERCISE

1) You will now repeat the previous exercise using larger volumes as outlined in the following chart:

Tube	Sol.I	Sol.II	Sol.III	Sol.IV
E	100	200	150	550
F	150	250	350	250

2) Now, pool the samples as above. Set your micropipettor to 1000 μL and withdraw the solution from each tube. Again, evaluate your micropipetting technique as above.

APPENDIX 6
USE OF THE pH METER

Prior to using the pH meter, ensure that you have a supply of Pasteur pipettes, Kimwipes, a squeeze bottle containing d_2H_2O and a waste beaker.

- Dissolve the solute in approximately half of the final desired volume of liquid. Place onto a stirring plate and stir gently.
- Remove the probe from the storage solution. Note that the probe is glass and is **VERY EXPENSIVE TO REPLACE**. Position the probe over a waste beaker and wash with a gentle stream of d_2H_2O.
- Carefully dry off the probe using ONLY Kimwipes (NOT paper towels). Place probe into the liquid in the beaker. Ensure that the stir bar will NOT hit the probe.
- Press the READ button on the pH meter.
- Choose the appropriate solution to pH your solution, and use a Pasteur pipette to add the solution in a dropwise fashion. Allow the needle to stabilise after each drop rather than adding an entire pipetteful of solution.
- Once the required pH is reached, press the STANDBY button on the pH meter.
- Lift the probe out of the solution and rinse off excess solution into the beaker using the d_2H_2O. Be careful that you don't add too much water.
- Dry the probe using a Kimwipe and return to the storage solution.
- Transfer your solution into a graduated cylinder and bring the volume up to the required amount.

APPENDIX 7
AGAROSE GEL ELECTROPHORESIS

Agarose = a linear polymer composed of alternating residues of D- and L-galactose joined by (1 3) and (1 4) glycosidic linkages. These residues form chains of helical fibres that then aggregate into supercoiled structures with a radius of 20-30 nm. When gelled, a meshwork of channels with 50 – 200 nm diameter channels is formed.

Note that commercial preparations of agarose are not homogenous. They may contain salts, proteins, or other polysaccharides; for the recovery of DNA, it may be necessary to use special purified grades of agarose.

Factors Determining Rate of Migration of DNA Through Agarose:

Size of DNA – ds DNA migrates at a rate inversely proportional to the $\log_{10}$ of molecular weight. Big particles move more slowly as they have more drag and cannot move as readily through agarose channels

Concentration of agarose – the higher the concentration, the slower the migration. Concentration of agarose will also affect separation and can be used to enhance separation of different sized fragments of DNA. For instance, the lower the concentration of agarose, the better the separation of larger sized DNA fragments.

DNA conformation – the three forms of DNA: supercoiled (form I), nicked circular (form II) and linear (form III) migrate at different rates through agarose – please see the following web page for an excellent explanation and animation of the three forms and of their migration:

http://bio.classes.ucsc.edu/bio20L/animate/anim2/topo.htm
Under the conditions we make use of, it is assumed that supercoiled migrates fastest through the gel while the other two forms may not be resolveable.

When ethidium bromide is incorporated into agarose, the ethidium bromide intercalates and will slow down (stiffen) the linear DNA molecules

Applied voltage – gels should be run at no more than 5-8 V/cm (note that cm refers to distance between electrodes)

Types of agarose:
Standard – from 2 species of seaweed –: *Gelidium* and *Gracilaria*. Standard agarose can be used for separation of DNA fragments from 1-25 Kb.

Low melting temperature – modified by hydroxyethylation and consequently melts at 65 °C – because of this, low melting temperature agarose can be used for recovery of DNA from the

agarose.
In terms of handling, this agarose is more delicate, and takes a longer time to set or gel.

Buffer – ions are required for DNA migration. Different buffers may be used. We use Tris Acetate EDTA buffer (TAE). Although TBE has one of the higher buffering capacities of the buffers of choice (much greater than Tris Acetate EDTA – TAE for instance, resolution of larger fragments is better in TAE, DNA will move faster in TAE than in TBE, and extraction of DNA from agarose may be more effective when performed on gels made with TAE.

Other Aspects To Consider:

Loading Dyes:

In this laboratory, we make use of mixtures containing Bromphenol blue. This dye migrates in 0.5x TBE at approximately the same rate as linear DNA of 300 bp in size. Often, xylene cyanol FF is used in conjunction with bromphenol blue, or separately. Xylene cyanol FF migrates at approximately the same rate as linear DNA of 4 Kb in size.

Markers:

Size standards are available as commercial preparations; for instance, 1Kb ladder, 100 bp ladder (New England Biolabs, Invitrogen). For an example please see:

http://www.invitrogen.com/content/sfs/manuals/15628019.pdf

As commercial ladder preparations may be expensive, often it is just as useful to use DNA from bacteriophage lambda that has been digested with one or two restriction endonucleases. In this laboratory, we generally make use of lambda digested with the enzyme *Hind*III

Create your own map of lambda *Hind*III by doing the following:

- Go to the web site: http://tools.neb.com/REBsites/index.php3
- At the bottom of the page, choose "**defined oligonucleotide sequences**"
- Under "**Name**", enter HindIII
- Under "**Oligonucleotide sequence**", enter AAGCTT
- Scroll back to the top of the page. On the right is a box containing names of standard sequences. Click on **Lambda**
- Print off the resulting picture showing fragment patterns in 0.7% agarose
- Click on the enzyme name to get exact fragment sizes. Print this information off also and include both pictures in your lab notebook for future reference.

