

MOLECULAR GENETICS OF PROKARYOTES

MBIO 4600

LAB MANUAL

2006

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

MBIO 4600 SCHEDULE 2006

LAB #	LAB DESCRIPTION	WEEK #	DATE	
			B02 Tuesday SLOT 23	BO1 Wednesday SLOT 25
1	The <i>lac</i> System: Review of Basic Genetics Techniques	1	Sept 12	Sept 13
2	Conjugation			
	F factor transfer	2	Sept 19	Sept 20
	Hfr factor transfer			
3	Transformation			
	Plasmid DNA preparation	3	Sept 26	Sept 27
	Preparation of competent <i>E. coli</i> cells			
	Transformation: Xgal detection system	4	Oct 3	Oct 4
4	Transduction	5	Oct 10	Oct 11
	P1 Generalized Transduction			
5	Transposition			
	λ 1098 Lysate Preparation	6	Oct 17	Oct 18
	λ 1098 Titration	7	Oct 24	Oct 25
	Tn10 Transposition with λ 1098	8	Oct 31	Nov 1
	Lab exam (class slot 4 - 8:30 am)	12	Nov 28	Nov 28

REPORT AND DATA DUE DATES for both Sections

Report #	Data Due ^a	Report Due ^b
Lab 1		Sept 27
Lab 2		Oct 11
Lab 3		Oct 18
Lab 4	Oct 17	Nov 1
Lab 5	Nov 6	Nov 15

^adue by 2:30 pm day requested

^bdue by 4:30 pm day requested

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GENERAL INSTRUCTIONS

Lab Instructor:	Dr. L. Cameron	Office: 414B
Demonstrators:	Vikash Jha	Lab: 410
	Brad Pickering	Lab: 105-7

Lab Location: 201 and 204 Buller Bldg.

WEBSITE: <http://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 4600 labs are continued throughout the week.

EVALUATION

[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. 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.
3. You must pass the lab to pass the course (10/20%).
4. The lab reports due dates are listed in the schedule.
5. Lab reports are to be handed-in as stated in the schedule by 4:30 pm of that day. Lab data is to be handed-in by 2:30 pm of the day requested. Hand in lab reports and data 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.**
6. 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.
7. Approximately two weeks prior to the lab exam, a brief outline of lab exam format and information content will be available on the website.
8. 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.

9. **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 (1 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, phrases and innovative**

terminology.” (Spatt¹, 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 (McMillan² 1997):

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 sources²:

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

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

²McMillan 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.

- 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 computer 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.

Genetic Nomenclature

Example:

Strain Number	Mating Type	Genotype	Important Properties
CSH121	HfrH	<i>ara ilvJ? zae::Tn10</i> <i>Δ(gpt-lac)5</i>	Pro ⁻ Lac ⁻ Str ^S ; donates Val ^r Tc ^r
CSH125	F ⁻	<i>ara leu lacY purE gal supE try</i> <i>his argG malA rpsL xyl mtl ilv</i> <i>metA</i>	Ara ⁻ Leu ⁻ Lac ⁻ Pur ⁻ Gal ⁻ Trp ⁻ His ⁻ Arg ⁻ Mal ⁻ Str ^r Xyl ⁻ Mtl ⁻ Ilv ⁻ Met ⁻ Su ⁺

- Each *E. coli* has a strain number.
- Genotype lists contains altered genes, usually defective, or some type of resistance which can be recognized by abbreviation for gene written in italics and lowercase letters. If a gene is not listed it is assumed it is functional (wild type) or wild type genes may be written with upper-case letters and italics.
- Important properties are usually phenotypic expression of genotype. Not necessarily all phenotypes are listed just those pertaining to a particular experiment.

There are several types of altered genes used in this lab. The following are examples of each type.

Genotype	Phenotype	Type of Mutant	Explanation
<i>ara,</i>	Ara ⁻	carbon source	<i>E. coli</i> strain cannot use arabinose as a carbon source.
<i>metA</i>	Met ⁻	amino acid	Methionine must be supplied in medium as the <i>E. coli</i> strain cannot synthesize methionine (auxotroph).
<i>rpsL</i>	Sm ^r	antibiotic resistance	<i>E. coli</i> strain resistant to antibiotic. It is assumed that a strain is sensitive to an antibiotic unless resistance stated.
<i>Δ(gpt-lac)5</i>	Lac ⁻ , Pro ⁻	deletion	Delta symbol means deletion of genes listed in brackets.

GENERAL REFERENCES

Sambrook J, & Russell D. 2001. *Molecular Cloning: A Laboratory Manual*. 3rd edition. New York: Cold Spring Harbor Laboratory Press. Three volumes.

Miller, J.H. 1992. *A Short Course in Bacterial Genetics*. New York: Cold Spring Harbor Laboratory Press. 456 p.

