RECOMBINANT DNA TECHNOLOGY

MBIO 4570

LAB MANUAL

2007

Lab manual is available as a pdf file on the website.

TABLE OF CONTENTS

Description	Page
SCHEDULES	3
GENERAL INSTRUCTIONS	4
GENERAL REFERENCES	8
LAB STANDARD OPERATION PROCEDURES	9
SAFETY	12
INTRODUCTION	15
LAB 1 INSERTIONAL MUTAGENSIS into rhamnose kinase of *Rhizobium*	27
Part I Cloning of rhamnose kinase fragment into pKNOCK	
Part II Transformation of *E. coli* S17-1 with pMR146 pKNOCK-kinase construct	
Part III Triparental Mating via Conjugation	
Part IV Screening of Triparental Mating Transconjugants	
Part V Genomic DNA isolation and Restriction Digestion	
Part VI Southern Blot	
Part VII Chemiluminescent Hybridizaton	
LAB 2 MEGAPRIMER MUTAGENSIS of *Rhizobium leguminosarum* rhamnose kinase	41
Part I PCR Mutant Primer design	
Part II Megaprimer PCR amplification	
Part III QIAquick Gel Extraction of PCR Product	
Part IV Restriction digest and Ethanol precipitation of PCR Product	
Part V PCR product ligation into pBluescript® and E. coli DH5α Ttransformation	
Part VI Culture set up for plasmid DNA preps	
Part VII Sequence analysis of rhamnose kinase mutant	
APPENDIX	
Media	56
Solutions and Functions	57
Pick Plate Grid	61
Pure Culture Methods	62
Determination of Probe Yield by Direct Detection Procedure	64
Pipetman Operation	65
Sample lab exam	67

MBIO 4570 SCHEDULE 2007

Date	Week #	Exp #	Experiment/Procedure
Jan 8	1	1	Part II Transformation of *E. coli* DH5α λ*pir* with pMR146 pKNOCK-kinase construct
Jan 9	1	1	Plating of pMR146 pKNOCK-kinase construct transformants
Jan 12	1	1	-culture inoculation
Jan 15	2	1	Part III Triparental Mating via Conjugation
		2	Part II Plasmid pM106 DNA preparation
Jan 22	3	1	Part IV Screening of Triparental Mating Transconjugants
		2	Part III PCR Megaprimer amplification
Jan 29	4	1	Part V Genomic DNA isolation and Restriction Digestion
		2	Part IV QIAquick Gel Extraction of PCR Product
Feb 5	5	1	Part VI Southern Blot
		2	Part V Restriction digest and Ethanol precipitation of PCR Product
Feb 12	6		MID-TERM BREAK
Feb 19	7	1	Part VII Chemiluminescent Hybridization
		2	Part VI PCR product ligation into pBluescript® and E. coli DH5α Transformation
Feb 23	7	2	Part VII Culture set up for plasmid DNA preps
Feb 26	8	2	Part VII Plasmid DNA preparation Part VIII Recombinant Verification by Restriction digestion and Agarose Gel Electrophoresis Part IX Sequence analysis of rhamnose kinase mutant
Mar 19	11		Question/Answer Lab (attendance is not compulsory)
Mar 26	12		Lab exam

REPORT DUE DATES - PLAN AHEAD reports are not due until end of term due to the length of each experiment - THERE ARE NO EXTENSIONS without subtraction of marks.

Lab	Week	Report DUE
1	9	March 5
2	10	March 12

GENERAL INSTRUCTIONS

Lab Instructor:	Dr. L. Cameron	Office: 414B
Demonstrators:	Chrystal Berry	Lab: 137

Lab Location: 201 Buller Bldg.

WEBSITE: www.umanitoba.ca/faculties/science/microbiology/staff/cameron/
OR via University of Manitoba Microbiology Homepage:
http://www.umanitoba.ca/faculties/science/microbiology/labinformation.htm
Information available at the website: changes/corrections, additional information, data, marks

REGULATIONS
1. Lab attendance is compulsory. Please inform the instructor if you are unable to attend a lab.
2. Students must wear a lab coat. Bring a permanent marker.
3. There is no smoking, drinking, or eating in the lab.
4. Students work in pairs.
5. The lab is opened Monday to Friday from 7:30 am to 5:00 - 5:30 pm. Check the lab schedule posted on lab door for open lab times as many of MBIO 4570 labs are continued throughout the week.

EVALUATION
1. The lab is worth 20% of the final course mark, 8% for lab reports and assignment and 12% for lab examination. There are no marks given for data handed in, but marks will be subtracted for data not handed in.
2. You must pass the lab to pass the course (10/20%).
3. The lab reports due dates are listed in the schedule.
4. Lab reports are to be handed in as stated in the schedule by 4:30 p.m. of that day. Hand in lab reports through slotted filing cabinet in room 204 Buller ONLY. Instructor and demonstrators do not accept lab reports. If handing in lab late, 1 mark will be subtracted for each class day late. Marked lab reports will be returned to students the next week. **A late report will not be accepted after that report has been returned to the class.**
5. Lab report marks are final unless an obvious error in addition of marks has been made. However, if a student feels they have a legitimate complaint, please direct attention to lab instructor.
6. Approximately two weeks prior to the lab exam, a brief outline of lab exam format and information content will be available on the website.
7. You must notify the lab instructor no later than two school days after the missed lab. A Doctor's certificate is required for a missed lab exam. All deferrals will write the lab exam at a scheduled time set by the instructor. Failure to comply will result in a zero on your lab exam.
8. **Plagiarism (copying another student's lab report (present or previous year) or copying published literature without citing is a violation of University regulations. Refer to the STUDENT DISCIPLINE BY-LAW in your student handbook (rule book) for action taken for plagiarism.**

LAB REPORT PRESENTATION

[Before handing in your report review report to ensure that all information is included. When printing Excel spreadsheets make sure you have selected all information before printing. If you are using text boxes, they must be completely within the selected area or they do not print.]

1. All reports must have an Honesty Declaration attached at end of report. Available as a pdf file on lab website.
2. A reference file is available in the science library (2 hour reserve).
3. Lab reports must **typed.** Up to 10% of the mark subtracted for reports not typed.
4. Number pages.
5. On the front page of the report state:
   - Course name and number
   - Experiment number and Title
   - Group # and section #
   - Individual or Group name(s). If handing in an individual report, also include lab partners name.
   - GROUP report or INDIVIDUAL report
   - Date
6. Lab report information is to be presented exactly as requested in lab manual. Number sections the same as the lab manual.
7. Lab report may be done as an individual effort or a group effort by the students in each group that carried out the experiment. Student in two different groups cannot submit a group report together. One report or more reports may be handed in per group. The decision on the number of reports per group is totally dependent on members of the group. This decision may be changed any time during the term. Therefore for each lab report the group has the option to hand in one or more reports exclusive of what has been done before or after that particular report. **Indicate on the cover page of the report if the report is a group report or an individual report.** If handing in an individual report also include lab partner's name.
8. Always include a sample of each type of calculation in your lab report.
9. If a group's data is not workable, borrow data from another group and reference. Non workable refers to data that cannot be plotted, used for calculations or required analysis. It does not necessarily mean the expected data.
10. Cite reference in text of lab report and record full reference at end of lab report. When should you cite and reference. The following is a good definition of plagiarism that explains when you should cite a reference. "**The unacknowledged use of another person's work, in the form of original ideas, strategies, and research, as well as another person's writing, in the form of sentences, phases and innovative terminology**." (Spatt[1], 1983, p.438) This is done by using bracketed reference number that you used when listing references at end of lab report or by bracketing first authors name and date. Quote text unless you paraphrase completely in your own words. But remember, quotes should only be a small part (~5%) of your work. If you are using the name year system, list the references alphabetically. Some examples are as follows (McMillan[2] 1997):

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[1]Spatt, B. (1983). *Writing from Sources*. New York: St. Martin's Press.

[2]McMillan V.E. 1997. Writing Papers in the Biological Sciences. 2nd ed. Boston: Bedford Books: 1997. 197 p. and McMillan, V.E. 2001. Writing Papers in the Biological Sciences. 3rd ed. Boston: Bedford Books. 123 p.

Binder V. Hendriksen C, Kreiner S. 1985. Prognosis in Crohn's disease - - based on results from regional patient group from county of Copenhagen. Gut 26:146-50.
Danforth DN, editor. 1982. Obstetrics and gynecology. 4th ed. Philadelphia: Harper and Row. 1316 p.
Petter JJ. 1965. The lemurs of Madagascar. In: DeVore I, editor. Primate behavior: field studies of monkeys and apes. New York: Holt, Rinehart and Winston. p 2920319.

If journal article assessed on the internet, site as journal. However, if available only on the web, reference as follows:
Kingsolver JC, Srygley RB. Experimental analyses of body size, flight and survival in pierid butterflies. Evol. Ecol. Res. [serial online] 2000;2:593-612. Available from: Colgate University online catalog. Accessed 2000 Oct 3.

11. Personal or Professional Electronic sources[2]:
Cite in-text by putting the following in parentheses, author's last name or file name (if no author's name is available) and publication date or the date of access (if no publication date is available).
At the end of report list: author or organization, publication date or date last revised, title of Web site,URL site in angle brackets, and the date accessed.
Cameron, L. 60.344 Microbial Physiology Lab Information
<http://www.umanitoba.ca/faculties/science/microbiology/staff/cameron/60_344.htm>. Accessed 2004 April 12.

Table presentation (if format not available on the website)

- Table number and title (legend) presented above the table body.
- Number tables using arabic numbers, even if only one table in a report.
- Include enough information in title to completely describe table, eliminating the necessity to search elsewhere in the lab report to understand information presented in table. Table title starts with an incomplete sentence. Additional complete sentences may be included to adequately describe the table (this also applies to figures).
- If abbreviations are used in table, indicate what abbreviations mean as a footnote. Other footnotes may be required to clarify material in the table.
- Like information should be in columns making it easier to view the table.
- Data in columns should be listed under the centre of each heading. Align decimal points and dashes. If a number value is less than 1 always include zero before the decimal.
- Column or Row headings should be complete and self explanatory. A heading is a separate entity from the title. It cannot be assumed information given in the title is adequate for a heading. The unit of measurement should only be included in the heading, not in column data.
- Group related column headings under larger headings.
- If information is the same for each column or row do not include but treat as a footnote.
- Make the table as concise as possible but include all necessary information. For example, any constant experimental conditions that would change the data presented.
- Tables should be properly set up with a straight edge. Horizontal lines must be included but it not necessary to always include vertical lines.

Figure presentation (graphs, diagrams, photographs, films)
(all graphs must be computed generated - where applicable, required Excel presentation procedure is given in the lab appendix)

- Hand drawn or computer generated are both acceptable.
- Figures are to be numbered separate from tables, using arabic numbers. Include figure number even if only one figure.
- Following the figure number a figure legend should be presented below graph. The figure legend, like the table, starts with an incomplete sentence describing the graph. For example, do not repeat just the labels of the x- and y-axis but present in a descriptive manner. Additional sentences should be included if additional information is required to completely describe figure, for example, abbreviations explanation, any constant experimental conditions, etc.
- All diagrams, photographs, and films are figures and should be completely labelled. For figures of graphs (use small grid graph paper), there is one dependent variable plotted and one or more independent variables plotted. The dependent variable is a function of the independent variable. It is accepted practise to plot the independent variable on the x-axis and the dependent variable on the y-axis. For example the measurement of absorbance (dependent) with increasing concentration of protein (independent). The size of the graph should fit the plot(s). The axis should not necessarily start at zero. Place graph completely within graph grid, this includes axis labels and legend. The overall size of graph should not be too large but should not be so small that information is obscured. Graph must be completely labelled (always include units). Use different symbols for each plot (not different coloured pens) on a graph. If more than one plot explain symbols in legend or in a key included in the body of the graph. Graph plots can be drawn in a number of ways (this depends on the plot): (a) best straight line, (b) join the points with a straight line, and (c) use a curved ruler or french curve.
  Note: Do not drawn a free hand line.
- Completely label diagram figures.

Note: When writing your lab reports you are frequently requested to present both a table and a figure for a given set of data, similar to keeping a research lab journal. This is not the accepted practice for papers published in journals or books. Usually either a table or a figure is presented for a given set of data and depending on nature of data; it may only be summarized in the text. How do you make a choice of data presentation? The aim is to effectively and efficiently demonstrate what you want to show, for example, correlations, comparisons, pattern, trends, etc.

Note: references listed below each experiment are those references used to prepare experiment. Majority of reference material required for lab reports are located in reference file (see below) or available on internet.

NUMBER	**REFERENCE DESCRIPTION**
1	Oresnik, IJ, Pacarynuk, LA, O'Brien, SA Yost, CR and Hynes, MF. 1998. Plasmid-Encoded Catabolic Genes in *Rhizobium leguminosarum* bv. *trifolii*: Evidence for a Plant-Inducible Rhamnose Locus Involved in Competition for Nodulation. Mol. Plant Microbe Interact. 11:1175-1185.[available on-line, UM NetDoc via Goggle Scholar, etc. Also linked on lab website.]
2	Richardson, JS, Hynes, MF, Oresnek, IJ. 2004. A Genetic Locus Necessary for Rhamnose Uptake and Catabolism in*Rhizobium leguminosarum* bv. trifolii. J. Bact. 186:8433-8442 [linked on lab website or available on-line, search ASM journals]
3	Alexeyev, MF. 1999. The pKNOCK series of broad-host-range mobilizable suicide vectors for gene knockout and targeted DNA insertion into the chromosome of Gram-negative bacteria. BioTechniques 26:824-828.
4	Sambrook, J, Russell, DW. 2001. Chapter 13 Mutagenesis. In Molecular Cloning A Laboratory Manual 3rd edition. Cold Spring Harbor: CSHL Press p 13.1- 13.10, 13.31-13.35

LAB STANDARD OPERATIONS PROCEDURE (SOP)
Safety information relevant to the Microbiology department is available at http://www.umanitoba.ca/faculties/science/microbiology/WHMISworkshop.htm

Bench area: Wash bench area before and after use with AIRx109.

Personal safety: You must wear a lab coat. Wear coat only in the lab, transport separately outside of the lab (in a plastic bag). Wash hands with antibacterial soap before leaving the lab. No eating or drinking in the lab. Use aseptic technique for transfer of bacteria. This is to protect yourself as much as to ensure the purity of your culture. Protect hands with gloves and eyes with glasses when needed. The gloves provided in the lab are to be disposed in Petri plate containers. . If you forget your lab coat, dark blue lab coats are available in the lab, sign IN/OUT sheet. Long hair must be tied back. Closed toed shoes must be worn.

Biohazards: Know biosafety risk groups. Handle all cultures as potential pathogens. Never mouth pipette. Always use a pro-pipette. If you spill a culture, cover the spill with paper towels. Pour AIRx109 over the towels to saturate. Gather up soaked towels and discard. Wipe area to dryness with fresh paper towels. Wash hands with soap and water. Place cultures on discard trolley. All cultures are autoclaved before disposing. Dispose of eppendorf tubes[a] in petri plate containers. Dispose of pipetman tips[a] in clear plastic lined basins along with glass or plastic Pasteur pipets, broken glassware, glass slides, brittle plastic objects, metal objects[a] (not needles or blades). Discard disposable pipettes (1 ml and 10 ml) in yellow plastic pails. Bacteria dilutions may to be poured down the sink and the tubes rinsed before placing on the discard trolley. Rinse sink with lots of water.
When handling level 2 microorganisms you must wear disposable gloves, make sure any cuts on your hands are covered with a bandage, and be aware of the possibility of bacteria aerosol when you flame your loop.
[a] due to the multi-use nature of the teaching lab, all eppendorf tubes, pipetman tips, Pasteur pipets, brittle plastic or metal objects will be treated the same as similar items contaminated with microorganisms.

Glassware (unbroken): Remove tape and pen markings (use alcohol) from glassware before placing on discard trolley. Used glassware should be rinsed and placed on the discard trolley. Rinsed test tubes should be placed in tray provided on the discard trolley. Used glass pipettes should be placed in pipette holders.

Petri plate culture and non-sharps solid culture material disposal: use covered plastic containers lined with clear plastic bags for contaminated petri dishes or any bacteria contaminated solid non-sharps material (eppendorf tubes, API strips, antibiotic strips, microtitration plates, etc)

Hazardous material disposal: Examples: radioactive material, ethidium bromide, sovents, etc. The lab demonstrator will instruct proper disposal methods for labs that contain hazardous materials. These materials must be disposed of in appropriately labelled containers and disposed via the safety office. Use fumehood when recommended. A MSDS binder available in lab gives information on all hazardous materials used in the lab. Use extreme care with flammable solvents. Alcohol used to flame spread rod should never be positioned within 40 cm of flame. Never put a very hot spread rod into a beaker of alcohol. The alcohol may catch fire. Many of the immunochemicals are preserved in 0.1% Na azide...handle with gloved hands. Handle caustic

(acids and bases) solutions with care. Never discard an acid or base greater than one molar down the sink. Discard in labelled glass containers provided. Use lots of water when discard caustic solutions (< 1M). These materials are disposed of through the university safety office. Never pour solvents down the sink (eg. phenol, ether, chloroform, etc). Discard in labelled containers provided. When handling Etidium Bromide wear N-Dex nitrile gloves as general supply of disposable gloves do not protect again EtBr. N-Dex nitrile gloves also protect against splashes up to 37% HCl and 50% NaOH.

Sharps disposal: Dispose of all sharps (needles, syringes, razors, scalpel blades) in specified container. Dispose of syringe with needle attached - do not take apart. Do not replace the needle cap before disposing (high frequency of accidents occur when replacing cap). Sharp's containers are autoclaved before disposing. .

Broken glass disposal: Dispose of broken glass in labelled plastic containers lined with clear plastic. Transferred to boxes before discarding.

Know location: Exits, fire extinguisher, eye wash, sink shower, and first aid kit. This information is given in the first pre-lab.

Equipment operation: Know how to operate equipment before use. DO NOT use equipment unless you know exactly how to operate the equipment. The demonstrator is always available to assist. Please follow instructions in appendix for proper clean up of Spectronic 20D. Ensure the spec tubes are thoroughly washed and rinsed with distilled water before replacing in rack upside down as you (hopefully) found the tubes.

Leave your bench area clean All equipment and supplies should be returned to original location.

LABORATORY BIOSAFETY GUIDE

In this lab you use only Level 1 bacteria risk group. However, level 2 bacteria may be used by other labs in this room. Follow standard operation procedures, SOP (see above).

The University of Manitoba Biosafety Guide (Feb 2000) and Health Canada Laboratory Biosafety Guidelines booklets are available in your lab. Biosafety information is also available at the Health Canada websites:

Guidelines: http://www.hc-sc.gc.ca/pphb-dgspsp/ols-bsl/lbg-ldmbl/index.html Health Canada http://www.umanitoba.ca/campus/health_and_safety/
MSDS (infectious agents):http://www.hc-sc.gc.ca/pphb-dgspsp/msds-ftss/index.html

There is no listing of level 1 agents in the guidelines or MSDS pamphlets
Risk group 1 bacteria are low individual and community risk and are unlikely to cause disease in healthy workers.
Risk group 2 bacteria are moderate individual risk and limited community risk. Bacteria in this group can cause human or animal disease but are unlikely to infect healthy laboratory workers. Effective treatment is available. Risk of spreading is limited.

CONTAINMENT LEVEL 1 (UM biosafety guide p. 11)

- microbiology lab with washable walls, countertops and hand wash sink
- established safe laboratory practices (hand washing and disinfection of countertops)
- general WHMIS safety training
- UM lab registration

CONTAINMENT LEVEL 2 (UM biosafety guide p.11)

- all of level 1 specifications
- biosafety permit
- biological safety cabinet (not required)
- biohazard sigage
- a written standard operations procedure
- MSDS for the infectious agent

HAZARDS AND SAFETY MEASURES

CULTURES: Handle all cultures as potential pathogens. Never mouth pipette. If you spill a culture, cover the spill with paper towels. Pour AIRx109 over the towels to saturate. Gather up soaked towels and discard. Wipe area to dryness with fresh paper towels. Wash hands with soap and water. In labs where bacterial cultures are used, it is advisable to always wash hands with soap and water before leaving.

KNOW LOCATION: Fire extinguisher, eye wash, and sink shower.

PROTECTION: Protect hand with disposable gloves and eyes with
glasses whenever recommended in the prelab. Wear a lab coat. Always use a pro-pipette.