Size Determination:

In order to determine sizes of unknown fragments, we can make use of the relationship between distance migrated (from the wells) and $\log_{10}$ of molecular weight. By plotting $\log_{10}$ of the nucleotide pairs vs distance migrated (in cm or in mm) of our known – for instance lambda cut with *Hind*III, we find that we end up with a curve that is linear for most of the relationship (beyond 1.5 cm migrated). Consequently, by measuring the distance that the unknown migrates, we can use the standard curve to determine size.

APPENDIX 8
QUANTIFICATION OF DNA

Using UV Spectrophotometry

At a wavelength of 260 nm, nucleic acids (DNA or RNA) may be quantified based on the fact that an OD (optical density) of 1 corresponds to a concentration of approximately 50 μg/mL for dsDNA and approximately 40 μg/mL for ssDNA and RNA. For single-stranded oligonucleotides, an OD of 1 corresponds to approximately 33 μg/mL. Spectrophotometric assessment is also used to evaluate purity of nucleic acid preparations. For this, a ratio of the OD_{260}:OD_{280} is used. At 280 nm, aromatic amino acids absorb strongly. Absorbance values between 1.8 and 2.0 indicate that a preparation of DNA is relatively pure. For RNA, a value of greater than 2.0 is desireable. Anything below this value may suggest contamination with guanidinium thiocyanate (found in, for instance, the commercial solution Trizol™). Values lower than 1.8 indicate contamination with phenol or with proteins. Readings at 230 nm (close to the maximum absorbance of peptide bonds and of Tris, EDTA, and other buffer salts) may also be performed to indicate purity of a preparation. Unfortunately, although spectrophotometric quantification is fast, it cannot distinguish between RNA and DNA, and larger concentrations (at least 1 μg/mL) are required for reliable results.

Using Comparitive Intensities on an Agarose Gel

If you have a very low concentration of DNA present in a solution, or you suspect contamination with RNA, quantitation using ethidium bromide may be more accurate. In general, the greater the mass of DNA present, the greater the amount of ethidium bromide that will intercalate. Consequently, the intensity of fluorescence will reflect the mass of DNA present. In the case of lambda digested with *Hind*III, sizes of fragments and proportion of each fragment of the total genome are known. As a result, comparison of intensities of unknown DNA samples in a gel with fragments of lambda may be used to provide a rough estimate of amount of DNA present. To do this, the unknown intensity is compared with that of the fragments of lambda.

For instance, let's say that the unknown band of DNA (5 μL was loaded) has the same intensity as that of the 9.416 Kb band of lambda *Hind*III. If we have loaded 0.25 μg of lambda, we can perform the following calculation:

$$\frac{9.416 \text{ Kb } (0.25\ \mu\text{g})}{48.502 \text{ Kb (= total molecular weight of the lambda genome)}}$$

$$= 0.0485\ \mu\text{g of unknown DNA in 5 } \mu\text{L} = 9.7 \times 10^{-3}\ \mu\text{g}/\mu\text{L}$$

References:

Sambrook, J. and Russell, D. W. 2001. Molecular Cloning: A Laboratory Manual, Third Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Ultrospec 211 *pro* UV/Visible Sprectrophotometer User Manual. Biochrom.

APPENDIX 9
MEDIA AND SOLUTIONS

BACTERIAL MEDIA - Generally, make up in a flask 2x larger than volume of media. Autoclave on liquid cycle, then cool while stirring until flask can be held. Then, add antibiotics to concentrations outlined in Appendix 4.

		LB Broth	
Compound	**Amount for: 1 L**	**500 mL**	**250 mL**
Bacto-Tryptone	10 g	5 g	2.5 g
Bacto-Yeast Extract	5 g	2.5 g	1.25 g
NaCl	10 g	5 g	2.5 g
For Solid Medium: Agar	12.5 g	6.25 g	3.13 g

Solutions

Alkaline Lysis Prep Solutions
Solution I (GTE)
Component (Final Conc.)
50 mM glucose
25 mM Tris/Cl (pH 8.0)
10 mM EDTA (pH 8.0)
d_2H_2O

Solution II (make up just prior to using)

Component	**Stock Solution**
0.2 M NaOH	2 M NaOH (store in a plastic container)
1% SDS	10% SDS (filter sterilise)

Solution III (KOAc/Glacial Acetic Acid)

Component	**Amount for 100 mL**
5 M Potassium acetate	60 mL
Glacial Acetic Acid	11.5 mL
d_2H_2O	28.5 mL

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate

Lysis Buffer

100 mM Tris-HCl pH 8.0
100 mM EDTA pH 8.0
1.5 M NaCl
1% CTAB (hexadecylmethylammonium bromide)

STET

10mM Tris-HCl pH8.0
100mM NaCl
1mM EDTA
5% Triton X-100

50x TAE

Tris Base	242g
Glacial Acetic Acid	57.1 mL
EDTA (0.5 M pH 8.0)	100 mL

Make up to 1L with dH_2O

10x TBE

Tris Base	108 g
EDTA	7.44 g
Boric Acid	55 g
Water	Up to 1L