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

LAB REFERENCES BINDER CONTENTS

#	Reference Description
1	Miller, JH. 1992. <u><i>A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria</i></u> . New York: CSH Press. The nomenclature in this reference does not discriminate between wild type and mutant genes, using the same gene symbol (lowercase italics) for both. p 45-54: The Lac Operon - Introduction p 57-61: Some Tools of the <i>lac</i> Geneticist p112-120: Mutagenesis p 167-171: Reversion Tests p 193-196: Mutator Stains p 215-224, 227-233: Gene Transfer, Introduction p 309-321: Experiments with Transposable Elements, Introduction p 343-346: Transposition of Tn10 from Phage λ Vehicles, Introduction
2	Sambrook, J, Russell, DW. 2001. Chapter 13 Mutagenesis. In <i>Molecular Cloning A Laboratory Manual</i> 3 rd edition. Cold Spring Harbor: CSHL Press p. 1.147 - 1.150

LAB STANDARD OPERATIONS PROCEDURE (SOP)

Bench area: Wash bench area before and after with BDD (Backdown Detergent Disinfectant containing nonyl phenoxy polyethoxy ethanol, alkyl-aryl ammonium chloride and ethyl benzyl ammonium chlorides).

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 of after use.

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 and disposable pipettes 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 1 ml and 10 ml pipettes, tip down, in yellow plastic pail. 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, solvents, 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.

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-ldmbi/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)

- 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)

- all of level 1 specifications
- biosafety permit
- biological safety cabinet (not required)
- biohazard signage
- 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.