EQUIPMENT: Know how to operate equipment before use. DO NOT use equipment unless you know exactly how to operate the equipment. The demonstrator is always available to assist.

UV LIGHT: Protect your eyes against UV light. Exposure to UV (near UV, 315-400 nm; mid-range UV, 280-315 nm; and far UV, 200-280 nm can cause acute eye irritation after a latent period of 0.5 h to 24 h. The latent period depends on the intensity of the exposure. Possible symptoms are sensation of sand in the eyes, tearing, sensitivity to light, difficulties in opening eyelids, temporary reduced visual acuity, etc. Usually disappearing in 48 hours. Students who wear plastic eye glasses do not need to wear protective glasses, although it is a good idea to still wear plastic goggles with side shields to further protect eyes. Skin is also sensitive to UV. Do not put your hands near strong UV light for any length of time and cover skin by wearing a lab coat. Keep exposure to UV light to a minimum.

DANGEROUS CHEMICALS: Use fumehood when recommended. Check label for necessary precautions if unfamiliar with the chemical. Use extreme care with flammable solvents. Alcohol used to flame spread rod should never be positioned within 40 cm of flame. Never put a very hot spread rod into a beaker of alcohol. The alcohol may catch fire.

> Chloroform: This chemical is a nonflammable, heavy, very volatile compound which is miscible with alcohol, benzene, and ether. Handle chloroform with care. Incorrect mixing of chloroform with other chemicals may result in serious accidents. Do not mix chloroform with acetone in basic solution. Mixing of chloroform with strong base or chlorinated hydrocarbons may result in explosion. Chloroform is an oral poison, poisonous to the central nervous system, and a skin and eye irritant. American Conference of Governmental Industrial Hygienists considers chloroform (10 ppm) a suspected human carcinogen.

WHMIS

The Workplace Hazardous Materials Information System (WHMIS) is a system for safe management of hazardous materials. WHMIS is legislated by both the federal and provincial governments.

Under WHMIS legislation, laboratories are considered to be a workplace, and students are workers. By law, all workers must be familiar with the basic elements of the WHMIS system.

The WHMIS program includes:
1. Cautionary labels on containers of controlled products. Consumer products, explosives, cosmetics, drugs and foods, radioactive materials, and pest control products are regulated separately, under different legislation.
2. Provision of a Material Safety Data Sheet (MSDS) for each controlled product.
3. A worker education program

1. A. **SUPPLIER LABELS**

Controlled products must have a label of prescribed design which includes the following information:

PRODUCT IDENTIFIER - trade name or chemical name
SUPPLIER IDENTIFIER - supplier's name and address
MSDS REFERENCE - usually, "See MSDS supplied"
HAZARD SYMBOL - (see illustration on next page)
RISK PHRASES - describes nature of hazards
PRECAUTIONARY MEASURES
FIRST AID MEASURES

B. **WORKPLACE LABELS**

All material dispensed in a workplace container must be labelled with the **Product Name, Precautionary Measures** (simplified) and **Reference to Availability of MSDS**.

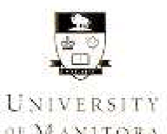
2. **MSDS**

Individual course MSDS are located in a binder in your lab (Room 201 binder located in 204). The main MSDS binders are located in the Microbiology preparation room, 307/309 Buller. MSDS are also available on the local area computer network (see your demonstrator, if necessary).

The MSDS will provide: relevant technical information on the substance, chemical hazard data, control measures, accident prevention information, handling, storage and disposal procedures, and emergency procedures to follow in the event of an accident.

3. **SAFETY**

The Laboratory Supervisor will provide information on the location and use of safety equipment, and emergency procedures.



UNIVERSITY OF MANITOBA

WHMIS

(WORKPLACE HAZARDOUS MATERIALS INFORMATION SYSTEM)

HAZARD CLASSIFICATION — SYMBOL

CLASS A - COMPRESSED GAS

CLASS B - FLAMMABLE AND COMBUSTIBLE MATERIAL
1. FLAMMABLE GAS
2. FLAMMABLE LIQUID
3. COMBUSTIBLE LIQUID
4. FLAMMABLE SOLID
5. FLAMMABLE AEROSOL
6. REACTIVE FLAMMABLE MATERIAL

CLASS C - OXIDIZING MATERIAL

CLASS D - POISONOUS AND INFECTIOUS MATERIAL
1. MATERIALS CAUSING IMMEDIATE AND SERIOUS TOXIC EFFECTS
2. MATERIALS CAUSING OTHER TOXIC EFFECTS
3. BIOHAZARDOUS INFECTIOUS MATERIAL

CLASS E - CORROSIVE MATERIAL

CLASS F - DANGEROUSLY REACTIVE MATERIAL

WORKPLACE LABELS

These labels are applied at the workplace on controlled products or containers of controlled products when

A. The original supplier label is missing or becomes unreadable.
B. The material is decanted or transferred from the supplier's container to another container.
C. The material is produced at the workplace for use within the workplace.

Workplace labels will include the following information

1. **Name/Identity of the Product**
2. **Safe Handling Information** - refers to any precautions that must be followed to minimize the risks of adverse health effect or injury. This also includes any personal protective equipment (PPE) e.g., type of gloves, eye or respiratory protection to be worn or other controls to be used through statements or pictograms (symbols)
3. **Reference to the MSDS.**

SUPPLIER LABELS

Supplier labels on a controlled product must be in English and French and include the following information

1. Product Identifier
2. Hazard Symbol(s)
3. Risk Phrase(s)
4. Precautionary Measures
5. First Aid Measures
6. Supplier Identifier
7. Reference to the availability of a Material Safety Data Sheet

This type of border (shown) is the indicator that the label identifies a controlled product.

MATERIAL SAFETY DATA SHEET

A MATERIAL SAFETY DATA SHEET (valid for three years) will include information relating to each of the following categories

1. Product Identification and Use
2. Hazardous Ingredients
3. Physical Data
4. Fire and Explosion Data
5. Reactivity Data
6. Toxicological Properties
7. Preventive Measures
8. First Aid Measures
9. Date and Source of MSDS

and any other hazard information of which the Supplier is aware or ought reasonably to be aware.

FIRE / MEDICAL EMERGENCIES

555 from 474, 789, 975, 977 exchanges or
#555 from a cell phone (MTS or AT&T)
or
911 from other exchanges. If 911 is called you must also call Campus Security at
474-9341

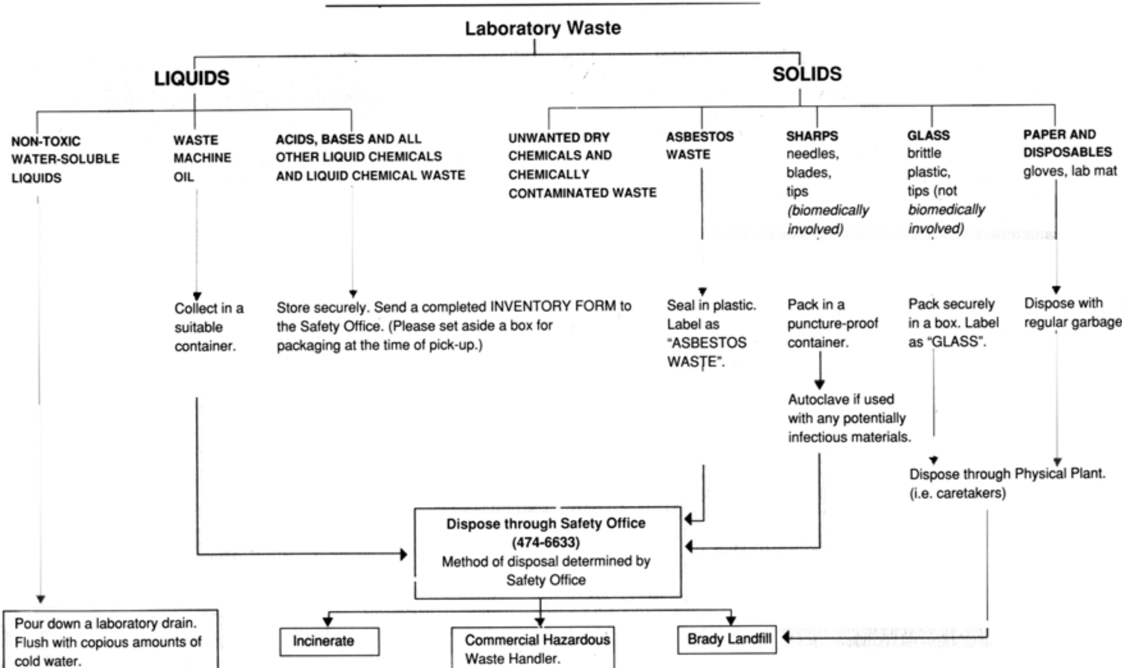
CHEMICAL / BIOHAZARDS EMERGENCIES

474-6633

(8:30 am - 4:30 Mon. to Fri.). If busy or after hours, call
555

For further information on WHMIS, contact the ENVIRONMENTAL HEALTH AND SAFETY OFFICE at 474-6633.

MANITOBA WORPLACE HEALTH HAZARD REGULATION 53/88 REQUIRES THAT A COMPLETE AND CURRENT CHEMICAL INVENTORY IS MAINTAINED AT ALL TIMES.



NO CHEMICALS ARE TO BE LEFT FOR THE UNIVERSITY OF MANITOBA CUSTODIAL STAFF. Empty reagent bottles are to be rinsed and have the labels de-faced. All potentially infectious materials (*biomedically involved*) must be autoclaved or de-activated using a chemical sterilizing agent prior to disposal. Animal carcasses are to be incinerated. Radioisotope users should consult the University of Manitoba "Waste Disposal Chart For Radioisotope Users".

THE ABOVE CHART IS A GUIDE, MORE INFORMATION IS AVAILABLE THROUGH THE OCCUPATIONAL HEALTH & SAFETY OFFICE (474-6633).

October 1993

INTRODUCTION

Rhizobium leguminosarum has the ability to utilize rhamnose as a sole carbon source. The ability to metabolise rhamnose is linked to the cells ability to compete for legume nodulation[3] in the rhizosphere. Richardson et al (manuscript in preparation[4]) have demonstrated that catabolism of rhamnose in *Rhizobium leguminosarum* is by a novel pathway. In *Rhizobium leguminosarum* rhamnose catabolism is initiated by the phosphorylation of rhamnose (rhamnose kinase, RhaK) before isomerization and dehydrogenation. Rhamnose transport is via ABC transport mechanism[5] and is dependent on a functional rhamnose kinase, RhaK[6].

In your lab you will carry out two mutagensis experiments, insertional and megaprimer, exposing you to a wide variety of recombinant DNA techniques. Richardson[4] used insertional mutagensis to create site-directed and double mutations in rhamnose catabolism genes. Using the pKNOCK double mutants they[4] were able to demonstrate that phosphorylation of rhamnose occurs first followed by isomerization and dehydrogenation. Megaprimer mutagenesis was used to demonstrate that transport of rhamnose is dependent on a functional kinase[4].

HOMOLOGOUS RECOMBINATION VIA TRIPARENTAL MATING

pKNOCK[7]

pKNOCK, a suicide vector (figure 1), allows the investigation of un-characterized DNA open reading frames. One way to specifically inactive a gene is to insert a suicide vector into the gene. The advantage of the suicide plasmid, pKNOCK, is that it has a polylinker with numerous restriction enzyme sites and can be introduced into the recipient strains via transformation, electroporation or conjugation. In our lab, conjugation is used as it is a very efficient method of DNA introduction into the recipient, virtually 100%. Another advantage is that DNA enters the recipient cell as a single strand which is much more effective at homologous recombination when compared to double stranded DNA (transformation and electroporation). Also conjugation is the only ‘easy’ way of transferring DNA from *E. coli* to *Rhizobium*. pKNOCK plasmid (2.2 kb) also contains a marker gene, in our case the marker is Tc (tetracycline) and origin of replication, R6K γ-ori. The suicide nature of the plasmid is contributed by the R6K γ-ori since

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[3]Oresnik, IJ, Pacarynuk, LA, O’Brien, SA Yost, CR and Hynes, MF. 1998. Plasmid-Encoded Catabolic Genes in *Rhizobium leguminosarum* bv. *trifolii*: Evidence for a Plant-Inducible Rhamnose Locus Involved in Competition for Nodulation. Mol. Plant Microbe Interact. 11:1175-1185.

[4]Richardson, JS, Hynes, MF, Oresnek, IJ. 2004. A Genetic Locus Necessary for Rhamnose Uptake and Catabolism in*Rhizobium leguminosarum* bv. trifolii. J. Bact. 186:8433-8442
[available on-line search ASM journals]

[5]Locher, KP, Lee, AT, Rees, DC. August 2002 ABC Transporter Architecture and Mechanism. http://www-ssrl.slac.stanford.edu/research/highlights_archive/rees_abc.pdf Accessed August 2004.

[6]Berg, JM, Tymoczko, JL, Stryer L. Covalent modification is a means of regulating enzymes. Biochemistry 5th ed. WH Freeman and Co. http://www.ncbi.nlm.nih.gov/books/bv.fcgi?rid=stryer.section.1363 accessed August 2004

[7]Alexeyev, MF. 1999. The pKNOCK series of broad-host-range mobilizable suicide vectors for gene knockout and targeted DNA insertion into the chromosome of Gram-negative bacteria. BioTechniques 26:824-828.

replication can only occur in *E. coli* strains that have the π protein encoded in *trans*. Mobilization (mob) site is RP4 oriT. This is the origin of transfer site for conjugation.

pMR146

An EcoR1 465 bp centre fragment[8] of *Rhizobium leguminosarum* rhamnose kinase (rhaK) from plasmid pMR106 was ligated into the polylinker site of pKNOCK plasmid. The ligation mixture was used to transform competent *E. coli* strain DH5α (Str^{s} Pro^{-}) resulting in the isolation

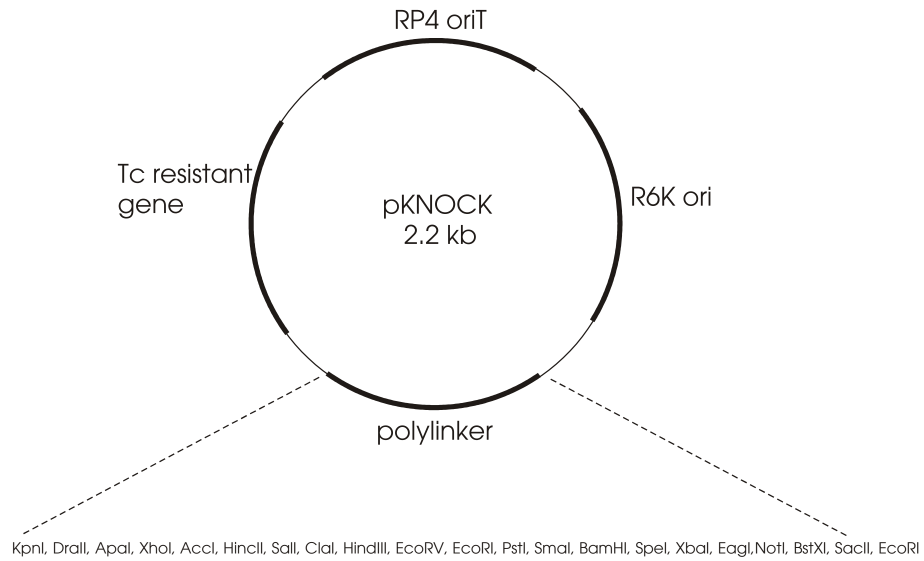

Figure 1. pKNOCK plasmid with tetracycline resistance gene (Tc), polylinker site with unique restriction enzyme sites except EcoR1I with Tc marker, R6K ori the plasmid origin of replication in permissive *E. coli* strains carrying □ gene in *trans* and RP4 oriT the origin of transfer of single stranded DNA during conjugation.

of Tc^{r} *E. coli* clone containing plasmid **pMR146 (pKNOCK-kinase fragment**).

pRK600 helper plasmid

E. coli MT616 ($Str^{s}Pro^{-}Tet^{s}$) containing the helper plasmid pRK600 is added to the conjugation mixture of *E. coli* donor containing plasmid pMR146 and *Rhizobium leguminosarum* recipient strain Rlt100 (wild type). Refer to any good molecular biology book for a review of conjugation, eg. Genes[9] series. The helper plasmid pRK600 is Mob^{+} Tra^{+}. pRK600 also has an ColE1 origin of replication and origin of transfer (mob). ColE1 only allows plasmid replication in *E. coli* not *Rhizobium*.

Recipient *Rhizobium leguminosarum* strain

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[8] The *Rhizobium leguminosarum* rhamnose kinase (rhaK) is available at the NCBI blastn site or experiment 2 in thi lab manual. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=Nucleotide&list_uids=37528956&dopt=GenBank It contains the complete sequence of the rhamnose catabolite genes and the gene of interest to use, rhamnose kinase rhaK coding sequence is bp 9686 to bp 11065. The 465 bp EcoR1 fragment is located at bp 10124 to bp 10589 in rhaK gene. EcoR1 only cuts at these sites in the rhaK gene.

[9] Lewin, B. 2004 Genes VIII Upper Saddle River: Pearson Prentice Hall. p.366-367, 380, etc.

Rhizobium leguminosarum Rlt100 is used in the triparental conjugational mating as recipient. *R. leguminosarum* Rlt100 is Str^r (streptomycin[10] resistant) and wild type with respect to rhamnose kinase.

Why is the helper plasmid required for conjugation?

What is really needed from the helper plasmid is the Tra transfer genes even though pMR146 is a mobilization plasmid the efficiency of transfer is greatly increased in the presence of helper plasmid. The Tra gene serves numerous functions that facilitate conjugation, phili formation, cell contact maintenance, unwinding of DNA and nicking the oriT (mob) which initiates linear transfer of single stranded DNA into recipient.

Conjugation

All three strains are added to the conjugation mixture (figure 2). *E. coli* containing the helper plasmid pRK600 cannot successful conjugation (no homologous regions) and cannot be maintained in *Rhizobium leguminosarum* with only the ColE1 origin of replication. The Tra^+ genes on plasmid pRK600 cause formation of phili and nick DNA at oriT(mob) and unwinds DNA. A single strand of plasmid pRK600 is transferred into *E. coli* DH5 containing pMR146 pKNOCK-kinase plasmid. Now there are two plasmid in *E. coli* DH5. The Tra^+ genes on pRK600 again are involved in phili formation on *E. coli* DH5 and nicking of RP4 ori T DNA originating transfer of pMR146 into recipient *Rhizobium leguminosarum*. In the recipient homologous recombination (figure 3) occurs between rhamnose kinase fragment on pMR146 plasmid and recipient genomic DNA inserting pKNOCK genes (Tc-mob-ori) between the 5' and 3' ends of rhamnose kinase. Homologous recombinants are selected on Str Tet medium. This selects against all individual strains as both *E. coli* strains are streptomycin sensitive and recipient *Rhizobium leguminosarum* is tetracycline sensitive.