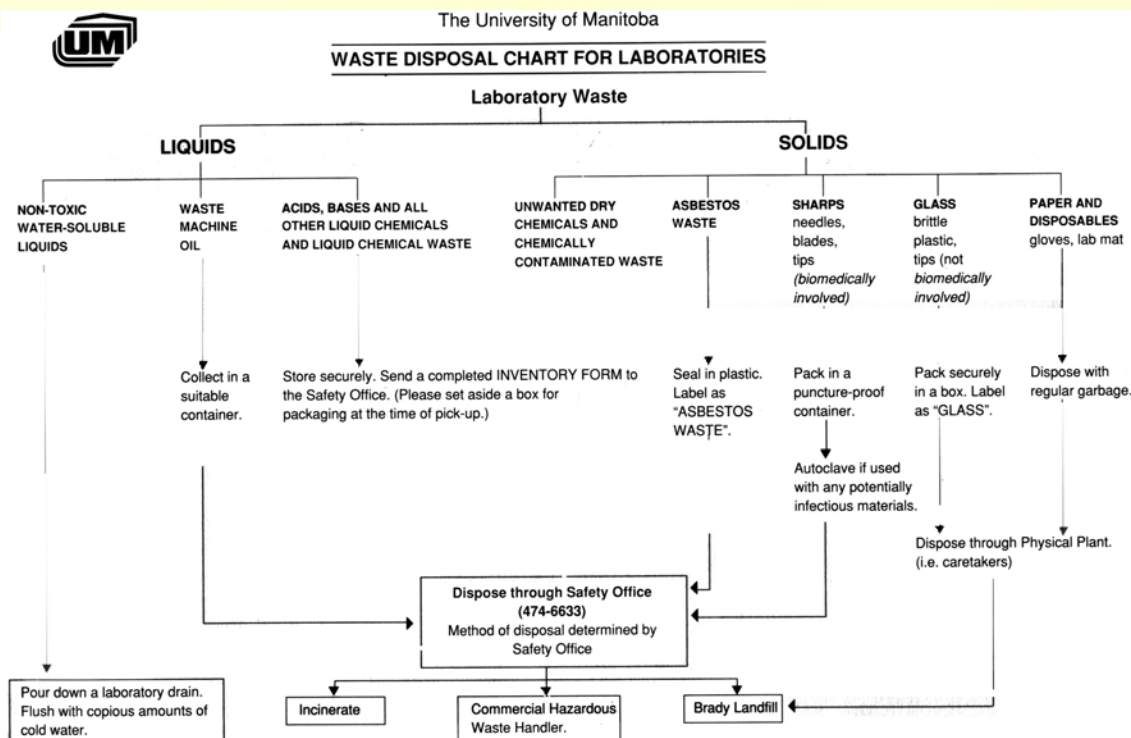
3. SAFETY

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



HAZARD CLASSIFICATION	SYMBOL	WORKPLACE LABELS	MATERIAL SAFETY DATA SHEET
CLASS A - COMPRESSED GAS		<p>These labels are applied at the workplace on controlled products or containers of controlled products when</p> <ol style="list-style-type: none"> The original supplier label is missing or becomes unreadable, The material is decanted or transferred from the supplier's container to another container, The material is produced at the workplace for use within the workplace. <p>Workplace labels will include the following information</p> <ol style="list-style-type: none"> Name/Identity of the Product 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) Reference to the MSDS. 	<p>A MATERIAL SAFETY DATA SHEET (valid for three years) will include information relating to each of the following categories</p> <ol style="list-style-type: none"> Product Identification and Use Hazardous Ingredients Physical Data Fire and Explosion Data Reactivity Data Toxicological Properties Preventive Measures First Aid Measures Date and Source of MSDS <p>and any other hazard information of which the Supplier is aware or ought reasonably to be aware.</p>
CLASS B - FLAMMABLE AND COMBUSTIBLE MATERIAL			
<ol style="list-style-type: none"> FLAMMABLE GAS FLAMMABLE LIQUID COMBUSTIBLE LIQUID FLAMMABLE SOLID FLAMMABLE AEROSOL REACTIVE FLAMMABLE MATERIAL 			
CLASS C - OXIDIZING MATERIAL			
CLASS D - POISONOUS AND INFECTIOUS MATERIAL			
<ol style="list-style-type: none"> MATERIALS CAUSING IMMEDIATE AND SERIOUS TOXIC EFFECTS MATERIALS CAUSING OTHER TOXIC EFFECTS BIOHAZARDOUS INFECTIOUS MATERIAL 			
CLASS E - CORROSIVE MATERIAL		<div style="border: 2px solid red; padding: 5px;"> <p style="text-align: center;">SUPPLIER LABELS</p> <p>Supplier labels on a controlled product must be in English and French and include the following information</p> <ol style="list-style-type: none"> Product Identifier Hazard Symbols Risk Phrases Precautionary Measures First Aid Measures Supplier Identifier Reference to the availability of a Material Safety Data Sheet <p>This type of border (shown) is the indicator that the label identifies a controlled product.</p> </div>	<p>FIRE / MEDICAL EMERGENCIES</p> <p>555 from 474, 789, 975, 977 exchanges or #555 from a cell phone (MTS or AT&T)</p> <p>OR</p> <p>911 from other exchanges. If 911 is called you must also call Campus Security at 474-9341</p> <p>CHEMICAL / BIOHAZARDS EMERGENCIES</p> <p>474-6633</p> <p>(8:30 am - 4:30 Mon. to Fri.). If busy or after hours, call 555</p>
CLASS F - DANGEROUSLY REACTIVE MATERIAL			

MANITOBA WORKPLACE 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).

LAB 1 THE *lac* SYSTEM: Review of basic bacterial genetics techniques

INTRODUCTION

PURE CULTURE METHODS

In a molecular genetics lab it is essential that pure culture methods be 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/in²), exposure to radiation, and filtration.

Aseptic transfer technique

This technique involves avoiding any contact of the pure culture, sterile medium, and sterile surfaces with contaminating microorganisms. This is accomplished by work area cleaned with BDD, 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) re flame 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