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[10]stretomycin may also be abbreviated as Sm similar to tetracycline, either Tet or Tc

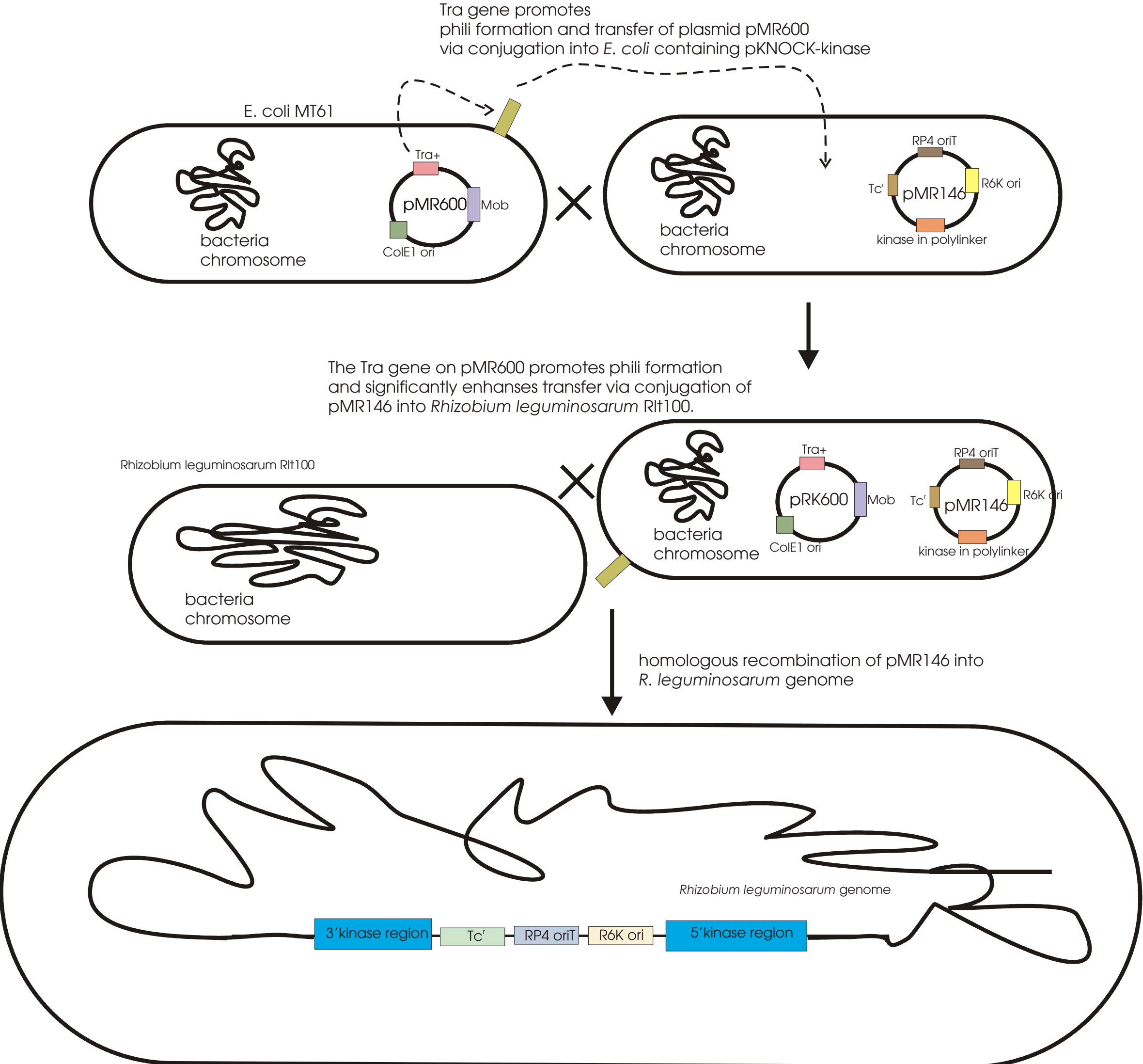

Figure 2. Triparental mating mixture, helper *E. coli* MT616 containing plasmid pRK600, *E. coli* containing pKNOCK plasmid pMR146 - rhamnose kinase construct and *Rhizobium leguminsoarum* Rlt100.

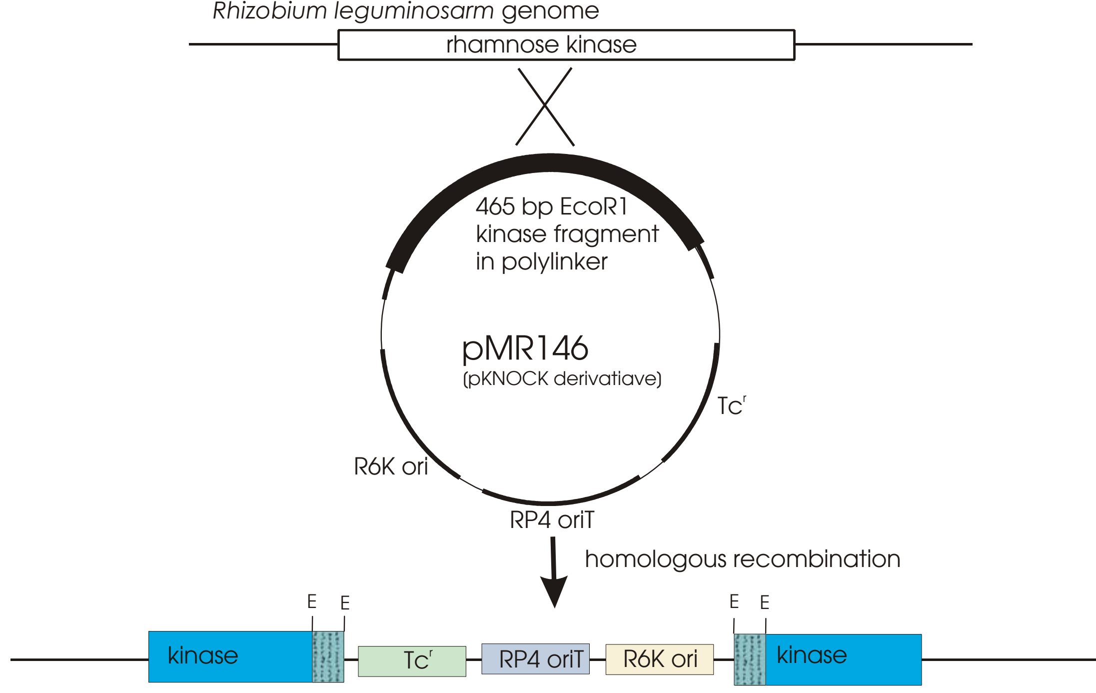

Figure 3. Recomination of homologous regions between *Rhizobium leguminosarum* genomic rhamose kinase and kinase 465 bp fragment on pMR146 after transfer into *Rhizobium* via conjugation. The 465 bp EcoR1 (E) kinase fragment is duplicated upon recombination, represented by hatched blocks.

RHAMNOSE CATABOLISM GENE LOCUS

In *Rhizobium leguminosarum* the rhamnose kinase gene, RhaK (accession number AF085687) is located in the rhamnose catabolism gene locus (1-11200 bp) at bp 9686 to 11065. Listed below are some relevant restriction sites located in the rhamnose gene locus.

Table 1. *Rhizobium leguminosarum* rhamnose catabolism gene locus (1-11200 bp) restriction map. RhaK located at **9686 to 11065.**

Restriction enzyme	Recognition site	Total #	bp position in rhamnose catabolism gene locus
BamHI	G|GATCC	0	
HindIII	A|AGCTT	1	3973
EcoR1	G|AATTC	12	1016, 1249, 2150, 2528, 2837, 3228, 5527,6665, 6974, 8902, 10124, 10589
Sma I	CCC|GGG	5	3165, 3223, 5286, 11017, 11072
Cla I	AT|CGAT	9	196, 505, 2057, 2282, 4122, 6089, 6257, 7540, 7970
Stu I	AGG|CCT	7	651, 1587, 2017, 5355, 7273, 8831, 10872

OLIGONUCLEOTIDE-DIRECTED MUTAGENSIS[10]

Oligonucleotide-direct mutagenesis creates mutations within the coding sequence of a protein that has "exquisite specificity and extraordinary breadth"[11]. This specific mutagenesis technique permits detection of specific protein function to a specific region of the protein, possibly elimination undesirable side activities and enhancement of protein desirable activities.

Design of mutagenic oligonucleotide primer: Size should be between 16 and 25 bp, low melting temperature between 42ºC and 48ºC and mutation should occur in the middle of the primer with 8-10 bp either side. Use the follow formula for small oligonucleotides, $T_m = 4(G+C)+2(A+T)$.
Design of exterior primers: The first external primer (R1) used in the megaprimer reaction should have a low T_m (42-46ºC) and low annealing temperature while the second external primer (F2) should have a high T_m (72-85ºC) and high annealing temperature (prevents annealing by residual first external primer). The external primer with the higher T_m should be larger (24-30 bp) than the other external primer (15-16 bp).

Other primer design criteria that should be considered is that there is no more than 3 of the same basepairs together. It is good idea that the 3' nucleotide should be either a G or C. This is not always crucial as long as the T_m is in the range required. It is important that there are no invert repeat areas (palidromes) that may form hairpins, especially 3' hairpins. This is another criteria that is difficult to estimate the effect on priming, best to consider, if you are experiencing difficulty getting the PCR reaction to work.

Megaprimer PCR site-directed mutagenesis

Richardson et al[4] used the megaprimer PCR system to introduce several specific basepair changes in rhamnose kinase (figure 4). The domain selected is the p-loop known to be the ATP binding site (most likely the active site). Any amino acid change should result in kinase mutant activity while still producing an open reading frame, ie. insertion of an alternate amino acid.

Megaprimer PCR (figure 4)[10] is a two in one PCR reaction. In the first PCR reaction M (mutant primer), R1 (reverse external primer) and plasmid pMR106 containing *Rhizobium leguminosarum* genomic rhamnose kinase (rhaK) open reading sequence (Bam HI/HindIII 1380 bp insert in pBluescript) are added along with required PCR components. The external primer (R1) should be reasonably close to M primer to reduce the size of the megaprimer. This produces a double stranded megaprimer. In the second PCR reaction, external primer F2 is added. Since F2 is a high annealing temperature primer, the reaction is carried out only at high temperature, 72ºC to 80ºC[10]. First the megaprimer extends on original DNA to produce a mutant strand. The F2 primer completes the second mutant strand. The mutant DNA is then amplified with these primers, megaprimer and external F2 primer. The original R1 external primer cannot prime as it has low annealing temperature and the original genomic DNA is not amplified efficiently in the second PCR reaction.

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[11] Sambrook, J, Russell, DW. 2001. Chapter 13 Mutagenesis. In Molecular Cloning A Laboratory Manual 3rd edition. Cold Spring Harbor: CSHL Press p 13.1-13.35. quote p.13.2.

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                                                Start |-------p-loop---------|
     ---R1 primer--->                        RBS           Met                   Gly Lys Thr                               BamHI
5'ATAAGTAAAACGACGGCCA..polylinker..AAGCTTGCAGAACTGCAG ATG..GTC CTC GAC ATC GGC AAG ACC AA..kinase..GCGATGGCATAGCTGCAGGGATCC 3'
3'    CATTTTGCTGCCGGT..polylinker..TTCGAACGTCTTGACGTC TAC..CAG GAG CTG TAG CCG TTC TGG TT..kinase..CGCTACCGTATCGACGTCCCTAGGTATA 5'
                                   HindIII                                                  <--F2 high melting primer-
                                                                  <-----M primer-------
                                                                              Met
     Mutant primer M1 (Lys ->Met)                               3' TG TAG CCG TAC TGG TT 5'(16 bp, Tm48°C)

     R1 external primer (M13-20 HindIII): 5'GTA AAA CGA CGG CCA 3'(15 bp, Tm46°C)
     F2 external primer (HiTmBamHI):      5'GGA TCC CTG CAG CTA TGC CAT CGC 3' (24 bp, Tm78°C)
     HindIII and BamHI site allow cloning of complete rhaK rhamnose kinase gene (1380 bp)(1408 bp fragment including linkers)
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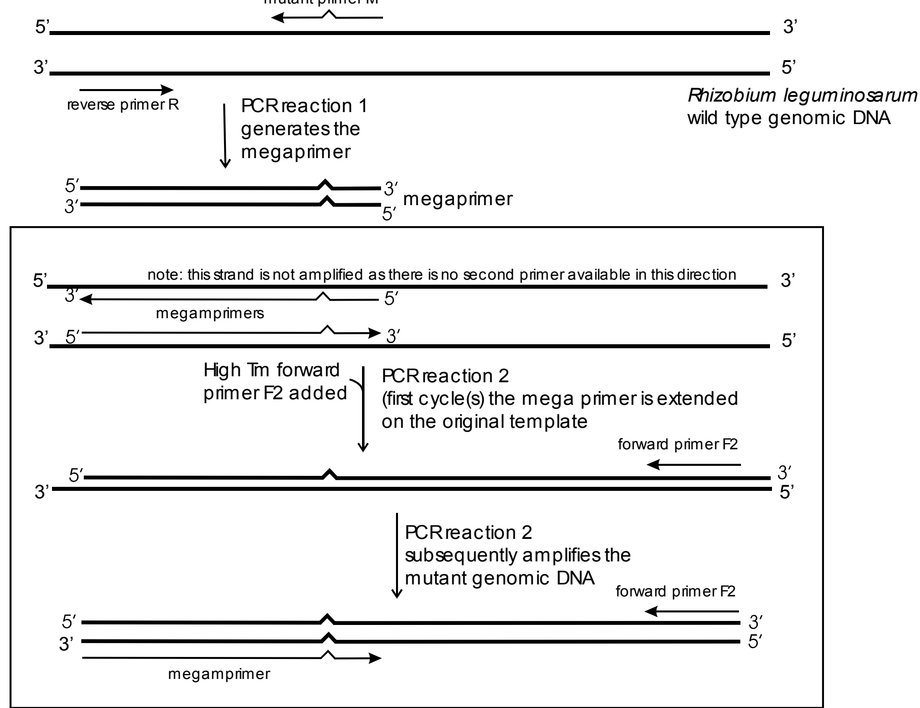
Figure 4. MegaPrimer PCR Mutagenesis in *Rhizobium leguminosarum* rhamnose kinase. Blip in line represents bp change. *R.leguminosarum* genomic DNA

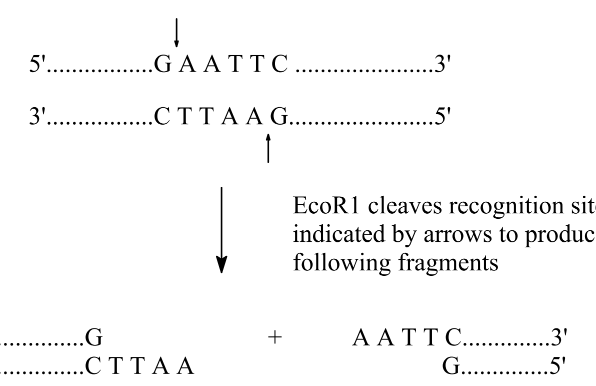


was cloned into bluescript plasmid prior to PCR facilitating the selection of external primers. The restriction sites BamHI and HindIII have been inserted using polylinkers not original to the rhamnose gene locus.

RECOMBINANT DNA TECHNIQUES

Restriction Enzymes (type II)

Restriction enzymes recognize a specific target sequence (tetra, penta, hexa, or heptanucleotides which have an axis of rotational symmetry) in double stranded DNA and make blunt ended or staggered cuts of the polynucleotide chains. The classic example of a restriction enzyme is Eco RI that cleaves in a staggered manner generating short single-stranded sticky ends. The cleavage sites are located on either side of a short palindromic sequence that is part of the enzyme recognition site.



Agarose Gel Electrophoresis

Agarose gel electrophoresis is the standard method to separate and identify DNA fragments. The technique quickly and easily resolves DNA fragments. Location of DNA fragment bands can be immediately detected by staining with the fluorescent, intercalating dye ethidium bromide.

The electrophoretic migration rate of DNA through agarose is dependent on molecular size of the DNA, agarose concentration, conformation of the DNA, and applied current. Base composition and temperature have very little effect. Linear double stranded DNA migrates at a rate that is inversely proportional to the log of their molecular weights. Agarose concentrations of 0.3% to 2.0% permit separation of linear DNA molecules from 0.1 kb to 60 kb. Closed circular, nicked circular, and linear DNA of the same molecular weight migrate through agarose gels at different rates. Closed supercoiled circular DNA generally migrates the fastest. Other forms that may be present are linear, nicked circular and dimer, trimer, concatenate forms of superhelical DNA. The relative mobilities are influenced by agarose gel concentration, strength of applied current, ionic strength of buffer, density of superhelical twists, and concentration of ethidium bromide. When considering the applied current, the rate of linear DNA migration is linear to the applied current at low voltage. As the electrical field strength is increased, high molecular weight DNA mobility is increased differentially resulting in effective range of separation decreasing as voltage increases. Generally maximum resolution is obtained at 5 V/cm.

Plasmid DNA Preparation

All procedures of plasmid DNA isolation involve (1) growth of bacteria with amplification of plasmid, (2) harvesting and lysis of bacteria, and (3) purification of plasmid DNA. Normally there are ten to two hundred plasmids (relaxed - not connected to chromosomal replication) per bacterial cell. The bacteria containing the plasmid are grown in LB medium plus antibiotic that selects for plasmid. The isolation procedures takes advantage of major differences between genomic DNA and plasmid DNA. By gentle bacterial lysis small molecules, including covalently closed supercoiled plasmids are released into solution. Large molecules such as

chromosomal DNA fragments are trapped in the cell debris. The cells can be lysed by boiling, alkali, detergents such as sodium dodecyl sulphate (SDS) or Triton and phenol. Each method is appropriate for different experimental expectations. Lysis by non-ionic detergent (Triton) is considered the most gentle and is especially good for isolation of plasmids over 10 kb. Heating or mild alkali (up to pH 12.5) treatment breaks most of the hydrogen bonds in DNA. Closed circular plasmids regain their native configuration when slowly cooled or returned to neutral pH while chromosomal DNA fragments remain in the denature state. The plasmid DNA can be purified by ethanol precipitation, ultracentrifugation in CsCl, and application to silica or resin column. The yield of plasmid DNA is dependent on the plasmid copy number, plasmid type, bacterial strain, and growth conditions.

Ligation and pBluescript

T_4 DNA ligase catalyses the formation of phosphodiester bonds between adjacent 3'-OH and 5'-P termini in double stranded DNA that has either cohesive ends or blunt ends. T_4 ligase requires both magnesium and ATP.

In your lab you ligate the PCR product that has been digested with BamHI/HindIII into pBluescript® that contains these restriction sites in the polylinker (multi-cloning site (MCS)). The advantage of pBluescript® 3 kb plasmid (see diagram) is many faceted. What is important to us is the ability to detect not only transformation (AMP antibiotic marker) but recombinant transformation. When rhaK is ligated into pBluescript β-galactosidase is inactivated. When the tranformants are plated on LB AMP X-gal/IPTG the white colonies are recombinants while blue colonies are only transformants. White colonies cannot metabolize X-gal in the presence of IPTG. If X-gal is metabolized (active Lac Z gene) blue colonies result.

pBluescript®SK (+/-) phagemid

The pBluescript® SK (+/-) phagemid is a 2958-bp phagemid derived from pUC19. The SK designation indicates the polylinker is oriented such that *lacZ* transcription proceeds from *Sac* I to *Kpn* I.

f1 (+) origin: (6-462 bp) f1 filamentous phage origin of replication allowing recovery of the sense strand of the *lacZ* gene when a host strain containing the pBluescript phagemid is co-infected with helper phage.

f1 (-) origin: (6-462 bp) f1 filamentous phage origin of replication allowing recovery of the antisense strand of the *lacZ* gene when a host strain containing the pBluescript phagemid is co-infected with helper phage.

ColE1 origin: (1032-1912 bp) Plasmid origin of replication used in the absence of helper phage.

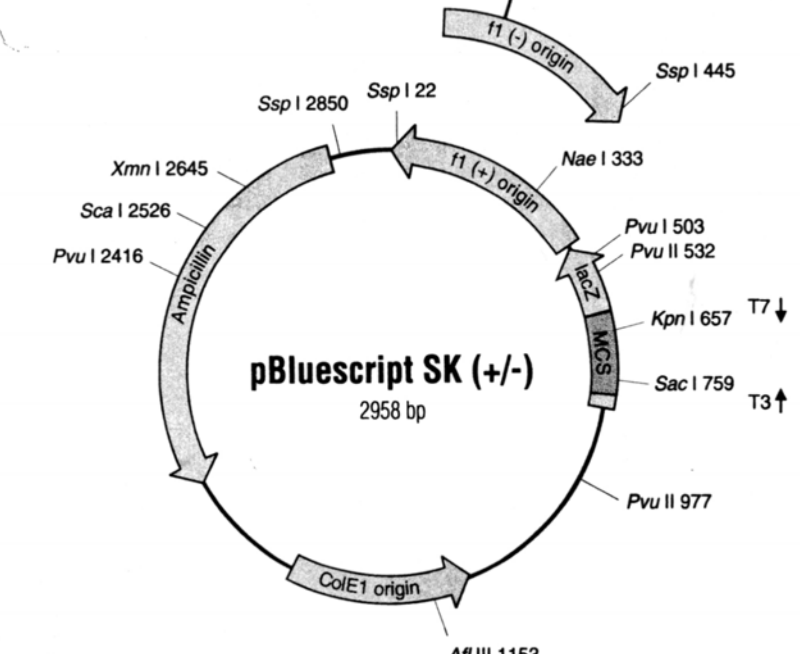
***lacZ* gene:** (*lac* promoter: 816-938 bp) This portion of the *lacZ* gene provides α-complementation for blue/white color selection of recombinant phagemids. An inducible *lac* promoter upstream from the *lacZ* gene permits fusion protein expression with the β-galactosidase gene product.

MCS: (657-759 bp) Multiple cloning site flanked by T3 and T7 RNA promoters (please see the polylinker sequence below).

Ampicillin: (1975-2832 bp) Ampicillin-resistance gene (Amp^r) for antibiotic selection of the phagemid vector.

Please Note: The upper strand is designated the (+) strand and the lower strand is designated the (-) strand.

GenBank® # 52325 [SK(+)] & #52324 [SK(-)]

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Reverse primer                     T3 primer                                SK primer
5' GGAAACAGCTATGACCATG 3'     5' AATTAACCCTCACTAAAGGG 3'            5' CGCTCTAGAACTAGTGGATC 3'

                                                         BstX I    Eag I            Spe I          Sma I          EcoR I
        MET               T3 promoter   +1 →       Sac I    Sac II   Not I    Xba I        BamH I          Pst I
5' GGAAACAGCTATGACCATGATTACGCCAAGCTCGAAATTAACCCTCACTAAAGGGAACAAAAGCTGGAGCTCCACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGG
3' CCTTTGTCGATACTGGTACTAATGCGGTTCGAGCTTTAATTGGGAGTGATTTCCCTTGTTTTCGACCTCGAGGTGGCGCCACCGCCGGCGAGATCTTGATCACCTAGGGGGCCCGACGTCCTTAA
           |                                                   |
          816                                                 759
        β-Galactosidase →

                             Bsp106 I                 Eco0109 I
                             Cla I      Hinc II       Dra II
                                        Acc I
                 EcoR V  Hind III       Sal I   Xho I  Apa I   Kpn I
AATTCGATATCAAGCTTATCGATACCGTCGACCTCGAGGGGGGGCCCGGTACCCAATTCGCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTCGTTTTACAA 3' (+)
    GCTATAGTTCGAATAGCTATGGCAGCTGGAGCTCCCCCCCGGGCCATGGGTTAAGCGGGATATCACTCAGCATAATGTTAAGTGACCGGCAGCAAAATGTT 5' (-)
                                                    |          ← +1 T7 promoter
                                                   657