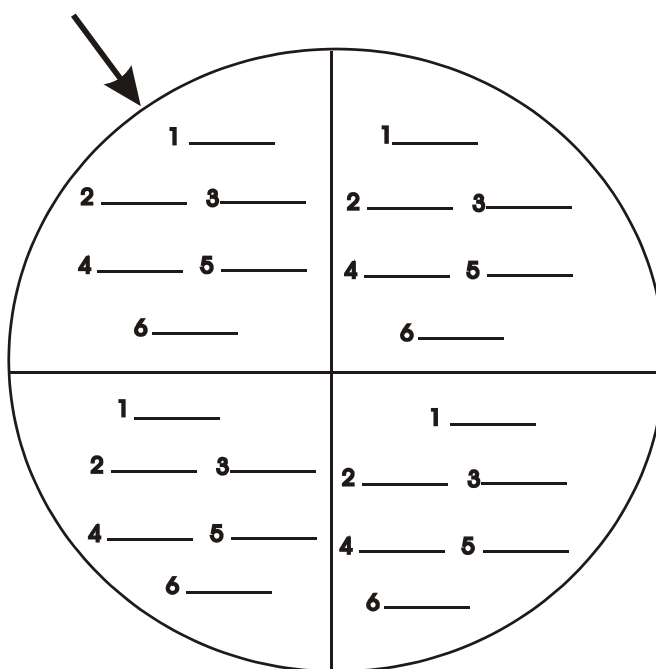
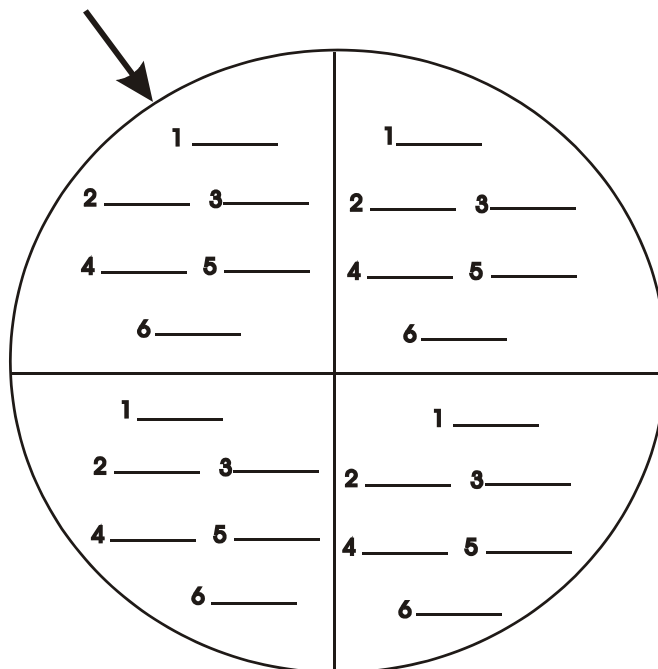
Pure cultures of microorganisms are isolated by several different methods; streak plate, spread plate, and pour plate. All the methods involve separation of single bacterium on solid (agar) media where it grows into a colony (clone). Individual colonies represent a single microorganism type. In the streak 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 agar plates using one of the grids provided. The first step involves the placing of duplicate agar plates onto the diagram* containing 2 grid circles. The agar plates must be orientated for quick identification of a particular colony or quick comparison of duplicate agar plates by placing a small mark on the bottom of the agar plates as shown by arrow on gridded circles. Another method of orientating involves drawing numbered lines on the bottom of the petri plate (this can be time consuming, but allows rapid location of a particular colony after storage). The agar plates used to screen the bacteria are usually indicator agar plates (e.g. Xgal, IPTG) or agar plates containing antibiotics (e.g. ampicillin and tetracycline) as selective markers. The technique is very reliable allowing numerous replica agar plates.

Preservation

For short term storage (2-4 weeks) colonies of most bacterial strains can be maintained at 4°C on the surface of agar media if the agar plates are tightly wrapped. Also liquid cultures can be maintained at 4°C 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 4°C. 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 loss of bacteria. The bacterial culture can be stored at -20°C for a few years or at -70°C for many years. Bacterial cultures may also be stored at -70°C 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 -70°C.

Duplicate pick plate grid for multiple strain pick plate.
Remember to include orientation mark or label the bottom of
each plate as below. Remember to label plate type before
you pick plate.

orientate



E. coli STRAIN LIST

STRAIN NUMBER	MATING TYPE	GENOTYPE	IMPORTANT PROPERTIES
CSH100	F ⁺	F' <i>lacproA</i> ⁺ , <i>B</i> ⁺ (carries I ^Q P mutations in the lac region) <i>ara</i> Δ(<i>gpt-lac</i>)5	I ⁺ P ⁻ Z ⁺
CSH101	F ⁺	F' <i>lacproA</i> ⁺ , <i>B</i> ⁺ (carries I ^Q Z mutations in the lac region) <i>ara</i> Δ(<i>gpt-lac</i>)5	I ⁺ P ⁺ Z ⁻
CSH140	F ⁺	F' <i>lacproA</i> ⁺ , <i>B</i> ⁺ (carries I mutations in the lac region) Δ(<i>lacpro</i>) <i>supE</i> , <i>thi</i>	I ⁺ P ⁺ Z ⁺
CSH141	F ⁺	F' <i>lacproA</i> ⁺ , <i>B</i> ⁺ <i>ara</i> Δ(<i>gpt-lac</i>)5 <i>supE rpsE</i>	I ⁺ P ⁺ Z ⁺

Note: genes listed after F' are carried on the F' episome.

PROCEDURE

STUDENTS WORK IN PAIRS FOR THIS LAB AND ALL SUBSEQUENT LABS.

Comment: Remember to treat all bacteria as possible pathogens. Read general instructions and introduction before starting this lab. USE ASEPTIC TECHNIQUE.

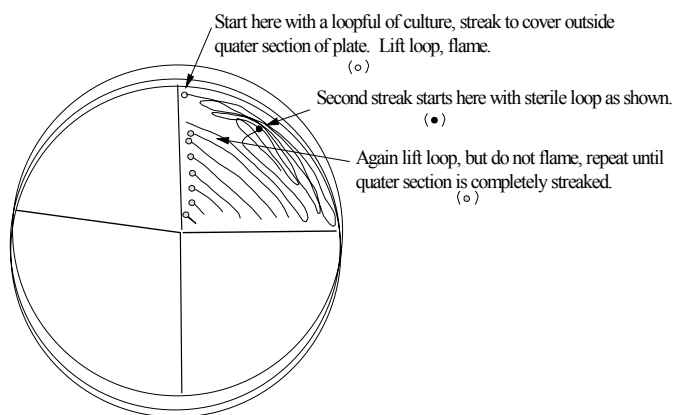
Week 1

One culture of each strain is available per two groups.