                  3' CTATGGCAGCTGGAGCT 5'        3' CGGGATATCACTCAGCATAATG 5'  3'TGACCGGCAGCAAAATG 5'
                        KS Primer                         T7 Primer                   M13 -20 Primer
```

Transformation of *E. coli* with Plasmid DNA

Mandel and Higa[12] (1970) first demonstrated that the uptake of lambda DNA by *E. coli* is enhanced by treatment of *E. coli* cells with calcium chloride under cold conditions followed by short treatment at 42ºC. A similar procedure was used by Cohen et al[13](1972) to transform bacteria with plasmid. This simple method similar to what we use in the lab generates 10^5 to 10^6 transformed colonies/µg plasmid DNA. Many techniques now exist for preparation of competent cells, for example, buffer of variety of divalent cations, adding reducing agent, growth temperature manipulation, freezing and thawing of cells, exposure to organic solvents, genetic manipulation of host, electrophoration, etc. All these techniques optimize the efficiency of transformation making it possible to obtain as many as 10^9 transformants/µg plasmid DNA. Once the plasmid DNA is inside the bacteria, the plasmid DNA replicates and expresses the drug-resistance marker that allows the transformed cell to survive in the presence of antibiotic.

Chemiluminescence DIG Hybridization

(Roche Molecular Biochemicals DIG application manual)

Hybridization

Hybridization is the formation of double stranded nucleic acid molecules by homologous complementary strands. Generally fixed single stranded nucleic acid (solid supports, southern blot or plaque blot) are screened with homologous single stranded radioactive probe. Hybridization parameters are varied depending on the degree of homology between the 'homologous or near homologous' strands of nucleic acid. The formation of nucleic acid hybrids is a reversible process. The melting temperature (T_m) of double stranded DNA is defined as the temperature when half the duplex molecules have dissociated. The T_m is affected by monovalent cation concentration (molar), base composition, length of nucleotides in shortest single strand, and concentration of helix destabilizing agents. The stability of duplex formation between strands with mismatched bases is decreased according to the number and location of mismatches. For duplex formation between single strands with high homology the stringency of the hybridization should be high to maintain a low background by adjusting the temperature and salt concentration. There is approximately 16ºC increase in T_m for each log increase in cation concentration. Hybridization stringency can be altered during hybridization or washing. For duplex formation with reduced homology the stringency of either the hybridization or the wash must be decreased by lowering the temperature or increasing the salt concentration. However, the wash must be stringent enough to allow differentiation between the positvie lamba clone - probe binding and nonspecific background binding.

Random Primed Labeling of DNA

As the name implies the DNA probes is randomly label. The probes is linearized with a restriction enzyme and denatured to single stranded DNA by boiling. Random hexanucleotides primers that have the capability to attach to any part of the DNA are annealed to the DNA probe. These hexanucleotides serve as primers for DNA synthesis (5' to 3') by the Klenow fragment of

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[12]Mandel M, Higa A. 1070. Calcium-dependent bacteriophage DNA infection. J. Mol. Biol. 53: 159-162.

[13]Cohen SN, Chang ACY, Hsu L. 1972. Nonchromosomal antibiotic resistance in bacteria: Genetic transformation of Escherichia coli by R factor DNA. PNAS 69: 2110-2114.

DNA polymerase I of *E. coli*. Klenow polymerase, which lacks 5' to 3' exonuclease activity of DNA polymerase 1, synthesis DNA incorporating the nucleotides plus some dUTP nucleotides that are labelled with a molecule of digoxygenin (DIG) conjugated to alkaline phosphatase (AP). UTP-DIG-AP is incorporated approximately every 10 nucleotides.

Chemiluminescent Detection

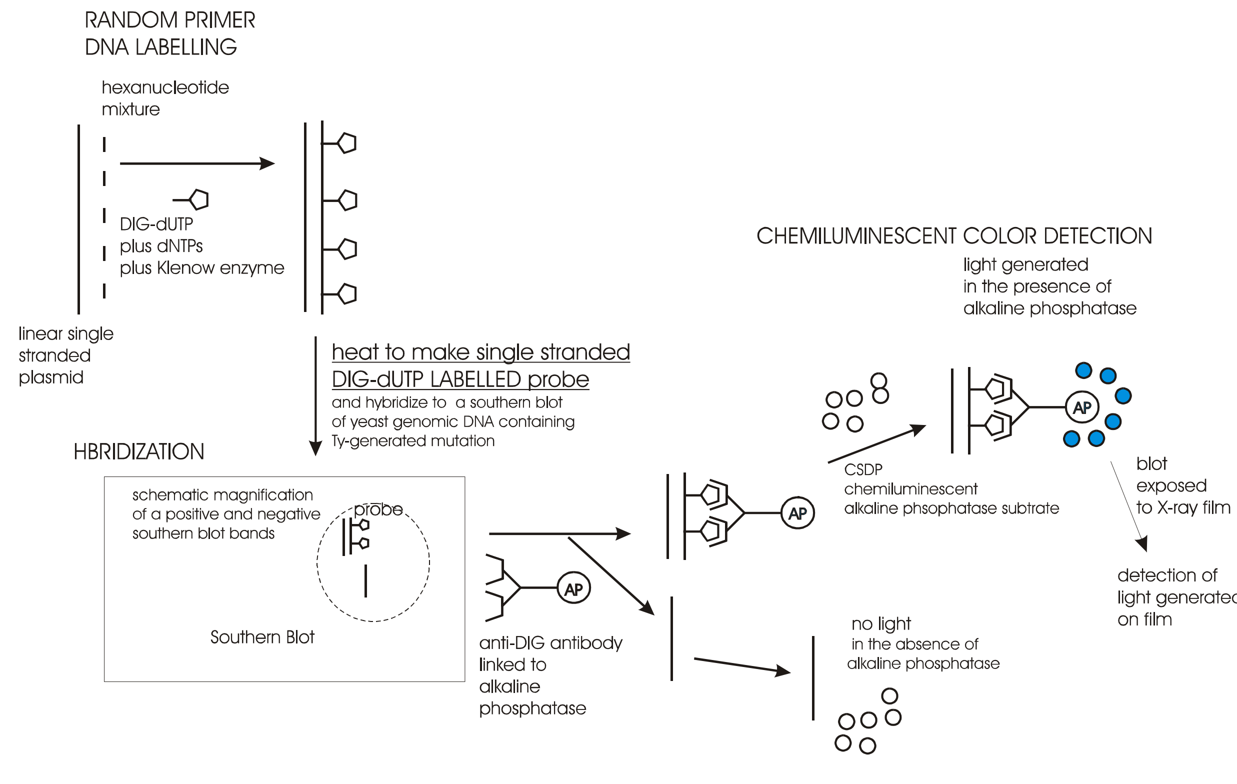

Figure 5. Digoxigenin (DIG) system of nucleic acid labelling and detection. In this lab a plaque blot of *Achlya* genomic DNA EMBL3 lambda library containing the calmodulin gene is probed with pCM116 (eel calmodulin cDNA plasmid) using the chemiluminenscent DIG hybridization system.

Once bound to the filter, the probe is detected by an immunological method that utilizes an antibody (rabbit IgG) specific for the digoxygenin molecule that is linked to alkaline phosphatase (figure 5). The chemiluminescent alkaline phosphate substrate, CSPD detects the probe-target DNA hybrid. The conjugated alkaline phosphatase reacts with CSPD substrate removing phosphate which generates light. The light is detected with X-ray film.

LAB 1 INSERTIONAL MUTAGENSIS into rhamnose kinase of *Rhizobium leguminosarum*

PROCEDURE

Week 1

Part I Cloning of rhamnose kinase fragment into pKNOCK

1. Plasmid pMR106 which contains the *Rhizobium leguminosarum* rhamnose kinase gene (1380 bp) was constructed by first amplifying the rhamnose kinase gene (rhaK) in *Rhizobium leguminosarum* using primers 5'HIndIII and 3'BamHI. Then the PCR fragment was cloned into plasmid pBluescript (pBS).
2. Plasmid pMR106 was digested with EcoRI. The 465 bp EcoRI fragment was isolated and ligated into plasmid pKNOCK polylinker site (digested with EcoR1) creating the new construct, pMR146.

Part II Transformation of *E. coli* DH5α/λ*pir* with pMR146 pKNOCK-kinase construct

1. Preparation of Competent *E. coli* DH5α/λ*pir* (1 ml of an overnight culture of *E. coli* J DH5α was added to 50 ml LB in a 500 ml flask. Shaken for 3-4 h at 37ºC. $Abs_{550\ nm}$ should be 0.4-0.5. It is important that a large surface area (oxygen supply) is present for log phase growth of *E. coli*. Centrifuged at 6000 rpm for 10 min at 4ºC. Supernatant discarded and cells resuspended 20 ml 0.1 M $MgCl_2$. Centrifuged at 6000 rpm for 10 min at 4ºC and supernatant discarded. Cells resuspended in 20 ml 0.1 M $CaCl_2$. Incubated on ice for 30 min. Again centrifuged at 6000 rpm for 10 min at 4ºC The cells were resuspended in ~4 ml $CaCl_2$ + glycerol. Incubated on ice overnight. 100 µl aliquots dispensed in Eppendorf tubes in the cold room using cold pipettes and immediately frozen at -80ºC.

> -λ*pir* permits plasmid replication of pKNOCK in *E. coli* DH5α *E. coli* DH5α/λ*pir* is Kan^r

STUDENT LAB STARTS HERE

2. If the competent cells are not thawed, thaw one tube containing 100 µl competent *E. coli* DH5α/λ*pir* at room temperature and put on ice (if not used immediately). Add 20 µl pMR146 plasmid (Tc^r pKNOCK- 465 bp kinase construct).

3. Heat shock at 43ºC for 1 min. Put on ice for 3 min.

4. Add 1 ml prewarmed LB broth (37ºC) and incubate overnight in a 37ºC waterbath.

5. Early the next morning prepare 10^{-1} dilution in saline by adding 100 µl transformation mixture to 900 µl saline in an Eppendorf tube. Mix. Repeat 10 fold dilution once more (10^{-2}).

6. Spread plate 100 µl of undiluted and each dilution (10^{-1} and10^{-2}) on LB-Tc plates.

7. Incubate plates at 37ºC for one days. Check for isolated colonies, ie. transformants. Often when plating the undiluted transformation mixture there will be very faint lawn of bacteria but the Tc resistant colonies will be obvious larger colonies above the lawn.

8. WEDNESDAY select two isolated colonies and streak single colonies on LB-Tc. Incubate at 37ºC for 1 day. Store your culture plates in student cold box (located in incubator room 203 off your lab) until Friday.

9 . **FRIDAY**. Set up two LB-Tc 5 ml broths by aseptically adding 25 µl 1 mg/ml tetracycline (dark bottle in coldbox) stock. Return tetracycline stock to cold box. To each 5 ml LB-Tc broth transfer a single colony from each of your streak plates. Clearly label each tube with student names (at least full last names) and group #. Place inoculated broth in labelled tray in STUDENT COLD BOX - located just off room 201 in room 203. The cultures will be transferred to the 37ºC culture rotator Sunday and incubated with rotation for Triparental mating during Monday's lab.

Tetracycline deteriorates rapidly once added to agar plates or broth. Tc is also light sensitive. Plates are prepared just before lab. There is possibility of satellite colonies forming (small colonies around a large colony). **Alway pick from the centre of the larger colony.**

Week 2

Part III Triparental Mating via Conjugation

E. coli MT616/pKR600 was grown overnight in LB-chloramphenicol and *Rhizobium leguminosarum* Rlt100 was grown in Ty broth with rotation at 30ºC for 2 days.

1. Set up the following mating mixtures ratios for each of your transformants and positive control, *E. coli* DH5α λpir/pMR146.

:

Mixture Ratio (donor, helper, recipient)	Combine in an Eppendorf tube (ml):		
	Donor (transformant or *E. coli* DH5α λpir/pMR146	Helper strain *E. coli* MT616/pKR600	Recipient *Rhizobium leguminosarum* Rlt100
1:1:1	0.5 ml	0.5 ml	0.5 ml
1:1:2	0.3 ml	0.3 ml	0.6 ml
1:1:5	0.2 ml	0.2 ml	1.0 ml

REPEAT THE FOLLOWING PROCEDURE FOR EACH MATING MIXTURE (total of 9)

2. Immediately microfuge for 1 min at room temperature.
3. Aspirate off or use P1000 to remove supernatant. Add 1 ml sterile saline and resuspend cells using P1000 by pipetting up and down.
4. Microfuge for 45 sec. Remove supernatant. Add 100 µl saline and resuspend.
5. Carefully spot the 100 µl suspension in the centre of a non-selective Ty plate. Keep the spot as small as possible.
6. Incubate upright at 30ºC until Friday.

Negative Controls:

Using a marker divide the bottom of a selection plate, TySmTc[14], into 5 pie sections. Label each section with bacterial name and stain number. Transfer 100 µl of each mating strain (3 donors, helper and recipient) to a section. Using a sterile stick to spread spot over each section. Allow to dry. Incubate at 30ºC. Check for growth on Friday. Record results. The expected result is no growth or few scattered colonies. Why? Discard control plates.

FRIDAY

7. Mating Mixture Plates (Repeat for each mating mixture plate):
   (i) Place 1 ml saline on triparental mating growth. Using a sterile stick or loop carefully mix until growth resuspended. Using Pipetman transfer entire amount to sterile Eppendorf tube (10^0). Prepare a 10^{-1} dilution using microtube by adding 100 µl 10^0 dilution and 900 µl saline. Mix. Spread plate 100 µl of undiluted and 10^{-1} dilution on TySmTc plates. TySmTc plates are located in STUDENT COLD BOX, covered with foil.

8. Incubate all plates at 30ºC until next lab, Monday.

Week 3
Part IV Screening of Triparental Mating Transconjugants

1. Using the grid (available in appendix or as pdf on website, set up three agar plates in the following order; VMM + Rha, TySmTc and VMM + Glu. When the plates are picked in this order it prevents unwanted carryover.
2. Pick plate total of 50 transconjugants in succession on plates VMM + Rha, TySmTc and VMM + Glu. For each colony use only one toothpick. Select transconjugants from all plates, make sure you include at least 20 transconjugants from positive control (mating mixture using *E. coli* DH5α/pMR146).
3. Incubate for 3 days at 30ºC. THURSDAY record the number of 'picks' that grew on each plate. A successful transconjugant should grow on TySmTc and VMM + Glu but not on VMM + Rha. Store plates in student cold box (room 203 off room 201) until tomorrow.
4. **FRIDAY.** Prepare two 5 ml TyTc broth tubes by adding 13 µl 1mg/ml tetracycline stock to each 5 ml Ty broth tube. Pick a single colony from TySmTc (colony must grow on TySmTc and VMM + Glu but not on VMM + Rha) and inoculate 5 ml TyTc broth (Tc added at ½ strength). Repeat inoculation with another single successful transconjugant (preferably triparental mating mixture that used DH5α/pMR146 previously transformed). Put the tubes on the culture rotator in the 28ºC incubator in the table top incubator by sink in room 201. Make sure you label your tubes with group number and full names. Balance your tubes on rotor - exactly opposite. Be careful when turning the culture rotator on and off as the switch is on the right side towards the back.

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[14] Ty medium promotes *Rhizobium* growth, Sm = streptomycin, Tc = tetracycline.

Week 4

Part V *Rhizobium leguminosarum* Genomic DNA Isolation and Restriction Digestion

QIAGEN DNeasy[15] Genomic DNA Isolation

-Repeat the following procedure for each of your transconjugants (should have two), *Rhizobium leguminosarum* Rlt212 (positive control) and *Rhizobium leguminosarum* Rlt100 (negative control).
-You will find the control cultures on the culture rotator with your group's transconjugants.
-Remember to keep tubes balanced on the culture rotator after you remove your cultures using blank 5 ml LB broth or equivalent.

Morning of lab (no later than 11:30 am)

1. Transfer 1 ml culture to an Eppendorf tube. Microfuge for 1 min. Discard supernatant (use an aspirator or pipetman to remove remaining supernatant).

2. Resuspend pellet in **180 µl Buffer ATL**. Add 20 µl proteinase K, vortex, place tubes in a floating tube holder and incubate in a shaking waterbath at 55ºC for ≥3 hours. The lysate should be viscous but not gelatinous.

Lab time (2:30 pm)

3. Vortex for 15 sec. Add **200 µl Buffer AL**, immediately vortex to mix, incubate at 70ºC for 10 min. Vortexing must be done immediately to ensure a homogenous solution.

4. Add **200 µl 95% ethanol**. Mix thoroughly by vortexing.

5. Add entire mixture* to DNeasy mini spin column that has been placed in a 2 ml collection tube. Microfuge for 1 min. Discard flow through and collection tube.
*includes white precipitate if present

6. Transfer the spin column to a new 2 ml collection tube. Add **500 µl Buffer AW1** to spin column and microfuge for 1 min. Discard flow through and collection tube.

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[15]**DNeasy DNA purification (GIAGEN)** (as process is patented many of buffer components are not available)
Proteinase K Digests proteins, activity of 600 mAU/ml, in the process releases genomic DNA from proteins and cellular components
Buffer ATL - buffer system optimized for tissue lysis in presence of proteinase K
Buffer AL and AW1 - contain the chaotropic salt, guanidine hydrochloride (degrades proteins)
Buffer AL contain optimum conditions for DNA binding to the spin column membrane
DNeasy spin column - silica-gel-membrane - selectively binds DNA (glass binds DNA), contaminants pass through, binds from 0.1 to 50 kb DNA fragments, predominantly 30 kb fragments (genomic)
Buffers AW1 and AW2 - both contain ethanol and salts when centrifuged remove enzyme inhibitors such as proteins and divalent cations.
Buffer AW2 contains sodium azide as a preservative

7. Transfer the spin column to a new 2 ml collection tube. Add **500 µl Buffer AW2** to spin column and microfuge for 6 min. Discard flow through and collection tube. Carefully remove column as it is essential that all ethanol is removed from the spin column.

8. Place the spin column in an eppendorf tube (cap cut off but saved). Pipet **100 µl ddH$_2$O** directly onto the DNeasy column membrane. Incubate at room temperature for 1 min. Microfuge for 1 min to elute. Cap and completely label tube. After setting up restriction digest store the remaining genomic DNA at -20ºC in student sample tray.

9. Set up Sma I digestions, one tube for each genomic DNA prep.
Clearly label tubes **ONLY** with group # and letter designation for sample type as listed below on **lid top and side**:
A. transconjugant 1
B. transconjugant 2 (preferably with DH5α/pMR146 previously transformed)
C. negative control (*Rhizobium leguminosarum* Rlt100)
D. positive control (*Rhizobium leguminosarum* Rlt212)

Addition	Amount added (µl)
genomic DNA	25
Invitrogen® #4 REACT buffer (10x)	4
ddH$_2$O*	10
Sma I restriction enzyme	1

10. Incubate in a 30ºC waterbath overnight. Stop each reaction by adding 8 µl agarose stop solution. Store at -20ºC in designated tray until next lab day. It is important to put your tubes in designated tray [labelled *Rhizobium leguminosarum* genomic DNA RE digests] as the TA needs to load your samples on an agarose gel prior to the start of next week's lab. Tubes should **ONLY be labelled with group # and letter designation for sample type on lid top and side**.

Restriction enzyme digestion information:
·Always keep restriction enzyme on ice and return to -20ºC as quickly as possible.
· Always handle restriction enzymes and all biological materials by the cap region of the tube. You do not want to kill the enzyme with the warmth of your hands. Restriction enzyme storage buffer: 50 mM Tris-HCl, pH 7.2, 300 mM NaCl, 0.5 mM EDTA, 5 mM EGTA, 5 mM mercaptoethanol, 500 µg/ml BSA (bovine serum albumin), 50% (v/v) glycerol, 0.2% (w/v) TritonX-100.
·Each restriction enzyme has a corresponding specific reaction buffer. For example, REACT Buffer #4: 20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 50 mM KCl.
·Add components using pipetman by touching the inside of the eppendorf tube about 0.5 cm below top of tube and releasing solution. A good idea is to place components at different spots around the inside of the tube.
·Add restriction enzyme last.
·Restriction enzyme: 1 unit of restriction enzyme is the amount of enzyme required to completely digest 1 µg of bacteriophage lambda DNA in 1 hour at 37ºC. Restriction enzyme may need to be added at a concentration of 1-8 units/µg plasmid DNA.

Week 5

Part VI Southern Blot of *Rhizobium leguminosarum* Transconjugants

A. Agarose gel electrophoresis of restriction enzyme digested genomic yeast DNA

1. The TA will load 25 µl aliquots of student RE digested *Rhizobium leguminosarum* DNA samples (4 samples/group) on a 1% agarose gel containing ethidium bromide. Student samples will be loaded in groups of six samples with a space between every two groups. The gels will be cut such that there is one Southern blot per two groups. Agarose gel electrophoresis carried out for ~4 hours at 100 volts, or until lab starts.

2. Photograph gel with a ruler using Alpha Innotech's MultiImage light cabinet, software Alpha EaseEF. Photograph will be saved and posted on lab website as soon as possible .

STUDENT LAB STARTS HERE (2 groups work together)

B. Southern Blot of agarose gel

CAUTION: Transfer buffer contains 0.4 M NaOH, corrosive both to you and the desk top. Wear gloves, rinse immediately if you spill it on yourself. Do not spill on desk top or if you do wipe immediately with a wet paper towel. Probably a good idea to set up transfer container on several sheets of paper towel. As the concentration of NaOH is less than 1 M, it is acceptable to pour the solution down the sink - flush with lots of water. Also all paper used for transfer may be discarded in the general waste container.

WEAR nitrile N-Dex[16] GLOVES FOR THIS ENTIRE PROCEDURE

1. Handle gel carefully as it easily breaks. Also there is EtBr in the gel - wear gloves. When working over the UV transilluminator wear gloves and eye protection.

Figure 4. Southern blot apparatus. Not shown is the saran wrap cover that prevents wicking around gel. Four pieces used to cover up to gel on all four sides and surface of transfer buffer tray. Remember transfer buffer is alkali, handle with care.

2. Using a plastic knife cut out your groups' agarose gel over a UV transilluminator.

3. Add 200 mM HCl to a small container at a height greater than the gel. Carefully place gel in the HCl and agitate for 10 min (either by hand or use flat top rotatory shaker). When the

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[16] When handling Ethidium Bromide wear N-Dex nitrile gloves (blue box) as general supply of disposable gloves do not protect again EtBr. N-Dex nitrile gloves also protect against splashes up to 37% HCl and 50% NaOH.

loading dye turns from blue to yellow you may proceed to the next step. During this time, one partner should cut one piece each of 3 MM chromatography paper and nylon[17] blot the same size of the gel. Notch upper left hand corner of nylon blot - for orientation. Make sure you only handle the nylon blot with gloved hands. Soak nylon in a small container of transfer buffer for 15 min. Also cut a 5 cm stack of paper towels the same size as your gel. **Caution**, the nylon blot is white. It is protected by paper on both side. Make sure you take the nylon blot not the paper protecting the nylon blot. Ask the TA if you are not sure.

4. Pour off HCl. Add sterile distilled water to rinse gel, pour off.

5. Add transfer buffer[18] until gel is immersed. Agitate gently for 10 min or until loading dye turns from yellow to back to blue.

HCl may be poured down the sink as it is less than 1 M but flush with lots of water.

6. Set up gel transfer (figure 4). Place container to back of your work area as other students use the lab. Label do not disturb. Fill bottom container with transfer buffer at 3-4 cm depth. Place gel stand in the container with transfer buffer. Place a strip of 3 MM paper on gel standard the same size as stand width and side flats that are long enough to extend into the buffer. This is the wick paper.

7. Invert the gel and place on 3 MM paper covered gel stand. Centre the wet nylon filter on top of the gel (notch - upper left hand corner). Roll with a 10 ml pipette to remove any possible air bubbles. Wet precut 3MM filter is transfer buffer and centre on the nylon covered gel, again roll with the pipette. Place the paper towel stack on top of the 3MM paper. Place a weight, double hockey pucks on top.

8. Since the DNA is transferred from the gel to the nylon by capillary action it is important that transfer buffer moves through the gel not around it. To prevent this, surround the gel using four strips of saran wrap, one for each side, press up close to the gel. This should cover the entire surface of the container forcing the buffer through the gel stack.

9. Allow transfer of DNA to proceed overnight.

NEXT DAY

10. Wearing gloves, dismantle blotting set up. Discard the paper towels and the 3MM paper. Turn over the dehydrated gel and the filter together and lay, gel side up, on a dry sheet of 3MM paper. Mark the position of the gel well slots on the filter with a pencil before removing the dehydrated gel. Peel off gel and discard.

11. Neutralize nylon membrane in a small container with 5X SSC[19] buffer for 10 min with gentle shaking.

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[17] specially designed nylon membranes for chemiluminescent detection - microporous, hybdrophilic, neutral nylon

[18] 0.4 M NaOH + 0.6 M NaCl

[19] all SSC solutions used are diluted using a 20X SSC stock: 3 M NaCl + 0.3 M sodium citrate

12. Place the nylon on the UV transilluminator for 3 min with DNA side down. After exposure to UV light, allow nylon to air dry on a piece of filter paper.

13. Label gel, in area provided above well using a pencil, with group number and last names.

14. Put filter under vacuum in desiccator provided at room temperature until required.

Week 7

Part VII Chemiluminenscent DIG-labeled Hybridization (1) using DIG-High Prime DNA Labeling and Detection Kit II[20]

A. Random primed DNA labeling of probe

PREPARED PRIOR TO LAB BY TA. See appendix for determination of probe yield procedure.

1. Plasmid pMR146 containing EcoR1 465 bp centre restriction fragment of *Rhizobium leguminosarum* rhamnose kinase, is boiled for 10 min to denature to single stranded DNA and immediately put in ice/water.

2. Add the following components in order to an Eppendorf tube on ice.
   -10 ng-1 µg ice cold denatured linearized plasmid
   -add sterile double distilled water to 15 µl
   -4 µl DIG-High Prime (contains hexanucleotide primers, dNTPs, dUTP-DIG-AP, Klenow and appropriate salts) - mix thoroughly before using
   OR add 2 µl hexonucleotide mix (vial 5) + 2 µl dNTP mix (vial 6) + 1 µl Klenow enzyme (vial 7)

3. Incubate at 37^{o}C overnight.

5. Add 2 µl 200 mM EDTA, pH 8.0 to stop the reaction.

6. Add 0.1 volume of 4 M LiCl and 2.5-3.0 volumes ice cold ethanol to precipitate the labelled DNA. Mix and put at -70^{o}C for 30 min.

7. Microcentrifuge for 15 min at 4^{o}C.

8. Discard the supernatant. Wash the pellet with 100 µl ice cold 70% ethanol. Microcentrifuge for 5 min at 4^{o}C. Remove and discard the supernatant.

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[20]**DIG-High Prime DNA Labeling and Detection Kit II (cat. no. 1 585 614)**
maleic acid buffer: 0.1 M maleic acid, 0.15 M NaCl, adjust to pH 7.5 with solid NaOH.
wash buffer: maleic acid buffer plus 0.3% w/v Tween20
blocking solution: blocking solution (purified fraction of milk powder) diluted 1:10 with maleic acid buffer
antibody solution: Anti-DIG-alkaline phosphatase conjugate vial 5 - centrifuge for 5 min at 10000 rpm in original vial. Remove an aliquot and dilute 1:10000 with blocking solution.
detection buffer: 0.01 M Tris-HCl, pH 9.5, 0.1 M NaCl
CSPD alkaline phosphatase substrate : dilute CSPD 1:100 in detection buffer

9. Dry the pellet and resuspend in 50 µl TE buffer. Store at -20ºC if not used immediately.

10. Quantitate the probe by "dot blotting" serial dilutions of probe and standard DNA.

6. Add DIG-UTP probe to 12 ml hybridization buffer in 15 ml screw capped tube and store at -20ºC until required.

B. Prehybridization and Hybridization of blot

1. Place one blot in a hybridization roller tube. Add 50 ml standard hybridization solution (5x SSC, 1.0% (w/v) Blocking, 0.1% sarcosyl (SLS), 0.01 % sodium dodecyl sulfate (SDS). Prehybridize for a minimum of 1 hour at 65ºC.

2. Boil 12 ml DIG-UTP probe - hybridization solution mixture for 10 min. Immediately place of ice. Note: probe may be boiled separately and added to blot in pre-hybridization solution.

3. Pour off prehybridization solution. Add 12 ml DIG- UTP probe - hybridization solution mixture to roller tube containing plaque blot.

4. Hybridize overnight at 65ºC in roller tubes.

STUDENT LAB STARTS HERE

5. Add ~40 ml 0.5x SSC + 0.1% SDS to hybridization bottle containing blot. First shake to wash in behind blot then put on roller. Wash for 5 min at room temperature. Discard wash solution. Repeat wash. This wash removes unbound probe.

6. Add ~40 ml ml 0.1x SSC containing 0.1% SDS (pre-warmed to 68ºC). Wash the blot on the roller for 15 min at 68ºC. Discard wash solution. Repeat wash. A more stringent wash to remove unbound probe.

C. Chemiluminescent Detection of probe-plaque hybrid[21]

It is important that the blot DOES NOT DRY during any of the following steps.

1. Place blot in 40 ml **wash buffer** (shake stock solution before adding) in a small plastic container. Gently shake for 5 min or place on a flat rotary shaker. Discard the wash buffer.

2. Add 40 ml **blocking solution**. Incubate for 30 min. Pour off and discard solution.

3. Add 20 ml **antibody solution**. Incubate for 30 min. Pour off.

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[21] **maleic acid buffer**: 0.1 M maleic acid, 0.15 M NaCl, adjust to pH 7.5 with solid NaOH.
wash buffer: maleic acid buffer plus 0.3% w/v Tween20
blocking solution: blocking solution (purified fraction of milk powder) diluted 1:10 with maleic acid buffer
antibody solution: Anti-DIG-alkaline phosphatase conjugate - centrifuge for 5 min at 10000 rpm in original vial. Remove an aliquot and dilute 1:10000 with blocking solution.
detection buffer: 0.01 M Tris-HCl, pH 9.5, 0.1 M NaCl
CSPD alkaline phosphatase substrate : dilute CSPD 1:100 in detection buffer

4. Wash twice (2x 15 min) with 40 ml amounts of **wash buffer**. Gently shake or place on a flat rotary shaker.

5. Equilibrate for 5 min in 20 ml **detection buffer**.

6. Place blot between two acetate sheets.

7. Apply 600 µl CSPD alkaline phosphatase substrate across the surface of the membrane (do not allow the membrane to dry). As you apply, cover area with upper inner side of clear acetate sheet - flip upper sheet up and down to spread the substrate. Cover smoothly, no air bubbles.

8. Incubate for 5 min.

9. If excess liquid present, squeeze out. Seal around membrane using tape.

10. Place acetate covered blot in large sealable plastic bag lined with moistened paper towels. Seal bag. Incubate membrane for 15 min at 37°C. This step is required for activation of CSPD.

THE REMAINING STEPS DONE BY TA.

11. Expose the blot (DNA side towards film) inside the clear acetate to X-ray film for 24 h. Develop film in dark room, 2 min developer, 30 sec water and 2 mix fixer (left to right in container).

12. The developed blots will be scanned with a ruler (possibly not the actual size) and available on lab website as soon as possible.

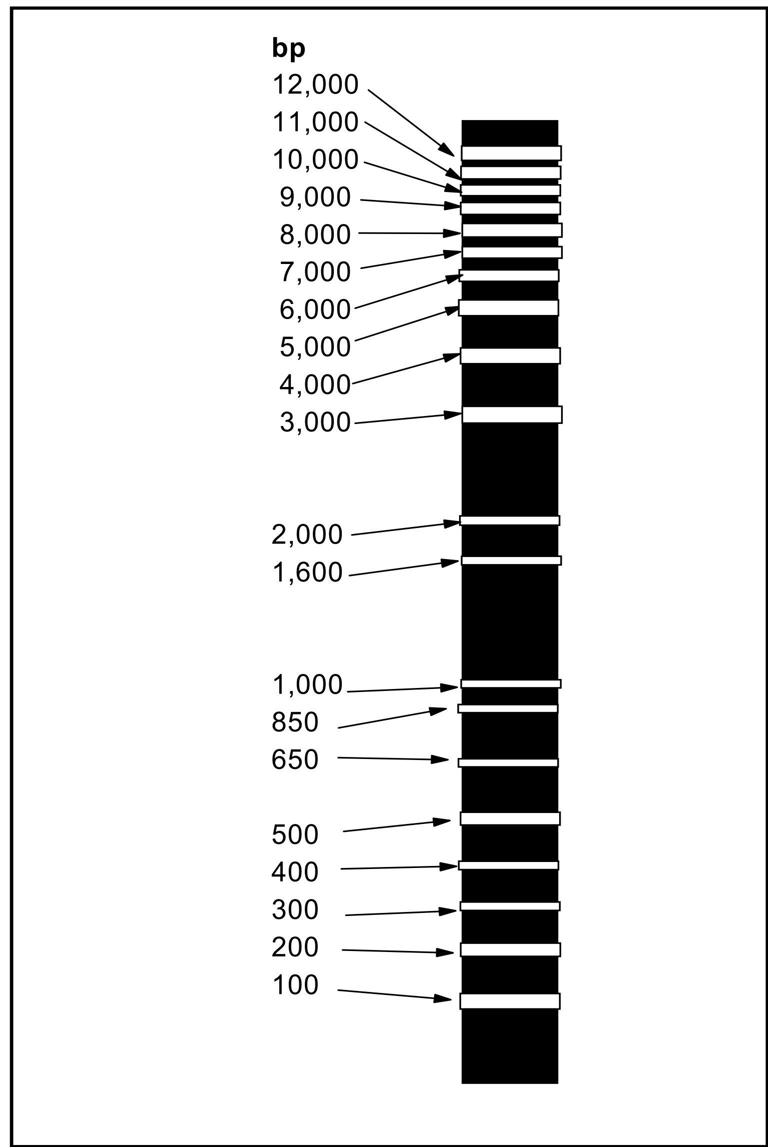

Figure 8a. 1 Kb Plus DNA Ladder Fragments. Fragments are linear double stranded DNA, therefore can only use as a standard for linear double stranded DNA. Bands are visualized by ehtidium bromide staining.

LAB 1 REPORT

Data presentation and analysis

2.0 1. Include a completely labelled figure (only one) of (a) Southern blot agarose gel and (b) chemiluminescent labelled x-ray film.
Presentation comment: see general instructions. Figure title should explain figure completely. Remember if you must label on the actual gel area do it discretely - use small markers or arrows between gel lanes, never on the lane itself. Discuss what your symbols mean in the figure title.
If the jpg. files are larger than required, just copy paste to Word document and resize.

4.0 2. Present an excel spreadsheet using the 1 kb Plus standard ladder curve to determine size of highlighted bands on Southern blot. Include all requested information. Include a standard curve. Measure distance migrated for 1 kb Plus bands starting at 300 bp and as high as 10000 bp (agarose gel). Enter the distance migrated by the major 'highlighted' band in each lane of Southern blot. Remember to use the photographed rulers by each picture to measure distance migrated (measure from well) as the photographs are most likely not the actual size. Use the standard curve **exponential regression formula** to determine the size of each major 'highlighted' band. Excel spreadsheet is available on website, save and open in Excel before entering data. See lab 1 appendix for Excel operations. State whether your presumed transconjugants 1 and 2 are actually transconjugants.
Refer to Table 1 in lab manual introduction for Restriction Enzyme map of rhamnose catabolism gene locus.

0.7 3. What band(s) would be highlight for *Rhizobium leguminosarum* Rlt100 if Stu I restriction enzyme was used to digest the genomic DNA and the probe was pMR106 (entire RhaK gene) while keeping all other experiment steps the same? State the number of bands and bp size (exact and/or estimate) for each. Refer to Table 1 in lab manual introduction for Stu I restriction enzyme map of rhamnose catabolism gene locus.

0.3 4. Ask a question with respect to procedures used in this lab. This is a compulsory question. Questions will be collected and answered during Question lab just before lab exam.

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7.0

EXCEL OPERATIONS AND STANDARD CURVE PLOT METHODS FOR MBIO 4570 Lab.

Procedures may vary depending on excel version. There are numerous ways to use Excel, only one described below. HINT: Right click on whatever you want to change and select appropriate item from the pull down menu.

Formula and Function calculations

Put the curvsor in the cell where you want to enter formula or function. Type in formula or select function button. In the spreadsheet just set up formula for one cell then copy and paste for similar calculations.

Cell Formulae Notations:

Remember to use correct mathematical brackets in your formulae.
start equation with an = sign, do not leave spaces between entries
* multiply
/ divide
+ add
- minus
^power or use the POWER function
EXP e raised to the power of a given number

R- squared value
The value is between 0 and 1. The R squared value is an indicator of how closely the estimated values used to draw the linear regression trendline correspond to the actual data. The linear regression trendline is most reliable when R value is close to 1.

DNA size (bp) Determination Standard Curve

(Normally the dependent variable is on the x-axis and independent variable is on the y-axis. For the determination of DNA bp size standard curve the axes are reversed to simplify solving the exponential equation for line best fit. Therefore DNA bp size is plotted on the y-axis and distance migrated is plotted on the x-axis. This simplifies determination of y (size in bp) given x, the known distance measured in mm for each double stranded linear DNA band.)

Select Insert pull down menu.
Select chart - (standard types), select (XY) Scatter chart type. Defaults to only markers present.
Click next.
Put cursor in data range box, click. Make sure there is no default information in box - delete if present as Excel will often automatically select data. Use the mouse to click and hold down to select y-axis data (molecular weight). Column should select - just check.
Select series tab. Series 1 plot is highlighted. Put the cursor in x values box, click. Again make sure there is no information in this box - delete if present. Use the mouse to click and hold down to select x-axis data (distance migrated) in the correct order as y-axis data. Under the legend tab, remove legend check mark - not required as only one plot per standard graph.
Click next.
Chart Options menu appears. Under default titles tab, enter title and axes labels. Unfortunately the title defaults to the top of the graph. After you are finished setting up graph, just select and move below graph. Mostly likely you will need to move the graph up. May require resizing, font size change, etc.

Click next. Keep the default setting (graph in data page).
Click finish.
Right click any empty area on graph area. Select format plot area. Change the background color to white. Click OK.
Put the cursor on any one of the plot markers. Right click. Select Add Trendline. Under the default Type tab select the **exponential regression box**. Select the Options tab. Select display equation on chart. Select display R-squared value on chart. Press OK. Move the display close to the Trendline in a open plot area.
Move the graph to specified area on data sheet.
Print to fit portrait page.

LAB 2 MEGAPRIMER MUTAGENSIS of *Rhizobium leguminosarum* rhamnose kinase

PROCEDURE

Part I PCR Mutant Primer Design

1. Follow the criteria outlined in lab manual introduction to design a mutant primer to megaprimer mutagenesis, much like the experiment that follows. Assume the two external primers (figure 4 in introduction) are the same as used by Richardson et al[4] but now create a bp(s) mutant primer ~70 bp further away from the 5' start of rhamnose DNA strand than the M1 designed by Richardardson.

2. The following sequence is the coding sequence of *Rhizobium leguminosarum* rhamnose kinase (rhaK) or can before found (cut and paste anywhere) at the NCBI blastn site.

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=Nucleotide&list_uids=37528956&dopt=GenBank It contains the complete sequence of the rhamnose catabolite genes[22] and the gene of interest to use, rhamnose kinase rhaK coding sequence is bp 9681 to bp 11065. Available on lab website with corresponding amino acid sequence (pdf file).