Part I: Multiple single colony isolation and application of selective indicator plates.

1. Prior to start of lab, an overnight culture of each *E. coli* strain was subcultured (1/50) into 5 ml LB broth and incubated with shaking at 37°C 3 hours to obtain an exponential culture.

2. Take four plates, LB agar, MacConkey, Xgal glucose and Xgal glucose + IPTG. Divide plates into four sections and streak each strain (CSH100, CSH101, CSH140 & CSH141) on one section. Refer to diagram for streaking method.



3. Incubate agar plates upside down at 37°C for 20-24 hours. Record results as requested in data sheet.

4. Next day use the LB agar plate for pick plate procedure (Part III).

Part II: Titration of *E. coli* CSH100

1. Prior to start of lab, an overnight culture of *E. coli* CSH100 was subcultured (1/50) into 5 ml LB broth and incubated with shaking at 37°C for 3 hours to obtain an exponential culture.
2. Prepare 10-fold serial dilutions 10^{-1} to 10^{-8} of *E. coli* CSH100.
 - Prepare dilutions in a total volume of 1 ml using 5 inch metal capped test tubes.
 - Mix tube by vortexing after each transfer.
 - Use a P200 (labelled on top of Pipetman piston) to transfer 0.1 ml (100 μ l)
 - Use a P1000 to transfer 0.9 ml (900 μ l).
 - **It is extremely important that you do not turn the dial of the P200 above 0.2 ml/200 μ l or P1000 above 1.0 ml/1000 μ l 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 μ l of vortexed culture to 0.9 ml saline (10^{-1} dilution), vortex, then transfer 100 μ l of 10^{-1} dilution to 0.9 ml saline (10^{-2} dilution), vortex - repeat this process until you have prepared dilutions up to 10^{-8} dilution.
3. Plate 0.1 ml 10^{-4} to 10^{-8} dilution in duplicate on LB agar plates. Total of 10 agar plates. Note: for all subsequent experiments 0.1 ml of culture or dilution is spread plated on agar medium unless otherwise specified.
4. 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.)
5. Allow plates to dry right side up at room temperature for 5 min before incubating upside down at 37°C overnight.
6. **Next day**: Count colonies on plates (all plates containing separated colonies) and record plate count data. Colony counters are available, see appendix for operation or ask your TA for assistance during scheduled lab time.

Day 2**Part III: Pick plate technique review**

1. From the LB plate containing the four *E. coli* strains, CSH100, CSH101, CSH140, & CSH141, pick plate each strain onto a quarter section of a glucose M9 minimal media plate and a lactose M9 minimal media plate. Use 6 colonies of each strain. You require only one plate of each medium.

Quarter section pick plate procedure (use multiple strain pick plate grid):

[Repeat the following pick plate procedure for each strain, ie. quarter section]

Colony one:

- First lightly touching the sterile tooth pick (use a separate tooth pick for each colony) to a pure colony
- next streak a line about 0.5 to 1.0 cm long (depends on space available) in number 1 grid or line of the first plate
- then without retouching the original colony streak in number 1 grid or line of the second plate
-

Colony two, etc:

- the next colony is picked to number 2 grid/line
- the process repeated until required number of colonies have been 'picked'.
- make sure the surface of LB plate is gently streaked so as not to cut through the agar.

2. Incubate plates at 37°C for 1-2 days. Record results as requested on data sheet.
Note for Wednesday slot: Check plates Friday (one day). If sufficient growth has occurred, record results. If not, put plates at room temperature over the weekend.

Molecular Genetics of Prokaryotes MBIO 4600

LAB 1 REPORT

Data Presentation and Analysis

Report tables available in Word format on lab website, save format, Open Word before entering requested information.

- Record requested indicator plate information in the following table

Table 1. Investigation of the <i>E. coli</i> Lac Operon phenotype using indicator plates.						
Incubation time and temperature:						
<i>E. coli</i> strain	Indicator Pate <i>E. coli</i> colony color					
	MacConkey (lactose)		Xgal M9 glucose		Xgal M9 glucose + IPTG	
	group data	expected ^a	group data	expected	group data	expected
CSH100 (I ⁺ P ⁻ Z ⁺)						
CSH101 (I ⁻ P ⁺ Z ⁻)						
CSH140 (I ⁻ P ⁺ Z ⁺)						
CSH141 (I ⁺ P ⁺ Z ⁺)						

^aexpected = theoretical color for *E.coli* strain genotype

Notes:

- Do not include detailed description of colony characteristics.
- Colony color is a subjective judgement.
- For MacConkey agar plates the literature states that Lac⁺ colonies are red, actual color more likely pink to deep rose pink.
- Lac⁻ colonies on the indicators agar plates are usually the color of medium (translucent).
- Xgal glucose agar plates: blue colonies range from pale blue to deep blue to white (translucent - color of medium). Record degree of blue color as this is pertinent to the discussion.