```
                  5'ATGccatgac cgccagttcc tatcgccgca tcgccgtcct
 cgacatcggc aagaccaatg ccaaggtcgt cgtgctcgac gccgggacag gcgccgagat
 cgccgtcctg aaacggccga acactgcgat caaaaccggt ccctatccgc attacgacat
 agaggcgctc tggtccttcg cgctcgattc gctgaagcgc cttgcgcagg cgcccggttt
 cgacgccatt tcgatcacca cccacggcgc cgcagccgcg ctgctcgatc gggacggcac
 gctcgccatg cctgtgatcg actacgaaca cgaatatccg caagaaatcc gcgatgccta
 tacggccttg cgcccctcct tcgacgaaac cttttcgcca cgcctttcga tgggcctcaa

 tgtcggcgcg cagctgcact accagaagag cgtctttccc gaagaattcg ccaaggtcgc
 gaccatcctc acctatgcgc aatactggac ggcgaggctg acgggagtgg ccgccaacga
 gttgacctct ctcggctgcc acaccgacct ctggaacccc agggcgggcg actattcctc
 gctcgtcgac aggctcggca tccgcgcgct gatggcgccg gtccgctccg ccttcgatgc
 gctcggcccg gttctgcccg aggtcgccgc ggagcttgcg ctcgcagccc ccgtgcaggt
 ctattgcggc attcacgatt ccaatgcctc gctgctgccg catctcgtcc atcgcgaggc
 cccttcgcc gtcgtctcca ccggcacctg ggtgatcaat ttcggcgtcg gcggcgatct
 cgatcacctc gatcagaaac gcgatgcgct cgccaacgtc gatgcctatg gccgggccgt
 tccctcctcg cgctttatgg gcgggcggga attcgagatc ctctcggccg aaatcggccc
 cgtcgacgaa caagccgccc aggcggcgat cggtcctgtc gtcgaaaaga gcatgatgct
 gctgccgaat atcgcccccg gttccggccc cttccctgat aaggcgagcc agtggatcgg
 cgctgaacag gctagccgcg aagagcgtca tgcggcggcc tgcctctatc tcgccctgat
 gaccgacgcc tgcctcggac tgatcggcgc caagggcccc atcatcgtcg aaggaccctt
 tgccctgaac gaggcctatc tgaagctgct cgccgccctt gccggccgtg aggtcctggc
 ccttccgggc acgaccggca ccagccaggg tgctgccctg ctgaccggca tccggccggt
 ctcgggggca gagactgacg tgccgccgca agatttcccg ggactggccg cctatcgcga
 tcgctggtac gcggcgatgg caTAG 3'
```

[22]Oresnik, IJ, Pacarynuk, LA, O'Brien, SA Yost, CR and Hynes, MF. 1998. MPMI 11:1175-1185.

3. Remember to consider opening reading frame, refer to any basic biochemistry book or internet for codon amino acid translation.
   http://archive.uwcm.ac.uk/uwcm/mg/docs/cd_amino.html

/gene="rhaK" CDS 9686..11065 /gene="rhaK" /note="putative sugar kinase"
/codon_start=1 /transl_table=11 /product="RhaK" /protein_id="AAQ92412.1"
/db_xref="GI:37528965"
/translation="MTASSYRRIAVLDIGKTNAKVVVLDAGTGAEIAVLKRPNTAIKT
GPYPHYDIEALWSFALDSLKRLAQAPGFDAISITTHGAAAALLDRDGTLAMPVIDYEH
EYPQEIRDAYTALRPSFDETFSPRLSMGLNVGAQLHYQKSVFPEEFAKVATILTYAQY
WTARLTGVAANELTSLGCHTDLWNPRAGDYSSLVDRLGIRALMAPVRSAFDALGPVLP
EVAAELALAAPVQVYCGIHDSNASLLPHLVHREAPFAVVSTGTWVINFGVGGDLDHLD
QKRDALANVDAYGRAVPSSRFMGGREFEILSAEIGPVDEQAAQAAIGPVVEKSMMLLP
NIAPGSGPFPDKASQWIGAEQASREERHAAACLYLALMTDACLGLIGAKGPIIVEGPF
ALNEAYLKLLAALAGREVLALPGTTGTSQGAALLTGIRPVSGAETDVPPQDFPGLAAY
RDRWYAAMA"

insert pdf printout of rhaK bp and a a seq.

Week 2
Part II Plasmid pMR106 DNA Preparation (QIAGEN[23] kit method)

Repeat the following procedure in DUPLICATE.

1. **Cell Harvesting**: Aseptically transfer 3 ml *E. coli* DH5α containing plasmid pBluescipt with a *R. leguminosarum* rhaK gene insert via BamHI/ HindIII (*E. coli* DH5α /pMR106 (AMP)) into two eppendorf tubes (each tube only holds 1.5 ml). DO NOT DISCARD CULTURE. Centrifuge at room temperature for 1 min (12,000 x g). **Remove supernatant by aspiration**. Aspiration must be used to completely remove all supernatant.
2. **Condensing two tubes to one tube and Cell Suspension**: Add 250 µl Buffer P1 to ONLY one pellet tube and **completely resuspending the cells** using the pipetman. Transfer the resuspended pellet to the second pellet tube and again completely suspend cells. The solution must be homogeneous or very little plasmid will be extracted. P1 buffer may have a blue dye added to help you determine complete cell lysis (P2 buffer) and neutralization (N3 buffer) of the cells. The solutions turns a homogeneous blue color after correct mixing of cell lysis solution (P2). After correct mixing of neutralization solution (N3) there should be no blue color remaining.
3. **Cell Lysis**: Add 250 µl Buffer P2 and mix gently by inverting the tube 4 -6 times. Do not vortex. If necessary continue inverting until the solution becomes viscous and slightly clear.
4. **Neutralization**: Add 350 µl Buffer N3 and mix immediately by inverting the tube gently 4 -6 times. Do not vortex. The mixture should become cloudy. Centrifuge for 10 min (12,000 x g) at room temperature. A white pellet forms.
5. **Cartridge loading**: Label cartridge. The cartridge is supplied housed in a round bottomed tube. Decant the supernatant into the cartridge. Decant by quickly tipping your tube over the cartridge with top edges touching. Do not remove remainder of supernatant with pipetman. It is important that none of the precipitate is transferred to the cartridge. Centrifuge for 1 min (12,000 x g). Discard the flow-through (liquid in round bottom tube).
6. **First Wash:** Return the cartridge to the round bottom tube. Add 500 µl Buffer PB to the cartridge. Centrifuge for 1 min. Discard the flow-through. This step is required for to destroy nucleases in nuclease rich bacteria.
7. **Second Wash** Return the cartridge to the round bottom tube. Add 750 µl Buffer PE to the cartridge. Centrifuge for 1 min. Discard the flow-through. Centrifuge again for 1 min to remove residual wash buffer. Discard round bottom tube.
8. **Plasmid Elution:** Cut the top off a1.5 ml eppendorf tube. Then place the cartridge into the tube. Add 50 µl distilled H2O directly to the centre of the spin cartridge. Incubate at room temperature for 1 min. Centrifuge for 1 min. Discard cartridge and cap tube.
9. Clearly label tube with **plasmid name**, DNA, concentration, group #, and your initials. Remove 5 µl plasmid from each plasmid prep and add to a new labelled eppendorf tube containing 1 ml ddH_2O for the spectrophotometer reading.
   Note: procedure may be changed to 200 µl ddH_2O and 1 µl sample using 0.1 cm pathway cuvet, you will be notified if this should be done.
10. **Plasmid DNA concentration determination spectrophotometrically**:
    Add entire diluted sample to 1 cm pathway quartz cuvet. The TA will assist you with the absorbance reading in room 313. Dilute your sample before going to room 313. Measure

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[23]QIAprep® Miniprep Handbook. QIAGEN March 2003.

absorbance at 260 nm to determine concentration of DNA. Write the plasmid DNA concentration on each tube, see note for calculation information. Actually it is easy to calculate due to dilution factor, eg if absorbance of 5 µl in 1.0 ml is 0.035 this means that you have 0.35 µg/µl plasmid DNA (ie. 0.035/1 x 50 x 201 = 351.7 µg/ml or .35 µg/µl). Store at -20ºC in student sample box.
If using 0.1 cm pathway cuvet a similar calculation is done: if absorbance of 1 µl in 0.2 ml is 0.0035 this means that you have 0.35 µg/µl plasmid DNA (ie. 0.0035/0.1 x 50 x 201 = 351.7 µg/ml or .35 µg/µl). Store at -20ºC in student sample box.

Return all QIAGEN plasmid DNA prep solutions to original rack - do not discard.

Notes: (i) An absorbance reading of 1 corresponds to approximately 50 µg/ml for double stranded DNA. Maximum yield expected is 30 µg/cartridge. (ii) The yield of plasmid DNA is dependent on the plasmid copy number, plasmid type, bacterial strain, and growth conditions.

Week 3
Part III PCR megaprimer amplification[24]

1. Select one pMR106 plasmid DNA prep. Calculate the volume required to add 0.5 ng to PCR mixture. Most likely you need to dilute your plasmid DNA prep before adding. For example, if the concentration is 0.2 µg/µl, first dilute to 10^{-3} in ddH_2O, ie . 0.2 ng/µl in 10^{-3} dilution. Add 2.5 µl to PCR reaction mixture.

2. Add the following components to a sterile 0.5 ml flat top Eppendorf tube. Label tube clearly with correct group # ONLY.

Component	Amount
10 x amplification buffer	10 µl
pMR106 template DNA [pBluescript - *R. leguminosarum* rhaK gene BamHI/ HindIII insert]	0.5 ng
2.5 mM dNTP solution	8 µl
mutagenic primer (10 pmole/µl)	1 µl
low T_m external primer (R1) (100 pmole/µl)	1 µl
Taq polymerase + pfx[25] DNA polymerase mixture (5 units/µl)	0.5 µl
ddH_2O	to 100 µl

Brief centrifuge to mix or stir with pipette tip.

3. Heat PCR lid to 110ºC. Put tube in thermocycler and close lid. Set the thermocycler

[24] 10x Amplification Buffer: 500 mM KCl, 100 mM Tris-HCl (pH8.3), 2.5 mM $MgCl_2$. Autoclave for 10 min at 15 psi on liquid cycle. Divide into small aliquots and store at -20ºC.
External primers: dissolve ddH_2O at 100 pmoles/µl.
Mutant primer: dissolve ddH_2O at 10 pmoles/µl.
genomic DNA: dissolved in TE, pH 8.0 buffer at 0.1 µg/ml
dNTP solution containing all four dNTPs, 2.5 mM each

[25] high PCR fidelity

program as follows:

PCR 1 -megaprimer production	Loop 1 (1x)	94ºC 4 min, 42ºC1 min, 72ºC 1 min
	Loop 2 (24x)	94ºC 40 sec, 42ºC1 min, 72ºC 1 min
	Loop 3 (1x)	94ºC 40 sec, 42ºC1 min
	Finish	72ºC 5 min, 4ºC hold

4. Add 1 µl high T_m external primer (F2) (100 pmole/µl), 0.5 µl Taq polymerase + pfu polymerase mixture (5 units/µl) and 3 µl 2.5 mM dNTP solution. Mix gently by pipetting up and down or brief microfuge to mix. Return to thermocycler for PCR 2.

PCR 2 -mutant production	Loop 1 (25x)	94ºC 40 sec, 72ºC 2 min
	Finish	72ºC 5 min, 4ºC hold

5. The TA will put the reaction mixtures in the PCR machines and after completion of PCR store at -20ºC.

Week 4

Part IV QIAquick Gel Extraction[26] of PCR Product

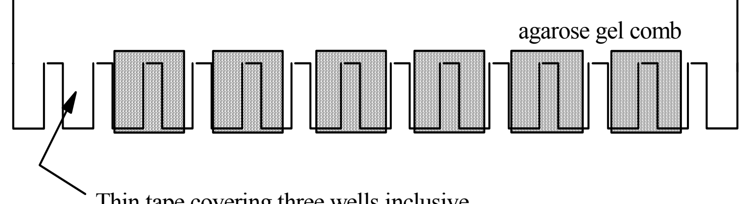


Figure 5. Well comb for preparative agarose gel electrophoresis.

1. The Entire PCR mixture is load into double wells of a large gel unit. Prepare 270 ml 1% agarose in 1x TAE buffer. Add ethidium bromide just before pouring (10 µl/250 ml agarose gel). The well comb is taped to enable loading of a large sample. Using plastic tape - tape off 2 wells (inclusive) and prepare agarose gel as usual. Use 1x TAE as the running buffer. Load 7 µl 1 Kb Plus Ladder at each end (single well). When cutting the DNA band out of the gel, the UV exposed time should be as short a time as possible. This helps reduce the nicks in double stranded DNA caused by ethidium bromide in the presence of UV light. Run at 80-100 volts for 4 hours.

[26]QIAquick Principle
Spin-Column containing silica-gel membrane selectively binds PCR DNA in the presence of high salts (required to neutralize the repulsion of PO_4^- groups, facilitating binding to silica-gel membrane). Binding is also dependent on pH. Isopropanol solubilizes dNTPs, etc and enhanses binding to silica gel membrane by exposing PO_4^- groups. The column binds PCR products as small as 100 bp while primers as large as 40 bp, nucleotides, EtBr, impurities, enzymes, salts, etc. are washed (PE buffer) through the column.
There is a pH indicator in both the solubilization buffer (QC), yellow ≤ pH 7.5 and orange or violet if higher. The pH must be lower than 7.5, add 3 M sodium acetate if orange or violet. The chaotropic agent, guandine thiocyanate solubilizes the agarose with heating.
EB buffer, low salt, alkaline pH 8.5 elutes PCR product from the column.

STUDENT LAB STARTS HERE

2. Wear disposable gloves. If gloves contain powder rinse gloved hands with water to remove any external powder. Cut out band and place in microtube keeping the size of the band as small as possible. The part of gel slice below the well bottom should be removed as it does not contain any DNA. Coarsely break gel up using a metal spatula. Centrifuge the sample for several seconds to bring down the gel slice. Estimate gel volume using graduated markings on the side of most Eppendorf tubes.

3. Add approximately 3 volumes **Buffer QC binding buffer (guanidine isocyanate)** to the gel slice and agitate gently to dissolve.

4. Place the tube in a 50ºC waterbath for 10 min or until completely dissolved. Shake every 2 or 3 minutes during the incubation. After the gel dissolves check that the color of the solution is still yellow. If orange or violet add 10 µl 3 M sodium acetate, pH 5.0 to turn mixture yellow.

5. Add 1 gel volume of **isopropanol**. Mix.

6. Place a **QIAquick spin column** in a provided 2 ml collection tube. Apply the sample directly to the center of column silica membrane. Microfuge 1 min. Discard flow-through and place column back in same collection tube.

7. Add 750 µl **PE wash buffer**. Microfuge 1 min. Discard the flow-through and place column back in the same collection tube. Microfuge 1 min.

8. Place **QIAquick spin column** in a clean 1.5 ml microtube. Add 30 µl **EB buffer** to the center of column silica membrane. Let column stand for 1 min. Microfuge 1 min.

9. Clearly label tube with group number, last names and description. Store at -20ºC until next week.

Week 5

Part V Restriction Digestion and Ethanol Precipitation of PCR product

1. To the 1.5 ml Eppendorf tube containing Prep-A-Gene purified PCR product add:
   11.5 µl   sterile distilled water
   2.5 µl   10x reaction buffer (Invitrogen® #3)
   1 µl each   BamHI and HindIII restriction enzymes (~10 units each) [add last]

2. Set up a second restriction enzyme reaction for pBluescript® digestion. To a 1.5 ml Eppendorf tube add ~5 µl for pBluescript® plasmid DNA, 1.5 µl 10x reaction buffer #3, 6.5 µl ddH_2O, and 1 µl each BamHI and HindIII restriction enzymes.

3. Mix by pipetting gently up and down. Spin for a few seconds if you have drops of liquid up the side of the tube.

Restriction enzyme digestion information:

·REACT buffer #3 is designed for BamHI, while HindIII requires REACT buffer #2, 1/2 NaCl concentration (50 mM). As both restriction enzymes are being used at the same time, buffer #3 is selected as it gives the 'best' digestion. However, the plasmid may not be completely digested due to sub-optimum digestion (only 40% active) conditions for HindIII.The expected PCR size is ~1380 bp.

4. Incubate for 1.5 hr at 37ºC.

5. Add 1 ml 95% ethanol and put at -20ºC for 10 min. Microfuge for 20 min at 4ºC. Aspirate off ethanol. Quick spin to bring down any remaining ethanol. Pipet off. Add 10 µl ddH_2O. Mix with pipette tip. Put open tube in a 65ºC waterbath for 10 min to evaporate off ethanol.

6. Completely label tubes (both partners should know how the tubes are labelled and where they are stored). Store at -20ºC until next week. **REMEMBER you need to start your ligation by 9:00 am the Monday after SPRING BREAK. Please do not forget.**

Week 7

Part VI PCR Product ligation into pBluescript and Transformation into *E. coli* DH5α

NOTE: The ligation reaction must be set up by 9:00 am the morning of the lab (4 to 6 hours incubation time at room temperature).

When you remove a small sample volume in an microtube from the freezer it is a good idea to give a quick spin to ensure all the sample is at the bottom of the tube.

Ligation (9:00 am)

1. Set up the ligation reaction in an microtube as follows:

addition	amount added
PCR product (BamHI/HindIII fragment)	4 µl
pBluescript (BamHI/HindIII digested)	1 µl
5x ligase buffer[27]	3 µl
T_4 ligase*	2 µl
distilled water	to 15 µl

*remember to keep the T_4 ligase on ice and return to freezer after using.
Incubate at room temperature for 4-6 hours.

Transformation

2. One tube of competent *E. coli* DH5α λpir cells (100 µl). Available on ice. Thaw if required and return to ice bucket if not used immediately. Add 10 µl ligation mixture. Competent *E. coli* DH5α prepared as previously described.

3. Heat shock at 43°C for 90 sec. Put on ice for 3 min.

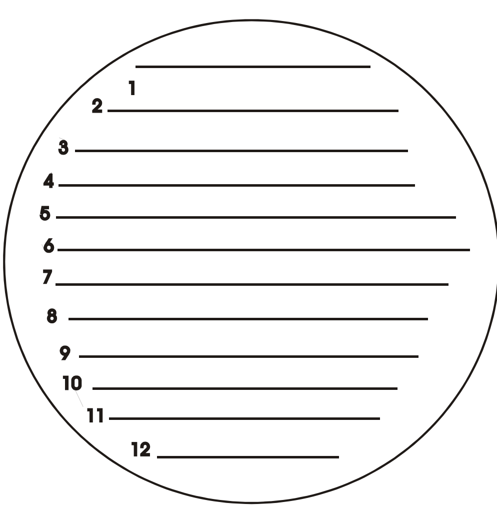
4. Add 1 ml prewarmed LB broth (37°C) and incubate for 1 h at 37°C.

5. Microfuge for 1 min at room temperature.

6. Resuspend cells in 100 µl LB broth.

7. Spread plate 90 µl and 10 µl on LB-AMP-Xgal IPTG plates.

8. Incubate plates at 37°C for 2 days. Check for isolated colonies, ie. transformants.



Re-streak method on LB AMP Xgal IPTG

9. WEDNESDAY select 30 isolated WHITE colonies (recombinants) and re-streak on LB-AMP-Xgal IPTG plates as shown in diagram. Use sterile sticks provided. Incubate at 37°C

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[27] 5x T_4 DNA ligase buffer: 250 mM Tris-HCl, pH 7.6, 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT, 25% (w/v) PEG, polyethylene glycol

for 1 day. Store at 4ºC. Do not incubate for more than one day as the ampicillin in agar quickly degrades. If you have no growth, streak more white colonies from original transformation plate. Incubate overnight. The more white colonies you streak the better as often what appears to be a white colony when re-streaked is really blue or growth doesn't occur. You need four white colonies with overnight growth to continue the experiment.

Part VII Plasmid DNA preparation and Recombinant Verification

10. **FRIDAY set up cultures.** First prepare four LB-AMP broths by adding 25µl 10 mg/ml ampicillin stock solution to 5 ml LB broth. Using sterile stick transfer a portion of your LB-AMP-Xgal IPTG streak plate recombinanat growth to 5 ml LB-AMP. Repeat using three more colony streaks with good growth. Clearly label each tube with student names (at least full lab names) and group #. Place inoculated broth tubes in labelled tray in STUDENT COLD BOX. Located just off room 201 in room 203. Sunday the cultures will be transferred to the 37ºC culture rotor and incubated with rotation for plasmid DNA isolation.

Week 8

Plasmid DNA Preparation(QIAGEN[28] kit method) and Restriction Digestion

1. Select two LB-AMP cultures (recombinant transformants) with growth. Do not discard extra cultures as you may need to give your extra cultures to another group. Repeat the following procedure for each.

2. **Cell Harvesting**: Aseptically transfer 3 ml transformed *E. coli* DH5α into two eppendorf tubes (each tube only holds 1.5 ml). DO NOT DISCARD CULTURE. Centrifuge at room temperature for 1 min (12,000 x g). **Remove supernatant by aspiration**. Aspiration must be used to completely remove all supernatant.
3. **Condensing two tubes to one tube and Cell Suspension**: Add 250 µl <u>Buffer P1</u> to ONLY one pellet tube and **completely resuspending the cells** using the pipetman. Transfer the resuspended pellet to the second pellet tube and again completely suspend cells. The solution must be homogeneous or very little plasmid will be extracted.
4. **Cell Lysis**: Add 250 µl <u>Buffer P2</u> and mix gently by inverting the tube 4 -6 times. Do not vortex. If necessary continue inverting until the solution becomes viscous and slightly clear.
5. **Neutralization**: Add 350 µl <u>Buffer N3</u> and mix immediately by inverting the tube gently 4 -6 times. Do not vortex. The mixture should become cloudy. Centrifgue for 10 min (12,000 x g) at room temperature. A white pellet forms.
6. **Cartridge loading**: Label cartridge. The cartridge is supplied housed in a round bottomed tube. Decant the supernatant into the cartridge. Decant by quickly tipping your tube over the cartridge with top edges touching. Do not remove remainder of supernatant with pipetman. It is important that none of the precipitate is transferred to the cartridge. Centrifuge for 1 min (12,000 x g). Discard the flow-through (liquid in round bottom tube).

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[28]QIAprep® Miniprep Handbook. QIAGEN March 2003.

7. **First Wash:** Return the cartridge to the round bottom tube. Add 500 µl Buffer PB to the cartridge. Centrifuge for 1 min. Discard the flow-through. This step is required for to destroy nucleases in nuclease rich bacteria.
8. **Second Wash** Return the cartridge to the round bottom tube. Add 750 µl Buffer PE to the cartridge. Centrifuge for 1 min. Discard the flow-through. Centrifuge again for 1 min to remove residual wash buffer. Discard round bottom tube.
9. **Plasmid Elution:** Cut the top off a 1.5 ml Eppendorf tube. Then place the cartridge into the tube. Add 50 µl ddH2O directly to the centre of the spin cartridge. Incubate at room temperature for 1 min. Centrifuge for 1 min. Discard cartridge and cap tube. You must use ddH_2O for plasmid DNA that is to be sequenced. Determine concentration of each plasmid DNA sample by measuring the absorbance at 260 nm of a 1/201 dilution (5 µl sample in 1000 µl ddH_2O*) as done previously.
   * procedure may be changed to 200 µl ddH_2O and 1 µl sample using 0.1 cm pathway cuvet, you will be notified if this should be done.

10. Completely label plasmid DNA preps - DNA type, group number, group last names, DNA concentration as the instructor needs to select the best ones (0.5 µg/µl) for sequencing. PLACE IN TRAY LABELLED DNA SEQUENCING IN THE -20°C FREEZER.

Part VIII Recombinant Verification by Restriction digestion and Agarose Gel Electrophoresis

1. Restriction enzyme digest each plasmid prep with two restriction enzymes, BamHI and HindIII. For each digestion set up the follow reaction mixture in an Eppendorf tube. RETURN ALL SUPPLIES TO FREEZER except distilled water.

Addition	Amoun t added (µl)
plasmid DNA (pMR106)	5
10x reaction buffer (Invitrogen® #3)	1.5
ddH_2O*	7.5
BamHI and HindIII restriction enzymes (~10 units each) restriction enzyme	1 µl each

*ddH_2O = double distilled water

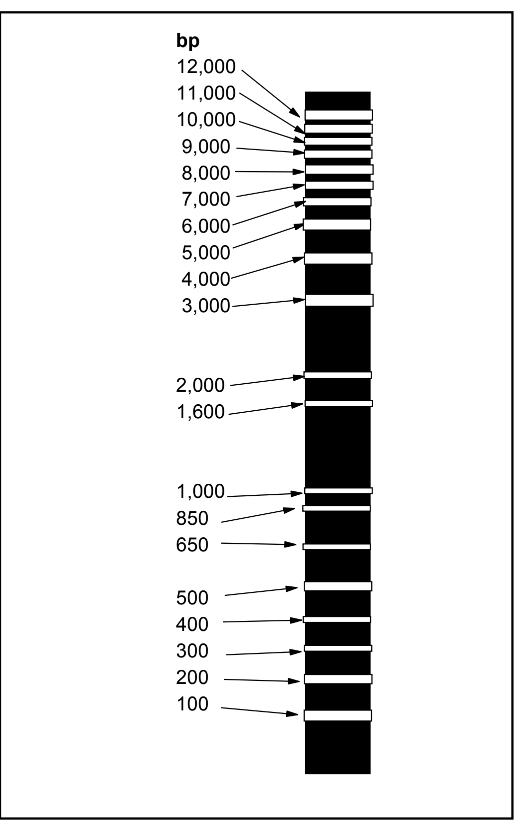

Figure 8a. 1 Kb Plus DNA Ladder Fragments. Fragments are linear double stranded DNA, therefore can only use as a standard for linear double stranded DNA. Bands are visualized by ehtidium bromide staining.

2. Incubate at 37ºC for 1 h. Stop reaction by adding 3 µl agarose stop solution.

3. For this experiment mini-gel electrophoresis

apparatus is used. Students are responsible for preparing a 1% agarose gel as described below. There are eight agarose gel electrophoresis units. As the maximum number of groups is 12, some groups must share gel with another group. All eight agarose gel units must be used. This is an essential recombinant DNA technique .

a) Prepare required percentage agarose in 1x Tris-acetate buffer. Add 60 ml 1x TAE buffer to a 250 ml Erlenmeyer flask. Add stirring bar. Place on heater stirrer. Turn on heat full and turn on stirrer until the bar rotates at medium speed. You do not want lots of bubbles forming while dissolving the agarose. Slowly add 0.6 g agarose (1%) while stirring. Cover loosely with overturned small beaker. Heat mixture until it comes to a boil and the agarose is completely dissolved. Remove from heater stirrer and cool at room temperature to ~55°C.
b) Put on nitrile gloves as ethidium bromide is a carcinogen. Add 3-4 µl 10 mg/ml ethidium bromide. Swirl gently to mix. Discard ethidium bromide tip in ethidium bromide waste container.
c) Pour agarose into gel holder that has been taped at ends with masking tape and well comb positioned. Allow to set at room temperature for ~20 min.
d) Carefully remove well comb and masking tape. Place gel in electrophoresis unit such that the well are toward the negative electrode (black).
e) Add 1x TAE buffer until the surface of gel is covered by ~3 mm of buffer using approximately ~300 ml 1x TAE (to fill line - makes it easy).
f) Load ~12 µl sample per well. Each sample is loaded using an eppendorf micropipet by holding the pipetman just above the well and releasing sample such that is sinks to the bottom of well. Good idea to steady your hand with the other hand.
In one lane add 5 µl 1 kb Plus DNA standard/ ladder (from Invitrogen).
g) The power supply should be connected such that the negatively charged DNA will migrate to the positive electrode. The black designates the negative connecting wire and red the positive electrode. Connect black to black and red to red. Turn on power supply and set at constant voltage (~100 volts). Electrophoresis for 1 hours. [Most likely the TAs will finish your experiment. Photographs of gels will be available on the website as soon as possible.] TURN OFF power pack before removing agarose gel.

4. Gels are photographed using Alpha Innotech's MultiImage light cabinet. The agarose gel is placed in an enclosed box on an UV transilluminator with a camera positioned on the top of the box. The box door is closed. Parameters selected to obtain the best photograph using the MultiImage software package. The file is saved, label and transferred to website as a jpg file.

5. Discard agarose gel in ethidium bromide waste container.

Part IX: Sequencing To identify gene in GenBank and To Confirm rhamose kinase bp Change (Site directed mutagenesis)

1. Plasmid DNA (10 µl containing 5 µg dsDNA) will be sent to University core sequencing service at the Faculty of Medicine, University of Calgary
3330 Hospital Dr. N.W., Calgary, AB T2N 4N1fax: (403) 283-4907. Sequence primer used is T7 primer (refer to pBLUESCRIPT diagram in lab manual introduction.. The data is posted for retrieval via FTP (WS_FTP) as pdf (*.pdf) and text sequence (*.seq). Files will be posted on website when available. Open the pdf file (all class data presented

together) and select only one sequence, either your group or another group if your DNA was not sequenced.

2. The sequence file (*.seq) may be opened in a sequencing program or Word or Wordperfect. Change font to Courier, 10 pt. Just copy and paste to blast search or contig alignment. DO NOT SELECT ALL THE SEQUENCE. Select only the accurate sequence as indicated by maximum height blue bars above the nucleotides. If red, yellow, short blue bar or designated as N, do not include any sequence in this area. That is, select only sequence in middle of printout that has basepairs showing blue maximum height bars.

3. BLAST SEARCH. Go to http://www.ncbi.nlm.nih.gov/
   - click on BLAST (top menu bar)
   - click on Nucleotide-nucleotide BLAST (blastn) under Nucleotide section
   - copy and paste sequence (or enter) in box
   - click on BLAST! button (leave subsequence blank and use default (nr[29]) database) - the program tells you how long you have to wait. After that time period click on Format!
   - scroll down. Select only the top bp alignment - has the highest score and lowest E value, best sequence match available, ie *Rhizobium leguminosarum* bv. *trifolii* rhamnose gene locus. Select alignment by holding down mouse button - click and drag. Either print or copy and paste to report. The sequence must be in a uniform letter format, eg. Courier - small print 10 pt.

4. To retrieve information about the best match click on link for top (BEST) alignment.

5. To retrieve additional information about the protein click on protein id number.

[29]nr database = All GenBank+RefSeq Nucleotides+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS, or phase 0, 1 or 2 HTGS sequences)

LAB 2 REPORT (Report format available as a Word document on lab website.)
Data Presentation and Analysis

3.0 1. **Primer Design**

Selection and characterization of a mutant primer for rhamnose ABC transporter; membrane-spanning protein (rhaP). Assume the rhaP coding sequence has been cloned into the same plasmid as has been done in lab with rhaK using the same R1 and F2 primers and polylinkers. Rha P sequence and amino acid translation available as a pdf file on 457 website (includes amino acid open reading frame) or download via NCBI Blastn Accession Number AF085687 for *Rhizobium leguminosarum* rhamnose catabolism gene locus, gene 7361-8362 bp, rhaP, ABC membrane transporter. Amino acid codon and abbreviations linked on lab website.

The following are two (GeneRunner software selected) possible mutant primers with original Tm values of 40°C[a] using nearest neighbour thermodynamic values and 50°C using lab simplified formula. All major primer requirements have been met, close to R1 external primer, size, etc. For each select a basepair to change to make M primer such that there is an amino acid change in the coding sequence of rhaP. Record all requested information in the following table.

Possible Mutant primer Underline/highlight bp to change	pb location of possible M primer in rhaP - 5' start	State bp change of M primer (eg. G –> A)	Amino acid change in the **coding sequence** of rhaP	
			original rhaP codon/ amino acid	mutant rhaP codon/ amino acid
5'CACGACGATGATGAGG	54			
5'CCACGATCATCACGAC	64			
State if primers are acceptable original primers to convert to mutant primer. 5'CACGACGATGATGAG G YES or NO 5'CCACGATCATCACGAC YES or NO				

[a] somewhat high but this depends on formula used to calculate Tm. In your lab you use a simplified formula, however Tm used by GeneRunner software (nearest-neighbour thermodynamic values method of Breslauer et al) is well in the range of M primer, 42-46°C.

1.0 2. State absorbance$_{260\text{ nm}}$ and concentration for each mutagenized recombinant plasmid. Show sample calculation.