2. Record requested information in the following table.

Dilution	<i>E. coli</i> CSH100 plate counts		<i>E. coli</i> CSH100 titer (bacteria/ml)
	plate 1	plate 2	
10 ⁻⁴			
10 ⁻⁵			
10 ⁻⁶			
10 ⁻⁷			
10 ⁻⁸			

TNTC = too numerous to count

0.1 ml of each dilution added per plate

*Put an asterisk by plate counts used to calculate titer.

Show Titer Calculation:

Use all counts ≥ 30 plate counts.

3. Record requested pick plate information in the following table.

<i>E. coli</i> strain	# colonies ^b that showed pick growth		State whether results ^c expected (yes or not)
	M9 ^a glucose	M9 lactose	
CSH100 (I ⁺ P ⁻ Z ⁺)			
CSH101 (I ⁺ P ⁺ Z ⁻)			
CSH140 (I ⁺ P ⁺ Z ⁺)			
CSH141 (I ⁺ P ⁺ Z ⁺)			

^asee MBIO 4600 lab manual appendix

^b 6 colonies picked for each strain on each plate type

^cIf only one, possibly two colonies grow consider this the same as zero. Possibly due to either experimental technique, carry over, or spontaneous mutation.

Growth at 37°C for 2 days.

4. MacConkey analysis: What is the carbon source for *E. coli* CSH101? State why peptone utilization does not change the color of the colony?
5. Record requested information in the following table.

Table 4. Explain the phenotypic Xgal glucose plate color results for <i>E. coli</i> strains CSH100 and CSH140			
<i>E. coli</i> strain	Re-state expected color (include intensity)	with respect to Inducer (I) and Promoter (P) genotype	with respect to Xgal and β -galactosidase
CSH100 (I ⁺ P ⁺ Z ⁺)			
CSH140 (I ⁺ P ⁺ Z ⁺)			

6. Xgal glucose + IPTG analysis: Remember to take into consideration differences in color intensity. Discuss the function of IPTG in Xgal glucose medium with respect to *E. coli* CSH141 expected results. Restate expected color.

REFERENCES

- Miller, J.H. 1992. *Experiment in Molecular Genetics*. Cold Spring Harbor Laboratories, Cold Spring Harbor, New York. (selected sections available on 2 h reserve in Science and Technology Library)
- Watson, J.D., Hopkins, N.H., Roberts, J.W., Argetsiner, J. and A.M. Weiner. 1987. *Molecular Biology of the Gene*. The Benjamin/Cummings Publishing Company, Inc.

factor (Hfr) genetic transfer will be investigated.

For the F plasmid transfer a recipient mutator *E. coli* strain is used to demonstrate an effective method to increase the mutation rate above spontaneous mutation rate. The F⁻ recipient *E. coli* CSH117 strain contains mutY mutator locus. The mutY strain lacks glycosylase active on G:A and 8-oxod G:A mismatches being specific for bp transversions G:C → T:A. This complements the *E. coli* CSH104 donor F⁺ strain (lac⁻ mutant that required only a C → A reversion to change the amino acid from alanine to glutamic, which is the amino acid required for active β-galactosidase (located in the active site)⁵. However, after transfer of the F plasmid to the mutator strain it, the strain will continue to mutate at a higher than normal rate after the desired mutation is found. Consequently, it is important to remove the gene under study from the mutator strain, for example, transfer of F plasmid to *E. coli* CSH109. *E. coli* CSH109 is used as the control strain as it is the background strain for *E. coli* CSH117 mutY mutator.

The Tc^r marker on Hfr strain, *E. coli* CSH121HfrH is used to demonstrate Hfr conjugation into recipient F⁻ strain, *E. coli* CSH125. The Tc^r marker is actually a Tn10 transposon inserted in the host chromosome *zae* gene, *zae*-502::Tn10 which is transferred at 4.75 min.

When performing a conjugation it is important to select against both parents by plating on appropriate media.

Strain List

Strain number	Mating Type	Genotype	Some Phenotype Properties
CSH104	F ⁺	<i>F'lacproA⁺,B⁺</i> (carries <i>I_Z</i> mutations in the <i>lac</i> region) <i>ara</i> Δ(<i>gpt-lac</i>)5	Lac ⁻ Str ^s Reverts to Lac ⁺ via a specific base substitution in <i>lacZ</i> . Donates <i>F'lacproA⁺,B⁺</i> episome
CSH109	F ⁻	<i>ara</i> Δ(<i>gpt-lac</i>)5 <i>rpsL</i>	Pro ⁻ Lac ⁻ Str ^r
CSH117	F ⁻	<i>ara</i> Δ(<i>gpt-lac</i>)5 <i>rpsL</i> <i>mutY</i> ::mini-Tn10	Pro ⁻ Lac ⁻ Str ^r MutY
CSH121	HfrH	<i>ara ilvJ?</i> <i>zae</i> -502::Tn10 Δ(<i>gpt-lac</i>)5	Pro ⁻ Lac ⁻ Val ^r Tc ^r Sm ^s
CSH122	Hfr KL16	<i>zed</i> -977::Tn10 <i>relA1 spoT1 thi1</i>	Thi ⁻ Tc ^r Sm ^s
CSH125	F ⁻	<i>ara leu lacY purE gal supE try his argG malA rpsL xyl mtl ilv meta</i>	Ara ⁻ Leu ⁻ Lac ⁻ Pur ⁻ Gal ⁻ Trp ⁻ His ⁻ Arg ⁻ Mal ⁻ Sm ^r Xyl ⁻ Mtl ⁻ Ilv ⁻ Met Su ⁺ Tc ^s

It is assumed that wild type *E. coli* are sensitive to all antibiotics.

PROCEDURE

⁵Cupples, C.G. & Miller, J.H. 1989. A set of *lacZ* mutations in *Escherichia coli* that allow rapid detection of each of the six base substitutions. PNAS 86:5345-5349.

Week 2

Part I: F plasmid conjugation

Day 1

1. An overnight culture of donor *E. coli* strain CSH104 was subcultured 1:50 in 5 ml LB medium. Also an overnight culture of each recipient *E. coli* strains CSH109 and CSH117 was subculture 1:10 in 5 ml LB medium.
2. The recipient *E. coli* strains were incubated for 3 hours with shaking (aeration) at 37°C. The donor *E. coli* strains were incubated in a 37°C waterbath without shaking for 3 or 4 hours, or until a density of $1-2 \times 10^8$ cells/ml is obtained.

Student Lab starts here

3. Prepare mating mixture by mixing 0.2 ml of **donor strain** *E. coli* **CSH104** with 0.2 ml **recipient strain** *E. coli* **CSH117** in an Eppendorf tube. Set up a second mating mixture by mixing 0.2 ml *E. coli* **CSH104** with 0.2 recipient *E. coli* **CSH109** in an Eppendorf tube. Incubate in 37°C waterbath for 1 hour.
4. Using a wire loop, streak each mating mixture on a glucose M9 + streptomycin (M9glucoseSm) agar plate. As controls streak the donor and each recipient on ONE M9glucoseSm agar plate divided into 3 sectors.
5. Incubate at 37°C for two days.

Day 3

6. Papillae observation: After two days, select a single colony from each mating plate, (*E. coli* CSH104xCSH109 and *E. coli* CSH104xCSH117) and streak each on a lactose MacConkey agar plate and a M9glucose Xgal IPTG agar plate. Use a streak technique that will give you as many single colonies as possible.
7. Incubate at 37°C. You should start to see papillae after 3 or 4 days. Once papillae have formed store plates in student cold box until lab day.

Day 7

8. *E. coli* CSH104 x *E. coli* CSH117 cross only: Take a picture of papillae on each selection plate for, lactose MacConkey and M9glucose Xgal IPTG using stereoscopic microscope camera set up (only one set up for both lab rooms). Record requested information by camera - must know your group # which is available on fridge or freezer door in your lab. The TA will be taking the pictures. Papillae pictures will be available on lab website as soon as possible.

Part II: Hfr (Genome integrated F plasmid) conjugation

Day 1

Donor Hfr *E. coli* strains, CSH121 and CSH122 were grown overnight in LB-Tc broth and recipient F⁻ *E. coli* CSH125 was grown in LB-Sm broth, both with rotation at 37°C.

Even numbered groups use *E. coli* CSH121 and odd numbered groups use *E. coli* CSH122 as the donor. Each group uses only one donor and one

1. Set up the conjugation mixture at a ratio of 1:5, donor:recipient. In an Eppendorf tube combine 0.2 ml donor (either *E. coli* CSH121 or CSH122) and 1.0 ml *E. coli* CSH125.
2. Mix by inverting several times and immediately microfuge for 1 min at room temperature. Why immediately?
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 LB plate. Keep the spot as small as possible.
6. Incubate upright at 32°C overnight.
7. Negative Controls: Using a marker divide the bottom of a selection plate, LBSmTc⁶, in half. Label each section with bacterial name and stain number. Transfer 100 µl of each mating strain to one section. Use a sterile loop to spread spot over each section. Allow to dry. Incubate at 32°C. Check for growth on next day. Record results. The expected result is no growth or few scattered colonies. Record results and discard control plates.