1.5 3. Present a completely labelled figure of restriction digested plasmid DNA preps on agarose gel. In figure title indicate which of your DNA preps is a **potential** mutant rhamnose kinase. State why.

0.5 4. Include a printout of your groups sequence profile (.seq file) available on website. Select

only one sequence reaction to print either your group's sequence if available or another group's sequence and reference. Label 5' and 3' ends if not present.

1 5. a) Using above sequence carry out a Blast nucleotide search. Include a printout of best match of your sequence - this is the entire first bp alignment. Indicate 5' orientation. Highlight site directed bp change. If not as expected, indicate where on the alignment it should of occurred and what the bp should be. See procedure for instructions. There may be additional errors near the end of the sequence due to incorrect sequencing (past limit of accurate sequence length).

1.2 b) Uging BLAST and Protein ID information record requested information in the following table about the *Rhizobium leguminosarum* rhamnose catabolism gene locus.

How many genes are in the *Rhizobium leguminosarum* rhamnose catabolism gene locus?	
What is the function of the stem_loop 5760-5784 in the rhamnose catabolism gene locus?	
What is the protein ID for rhaK	
What is the amino acid size of the rhaK protein?	
What conserved domain is located in rhaK protein?	

1.0 6. Why was this particular bp change in the rhaK gene selected by Richardson et al[30]?

0.5 7. Explain why the T7 primer is the best choice of primer for sequencing in your MBIO 4570 lab, ie better than T3 primer. The question is **not asking** relative to KS primer or M13 -20 primer (both are acceptable, however, KS primer is risky).

0.3 8. Ask a question with respect to procedures used in this lab. This is a compulsory question. Questions will be collected and answered during Question lab just before lab exam.

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10

APPENDIX

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[30] Richardson, JS, Hynes, MF, Oresnek, IJ. 2004. A Genetic Locus Necessary for Rhamnose Uptake and Catabolism in*Rhizobium leguminosarum* bv. *trifolii*. J. Bact. 186:8433-8442 [linked on lab website or available on-line, search ASM journals]

MEDIA

LB (Luria-Bertani) Medium: dissolve 10 g Bacto-tryptone, 5 g yeast extract, and 5 g NaCl in 800 ml distilled water. Adjust to pH 7.5 with NaOH and bring up volume to 1 liter with distilled water. For agar plates add 15 agar/litre.
Add 7 g agar/liter for top LB agar.
LB-Tc: Add 5 ml per liter of stock 1 mg/ml tetracycline solution. (prepared in ddH_2O and filter sterilized).

Ty agar plates: 2.5 g Tryptone, 1.5 g Yeast Extract, 0.25 g $CaCl_2.2H_2O$, Distilled water to 500 ml. Add 7.5 g agar per 500 ml.

TySmTc AGAR PLATES
Streptomycin Tetracycline
Add 2.5 ml tetracycline stock (1 mg/ml) per 500 ml Ty agar.
Add 5.0 ml streptomycin stock (20 mg/ml) per 500 ml Ty agar.

VMM defined medium
Salt Mixture: dissolve 1 g K_2HPO_4, 1 g KH_2PO4 and 0.6 g KNO_3 bring up to 1 liter with double distilled water (ddH_2O). Dispense in 270 aliquots. Autoclave.
Trace Element Mixture: dissolve 0.05 g $FeCl_3$, 1.25 g $MgSO_4$ and 0.5 g $CaCl_2$ bring to 500 ml with ddH_2O. Dispense in 27 ml aliquots. Autoclave.
Vitamin mixture: dissolve 0.01 g biotin, 0.01 g thiamine-HCl, 0.01 g Ca pantothenate (pantothenic acid) dissolve and bring to 100 ml with ddH_2O. Filter sterilize.
Prepare 1.5 M carbon source. Autoclave.
After media cools add 27 ml trace elements, 3 ml carbon source and 0.3 ml vitamin mixture to 270 ml salt mixture.

SOLUTIONS and FUNCTIONS

General

Saline: dissolve 8.5 g NaCl in a total volume of 1 liter distilled water. This is physiology isotonic solution. Used to prepare bacterial dilutions, prevents cells lyses.

TAE buffer (10x): 48.4 g Trisma base, 11.4 ml glacial acetic acid, 20 ml 0.5 M EDTA, pH 8.0, and distilled water to 1 liter. Buffers at desired pH (added acetic acid until pH 8). DNA is stable at pH 8 and more soluble.

Agarose stop solution: Dissolve 12 g urea (denatures protein), 25 g sucrose (inert molecule that increases the sample density so the sample sinks to the bottom of the well), 0.95 g Na_4EDTA (chelates divalent cations, stops digestion as restriction enzymes require divalent cations for activity), and 0.5 g bromophenol blue (small colored molecule that runs at the front during gel electrophoresis, tells you how long to run the gel) in 40 ml distilled water. Adjust to pH 7.0. Bring volume up to 50 ml with distilled water.

5x T_4 DNA ligase buffer: 250 mM Tris-HCl, pH 7.6, 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT, 25% (w/v) PEG, polyethylene glycol

DNeasy DNA purification (GIAGEN)
(as process is patented many of buffer components are not available)
Proteinase K Digests proteins, activity of 600 mAU/ml
Buffer ATL - buffer system optimized for tissue lysis in presence of proteinase K
Buffer AL and AW1 - contain the chaotropic salt, guanidine hydrochloride (degrade proteins)
Buffer AL contain optimum conditions for DNA binding to the spin column membrane
95% ethanol - allows DNA to bind to the column, ie., optimum conditions for DNA binding to the column
DNeasy spin column - silica-gel-membrane - selectively binds DNA (glass binds DNA), contaminants pass through, binds from 0.1 to 50 kb DNA fragments, predominantly 30 kb fragments (genomic)
Buffers AW1 and AW2 - both contain ethanol and salts when centrifuged remove enzyme inhibitors such as proteins and divalent cations.
Buffer AW2 contains sodium azide as a preservative

QIAGEN Plasmid DNA preparation:
Cell Suspension Buffer P1: 150 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 μg/ml RNase A
-centrifuge to remove media components, and resuspend in buffer to give a homogenous suspension of bacteria cells that is appropriate for lysis of cells in the next step.
150 mM Tris-HCl, pH 8.0 optimum ionic and pH for stability of cells- DNA is more soluble at pH 8.0.
10 mM EDTA - chelates divalent cations may be involved in initial destabilization of cell walls but does not lyse the cells, available divalent cations removed from cell surface. Inhibits the activity of nucleases (DNase).

100 μg/ml RNase A - degrades RNA, really required for next step, cell lysis, to degrade RNA released from the cell. Usually added at 1 μg/ml or less.
Cell Lysis Solution P2: 0.2 M NaOH, 1% SDS
Lysis occurs under controlled conditions, the cell membrane should remain attached to the genomic DNA so when the alkaline solution is neutralized with potassium acetate the cells debris traps the genomic DNA and is precipitated out of solution
0.2 M NaOH - alkaline lysis of cells, also degrades DNA to single strands, both genomic and plasmid DNA
1% SDS - dissolves cell membranes, lysis of cell, solubilizes phospholipids
Neutralizing Buffer N3: contains high concentration potassium acetate, pH 4.8 and guanidine hydrochloride
Precipitate protein and neutralizes alkaline conditions of cell lysis solution. As stated above the precipitating protein traps other cell debris including degraded genomic DNA. Genomic DNA cannot reanneal but the closed circular plasmid DNA can reanneal as attached. The plasmid DNA is release in solution. This solution also contains guanidine hydrochloride which denatures proteins, inhibits DNase activity and enhanses binding of the DNA to the silica gel
-adjusted to high salt
Cartridge
- spin cartridge contains silica based membranes that selectively bind plasmid DNA at high salts and pH ≤7.5. Polar stationary phase, sieves - selectively retains (trapped) range of DNA wanted.
Wash Buffer PB: contains acetate, guanidine hydrochloride, EDTA and isopropanol
-second chance at destroying any remaining nuclease activity. DNA remains attached to cartridge, removes any contaminants soluble in isopropanol.
Wash Buffer PE (200 mM NaCl, 20 mM Tris-HCl, pH 7.5, dilute 1:1 with 95% EtOH)
remove salts and other impurities (nucleotides, proteins, etc), while not removing the DNA bound to the resin
200 mM NaCl - stablility of DNA
20 mM Tris-HCl, pH 7.5 - optimum ionic and pH
5 mM EDTA - prevent degradation of DNA, chelates divalent cations, prevents nuclease activity as requires divalent cations.
dilute 1:1 with 99.9% EtOH - solubilizes salts etc but not the DNA on resin
ddH$_2$O
elutes DNA from resin. It is important that the pH of the water is between pH 7.0 and pH 8.5 to efficiently remove the plasmid DNA from the resin.

Southern blotting:
Reference: protocol from Boehringer Mannheim, The DIG System User's Guide for Filter Hybridization

0.25 M HCl - nick DNA thus chops DNA into smaller pieces - this is time dependent. This facilitates the binding of probe as there is more DNA/band for larger size fragments on agarose gel consequently the probe is not physically able to reach all DNA, but by treating with HCl, DNA band is more accessible to the probe.

Transfer buffer:0.4 N NaOH, denatures double stranded DNA to single strands (DNA must be single stranded to bind single stranded probe) and allows the transfer single stranded DNA from the agarose gel to the nylon blot via capillary action.
0.6 M NaCl - high concentration of NaCl is to stabilize the denatured DNA by Na-phosphate

binding

Neutralization solution, 5x SSC: .75 M NaCl, 75 mM sodium citrate, pH 7.0.- neutralizes the blot, same solution as hybridization solution, stable DNA, optimum conditions for annealing of single stranded DNA.

Hybridization
4 M LiCl: added for ethanol precipitation of randomly labelled probe. Increases the efficiency of DNA precipitation especially for small amounts of DNA.
5x SSC: 750 mM NaCl, 75 mM sodium citrate, pH 7.0.
Standard hybridization solution: 5x SSC, 1.0% (w/v) Blocking Reagent for nucleic acid hybridization, 0.1% sarcosyl, 0.01% sodium dodecyl sulfate (SDS).
NaCl stabilizes the DNA as the melting temperature (T_m) of double stranded DNA is affected by the monovalent cation concentration. There is a 16°C increase in T_m with each log increase (10-fold) in monovalent cation concentration. Decreases stringency and promotes formation of double stranded DNA, ie., promotes annealing of single stranded DNA.
Na citrate - in addition to presence of sodium has some buffering capacity. Citrate chelate divalent cations but does not have a strong dissociation constant as compared to EDTA, ie reversible.
0.01% SDS and 0.1% SLS (sarcosyl) - ionic detergents, acts like a detergent solubilizes non-specifically bound molecules keeping everything in solution. As a result reduces background.
blocking reagent - patent, inert protein that binds the membrane wherever the DNA is not bound. This effectively reduces nonspecific binding of the probe to membrane filter. As a consequence reduces background binding of the probe. As the probe only binds to homologous DNA dependent on the stringency of the hybridization. The blocking reagent also functions to increases the density of the solution, effectively reducing the volume of the hybridization solution. This increases the concentration of the probe as there is less liquid so the probe has a better chance of coming into contact with the immobilized DNA on the blot.

Hybridization Blot Wash solutions: 0.5x SSC containing 0.1% SDS and 0.1x wash solution containing 0.1% SDS: 0.1x SSC containing 0.1% SDS: Both wash solutions are used to reduce background binding such that there is a significant difference between background and homologous DNA band. The salt concentration is decreased (from 5x SSC during hybridization) to increases stringency. Stringent conditions, DNA annealing accuracy 70% to 100%, as compared to relaxed DNA annealing conditions during hybridization. 0.5x SSC is the less stringent wash while 0.1x SSC is the high stringency wash, both should remove non-homologous binding of probe. Washing is done in steps to prevent over washing, ie, the removing of homologous bound probe. Temperature also plays a part in washing the blot the higher the temperature the more stringent the wash conditions.

Chemiluminescent Detection
Wash buffer: 100 mM maleic acid, pH 7.5 (buffers at optimum pH), 150 mM NaCl (optimum ionic conditions, stabilizes DNA). Tween 20 - non-ionic detergent that behaves as a soap, solubilizes, reduces background (non-specific binding), but does not degrade protein.
Detection buffer: 100 mM Tris-HCl, pH 9.5 (+20°C) (buffers at alkaline conditions required by the alkaline phosphatase to produce light, 100 mM NaCl (optimum ionic strength for the reaction)

Restriction enzyme storage buffer:

50 mM Tris-HCl, pH 7.2, buffers at optimum pH
300 mM NaCl - ion concentration
0.5 mM EDTA - chelator of divalent cations, many degradative enzymes require divalent cation. Prevent degradation of enzyme.
5 mM EGTA, - weak chelator of divalent cations, as above
5 mM mercaptoethanol, - reducing agent when added at low concentration maintains a reducing environment similar to *in vivo*. In vitro, enzymes may be oxidatively degraded thus no longer active.
500 μg/ml BSA (bovine serum albumin), inert protein that increases the protein density. Proteins are more stable in a concentrated solution maintaining the enzyme activity of the restriction enzyme as long as possible.
50% (v/v) glycerol, prevents freezing at -20ºC. It is a cyrogenic agent, ie. preserves the enzyme upon storage at -20ºC by preventing ice crystal degradation of the enzyme.
0.2% (w/v) TritonX-100 - non-ionic detergent, does not degrade the protein. Solubilizes components, keeps everything in solution.

Restriction Enzyme Reaction Buffer #3:
50 mM Tris-HCl (pH 8.0) - buffers at optimum pH
10 mM $MgCl_2$ - Mg cofactor for restriction enzyme
100 mM NaCl. - optimum ionic concentration

Pick plate grid for maximum 61 colonies .

Remember to include orientation mark or label the bottom of each plate as below. Remember to label plate type before you pick plate.

orientate

1 2 3
4 5 6 7 8 9 10
11 12 13 14 15 16 17
18 19 20 21 22 23 24 25 26
27 28 29 30 31 32 33 34 35
36 37 38 39 40 41 42 43 44
45 46 47 48 49 50 51
52 53 54 55 56 57 58
59 60 61

1 2 3
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18 19 20 21 22 23 24 25 26
27 28 29 30 31 32 33 34 35
36 37 38 39 40 41 42 43 44
45 46 47 48 49 50 51
52 53 54 55 56 57 58
59 60 61

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18 19 20 21 22 23 24 25 26
27 28 29 30 31 32 33 34 35
36 37 38 39 40 41 42 43 44
45 46 47 48 49 50 51
52 53 54 55 56 57 58
59 60 61

1 2 3
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18 19 20 21 22 23 24 25 26
27 28 29 30 31 32 33 34 35
36 37 38 39 40 41 42 43 44
45 46 47 48 49 50 51
52 53 54 55 56 57 58
59 60 61

PURE CULTURE METHODS

In a molecular genetics lab it is essential that pure culture methods by practised. The growth of a microorganism in pure culture means that all other microbes must be eliminated.

Sterilization

All materials that come into contact with the pure culture must be sterilized. A variety of ways are used to sterilize liquids, containers, and instruments: autoclaving (steam at 15 lb/in^2), exposure to radiation, and filtration.

Aseptic transfer technique

This techniques involves avoiding any contact of the pure culture, sterile medium, and sterile surfaces with contaminating microorganisms. This is accomplished by work area cleaned with AIRx109, the transfer loop sterilized by heating with a bunsen burner before and after transferring, and the work performed quickly and efficiently to minimize the time of exposure during which contamination of the culture or laboratory worker can occur. The steps for transferring a culture from one container to another are (a) flame the transfer loop and allow to air cool, (b) open and flame the mouths of the culture tubes/flasks, (c) pick up some of the culture growth and transfer to fresh medium, (d) flame the mouth of the culture vessels and reseal them, and (e) reflame the inoculation loop. Similar technique is used to transfer culture from a petri plate (only the petri dish is not flamed) and to transfer cultures using sterile pipettes (the pipette canister is flamed after removing top and then flamed again after removal of pipette before replacing the top). It is essential after removal of sterile caps, plugs, or pipette canister tops that they are kept in your hand sterile side down before replacing. DO NOT place on bench area.

Isolation

Pure cultures of microorganisms are isolated by several different methods; streak plate, spread plate, and pour plate. All the methods involve separation of single bacterium on solid (agar) media where it grows into a colony (clone). Individual colonies represent a single microorganism type. In the streak plate method a loopful of bacterial cells is streaked across the surface of nutrient agar plate. The method of streaking established a dilution gradient so that single colonies develop. In the spread plate method a drop of microbial suspension (for this lab 0.1 ml) is placed on the centre of an agar plate and spread over the surface of the agar using a sterile glass rod. Usually culture dilutions are plated to obtain an appropriate dilution permitting separated single colonies. For the pick plate method of replication transfer required number of colonies to duplicate plates using grid provided. The first step involves the placing the required number of plates onto the grid diagrams. The plates must be orientated for quick identification of a particular colony or quick comparison of duplicate plates by placing a small mark on the bottom of the plates as shown by arrow on grid circles. Use a toothpick to successively transfer a colony to each selective plate. The plates used to screen the bacteria are usually indicator plates (e.g. X-gal, IPTG) or plates containing antibiotics (e.g. streptomycin and tetracycline) as selective markers. The technique is very reliable allowing numerous replica plates.

Preservation

For short term storage (2-4 weeks) colonies of most bacterial strains can be maintained at 4ºC on the surface of agar media if the plates are tightly wrapped. Also liquid cultures can be maintained

at 4ºC for short term storage. For medium term storage (1-2 years) most strains of bacteria can be maintained in stab cultures. Such cultures are usually prepared in small screw capped bottles containing 2-3 ml agar medium. The culture is inoculated with sterile straight wire using a dense liquid bacterial culture, then stabbed deep into medium, and incubated overnight with a loose fitting cap. The cap is then tightened, wrapped with parafilm, and stored in the dark at room temperature or 4ºC. For long term storage, bacteria can be maintained in 15-50% glycerol (1 ml in a small screw capped vial) at low temperature without significant lose of bacteria. The bacterial culture can be stored at -20ºC for a few years or at -70ºC for many years. Bacterial cultures may also be stored at -70ºC in 8% DMSO, dimethylsulfoxide. Mix 0.8 ml of a fresh liquid culture with 0.07 ml DMSO in screw capped vial with rubber liner. Freeze the vial on dry ice and then store at -70ºC.

Dilution and Plating Procedure

- Prepare dilutions in a total volume of 1 ml using 5 inch metal capped test tubes or microtubes.
- Mix tube by vortexing after each transfer.
- Use a P200 (labelled on top of Pipetman piston) to transfer 0.1 ml/100 µl
- Use a P1000 to transfer 0.9 ml/900 µl.
- **It is extremely important that you do not turn the dial of the P200 above 0.2 ml/200 µl or P1000 above 1.0 ml/1000 µl as it causes permanent damage. Get assistance from the demonstrator if you are not sure of pipetman operation.** Refer to the appendix for operation of pipetman.
- Use separate tips for each dilution and each different dilution plating.
- Preparation of 10 fold- serial dilutions: transfer 100 µl of vortexed culture to 0.9 ml saline (10^{-1} dilution), vortex, then transfer 100 µl of 10^{-1} dilution to 0.9 ml saline (10^{-2} dilution), vortex - repeat this process until you have prepared the required number of dilutions.
- Generally 0.1 ml of culture or dilution is spread plated on agar medium unless otherwise specified.
- Use spread plate technique to distribute the bacteria evenly over the surface of the LB plate.
  1) Aseptically transfer 0.1 ml of culture or dilution to centre surface of agar plate. Never lift the lid of agar plate completely off the plate or place on bench surface. Best method is with the lid tilted above the plate.
  2) Dip hockey stick spreader in bottle of alcohol.
  3) Flame the spreader until alcohol ignites. Immediately remove spreader from flame and wait until alcohol completely burns off. Cool slightly.
  4) Open lid keeping it tilted over the plate and touch spreader to surface of plate that does not contain culture drop...if spreader is still hot it will kill bacteria. Still holding the lid tilted over plate, move the plate around spreading bacteria evenly over agar surface. (Use turntable to rotate plate if available.)
- Allow plates to dry right side up at room temperature for 5 min before incubating upside down for specified time and temperature .

DETERMINATION OF PROBE YIELD BY DIRECT DETECTION PROCEDURE
Require to ensure that you add the correct amount of probe to the hybridization.

1. Serial dilution of probe: Add 2 µl DIG-labeled probe to 38 (1/20) DNA dilution buffer. Mix and transfer 5 µl to 45 µl (1/10 x 1/20) DNA dilution buffer. (Repeat 1/10 dilution five more times.
2. Serially dilute DIG-labeled control DNA (stock is 5 ng/µl - dilute to 1 ng/µl) as step 1 starting with 1 ng/µl stock.
3. Using positively charged nylon filter (3 x 5 cm), apply 1 µl spots of each dilution of probe in a row. Directly below and in the same order apply 1 µl spots of each control DNA dilution. Mark location of each spot with a pencil.
4. Crosslink DNA to blot by placing a UV transilluminator for 3 min.
5. Place blot in 20 ml **wash buffer** (shake stock solution before adding) in a small plastic container (top of tip box). Incubate for 2 min with shaking on flat rotary shaker. Discard the wash buffer.
6. Add 10 ml **blocking solution**. Incubate for 30 min. Pour off and discard solution.
7. Add 10 ml **antibody solution**. Incubate for 10 min. Pour off.
8. Wash twice (2x 15 min) with 10 ml amounts of **wash buffer**.
9. Equilibrate for 5 min in 10 ml **detection buffer**.
10. Place blot between two acetate sheets.
11. Apply 0.1 ml (about 4 drops) of ready-to-use CSPD alkaline phosphatase substrate quickly across the surface of the membrane (do not allow the membrane to dry). As you apply, cover area with upper inner side of plastic bag - flip upper sheet up and down to spread the substrate. Cover smoothly - no air bubbles.
12. Incubate for 5 min at room temperature.
13. If excess liquid present, squeeze out.
14. Place acetate covered blot in sealable container on moistened paper towels. Seal lid. Incubate membrane for 15 min at 37°C. This step is required for activation of CSPD.
15. Expose the blot inside the clear acetate to X-ray film for 30 min. Develop film. If no highlighted plaques are apparent. Expose to film for 1 or 2 days. Develop film.
16. Compare dot intensity of your probe to control DNA. An excellent concentration is 20 ng/µl. For best results your DIG-labelled probe should have similar intensity to control DNA.

PIPETMAN OPERATION

In your lab, you have available three different pipetmen depending on the lab. If you look at the top of the plunger it states the size of the pipetman.
P20 measures accurately from 2 µl to 20 µl.
P200 measures accurately from 20 µl to 200 µl.
P1000 measures accurately from 100 µl to 1000 µl.
Never turn the pipetman above the maximum volume; 20 µl for P20, 200 µl for P200, and 1000 µl for P1000 as this breaks the pipetman. The scale on the pipettor is read different for each type - refer to Figure 5 for an example of how to read the scale.

(Excerpted from Gilson pipetman operation manual.)

1. Setting the volume: The required volume is set on the digital volumeter by turning the knurled adjustment ring (Figure 3-2A). The volumeter display is read from top to bottom in µl for P20 and P200 and ml for P1000 (Figure 3-2).

2. Place a disposable tip on the shaft of the Pipetman. Press on firmly with a slight twisting motion to ensure an airtight seal. Depress the push-button to the first positive stop (Fig. 3-3A). While holding the Pipetman vertical, immerse the tip 2-4 mm into the sample liquid. Release the push-button slowly to draw up the sample (Fig. 3-3B). Wait 1 to 2 seconds, then withdraw the tip from the sample.

3. To dispense the sample, place the tip end at a 10-45° angle against the inside wall of the vessel and depress the push-button SMOOTHLY to the first stop (Fig 3-3C). Wait 1 to 2 seconds and then depress the push-button completely to expel any residual liquid (Fig. 3-3D). With the push-button fully depressed, carefully withdraw the Pipetman, sliding the tip along the inside wall of the tube. Release the push-button. Remove the used tip by depressing the tip ejector button (Figure 3-1F).

pipetman diagaram

SAMPLE LAB EXAM
Recombinant DNA Technology **MBIO 4570 FINAL LAB EXAM**
DATE: sample **PAGE: 1 of 2** **TIME: 1.5 h**
INSTRUCTOR Dr. L. Cameron
Student Name ___________________________ Student Number ______________________

Answer exam questions in PEN ONLY.
Answer QUESTIONS ON EXAM PAPER.
Spacing has been removed for example exam.

1 1. List the relevant experimental steps that make *E. coli* cells competent. All steps serve the same purpose, what is it?

2. Explain the function of each the following experimental steps or solutions.

0.5 a) Heat shock at 43ºC for 90 sec. Put on ice for 3 min.(*E. coli* transformation)
0.5 b) Plate on LB-Tc ((*E. coli* transformation)
c) Plasmid DNA preparation solutions:
1 (i) 0.2 M NaOH, 1% SDS
1 (ii) 200 mM NaCl, 20 mM Tris-HCl, pH 7.5, dilute 1:1 with 95% EtOH
0.5 (iii) cartridge
1 d) Genomic DNA preparation solutions:
(i) sarcosyl-protease solution
(ii) chloroform
0.5 e) 0.4 M NaOH + 0.6 M NaCl (Southern Blot)
1 f) DIG-High Prime (contains hexanucleotide primers, dNTPs, dUTP-DIG-AP, Klenow and appropriate salts
1 g) standard hybridization solution (5x SSC, 1.0% (w/v) Blocking, 0.1% sarcosyl, 0.01 % sodium dodecyl sulfate (SDS).
0.5 i) sodium perchlorate in prep-a-gene purification binding buffer
0.5 h) VMM medium

1 3. How do you confirm that *E. coli* has been transformed with pKNOCK-kinase construct?

0.5 4. Explain the function of the helper phage pRK600.

1.5 5. For a conjugation experiment (*E. coli* DH5α (Str^s Pro^-) containing pKNOCK rhamnose kinase fragment plasmid construct x *Rhizobium leguminosarum* Rlt100 Str^r) state the medium (be precise) required to select for homologous recombinants. Explain why.

CONTINUED ON PAGE 2...

Recombinant DNA Technology **MBIO 4570 FINAL LAB EXAM**
DATE: sample **PAGE: 2 of 2** **TIME: 1.5 h**
INSTRUCTOR Dr. L. Cameron

2	6.	a) What is the purpose of the Southern blot hybridization experiment (*Rhizobium leguminosarum* construct)? Answer question by presenting a completely labelled schematic diagram of experiment results. Include a figure title with all relevant information.
1		b) Why is it important that *Rhizobium leguminosarum* construct genomic DNA be isolated with care (ie fragment size >50 kb)? That is, how would the hybridization results change?
1.5	7.	After hybridization the final wash is ~40 ml ml 0.1x SSC containing 0.1% SDS (pre-warmed to 68ºC). The blot is washed on the roller for 15 min at 68ºC. Explain the function of the wash components and temperature with respect to DNA hybridization.
1	8.	During agarose gel electrophoresis, linear double stranded DNA migrates at a rate that is inversely proportional to the log bp size. Explain why.
0.5	9.	a) What is the purpose of megaprimer amplification as used in your lab?
1		b) How does the megaprimer amplification differs from basic PCR amplification?
1		c) State the experimental steps that ensure there is only one PCR product.
1	10.	Explain why the megaprimer product is cloned into pBluescript plasmid.
1	11.	What is a blastn search? Why did you analyze your data with a two sequence contig?

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22

- END -