Day 2

8. Place 1 ml saline on conjugation mating growth. Using the side of the Pipetman tip carefully mix until growth is suspended. Try not to gouge the agar. Tilt plate to allow suspension to run to side. Take up suspension several times with Pipetman each time releasing on growth to obtain a homogenous suspension. Then transfer the entire amount to a sterile Eppendorf tube (10⁰). Prepare 10⁻¹ dilution by adding 100 µl 10⁰ dilution to 900 µl saline. Mix. Repeat for 10⁻² dilution. Spread plate in duplicate 100 µl of 10⁰ (undiluted), 10⁻¹ and 10⁻² dilutions on LBSmTc agar plates. LBSmTc agar plates are located in STUDENT COLD BOX, covered with foil. Incubate all plates at 32°C overnight. You do not need to cover your plates, plastic container should be fine.

Day 3

9. Record colony counts for each conjugation mixture.
10. Pick plate any 6 conjugants onto LBSmTc plate, LBSm plate and LBTc plate to confirm conjugants. Use the same grid as lab 1.
11. Tuesday's section incubates overnight at 32°C while Wednesday's section incubates at room temperature over the weekend. Record pick plate results.

⁶ Sm = streptomycin, Tc = tetracycline.

LAB 2 REPORT

Report Format available as Word document on lab website.

Data Presentation and Analysis**Part I: F plasmid conjugation**

1. a) Record requested information in the following table.

Table 1. Initial M9glucose + streptomycin streak plate results for individual <i>E. coli</i> strains and mating mixture.		
<i>E. coli</i> strain	Degree of growth ^a	Expected degree of growth
CSH104		
CSH117		
CSH109		
mating mixture (CSH104 x CSH109)		
mating mixture (CSH104 x CSH117)		

^a - no growth, +/- few colonies, + growth, ++ heavy growth

1. b) State media component (presence or absence) in the following table that prevents growth of donor and recipient while allowing only the conjugant to grow. State the genotype of *E. coli* strain that supports the presence or absence of the media component.

Table 2. Analysis of donor and recipient <i>E. coli</i> strains with respect to media selection and parent genotype.		
<i>E. coli</i> strain	Media component (present or absence)	<i>E. coli</i> strain genotype and phenotype that matches media component
CSH104 donor		
CSH117 or CSH109 recipient		

2 2. a) Present one figure of F plasmid conjugant (*E. coli* CSH104 x *E. coli* CSH117) papillae results on Xgal M9glucose IPTG and MacConkey (lactose) streak plates. The jpg files available on website may be too large, just copy and paste into Word document and resize to fit two pictures in one figure.

0.5 b) Did you observe papillae for the *E. coli* CSH104 x *E. coli* CSH109 cross?

Circle one: YES or NO

0.5 c) On occasion, papillae formation does occur for *E. coli* CSH104 x *E. coli* CSH109 cross. State why.

Part II: Hfr (Genome integrated F plasmid) conjugation

1 3. a) Record requested information in the following table.

Table 3. Initial LBSmTc streak plate results for individual <i>E. coli</i> strains and Hfr mating mixture.		
<i>E. coli</i> strain	Degree of growth ^a	Expected degree of growth
CSH121 or CSH122 circle one		
CSH125		
mating mixture		

^a - no growth, +/- few colonies, + growth, ++ heavy growth

0.5 b) State the phenotype of the Hfr conjugant.

- 1.5 4. Record initial Hfr conjugation results in the following table.

Dilution	Duplicate plate counts		Titre of conjugant mixture (bacteria/ml)
	plate 1	plate 2	
10^0			
10^{-1}			
10^{-2}			

TNTC = too numerous to count

Indicate in table with asterisk values used to calculate titre

Titre calculation:

- 0.5 5. Record pick plate results in the following table.

Table 5. Hfr conjugant (<i>E. coli</i> CSH121 x CSH1125) pick plate results.	
Media	# colonies ^a that showed pick growth
LBSmTc	
LBSm	
LBTc	

^a 6 conjugant colonies picked

Question

- 1.5 1. Diagram pick plate results that demonstrate the isolation of a colony that is both a histidine auxotroph and can no longer utilize rhamnose from a mutagenized wild type *E. coli* culture that has been previously spread plated to give individual colonies on LB medium. Diagram must be labelled. Answer must be concise, no text other than labelling of diagram accepted (ie., not marked).

LAB 3 TRANSFORMATION

Introduction

Plasmid Isolation

Plasmid DNA isolation involves (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 grow in LB medium plus antibiotic that selects for plasmid (antibiotic resistant gene located on plasmid). pBluescript has the AMP^r gene, *bla* that codes for TEM β -lactamase which inactivates β -galactosidase.

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. The cells may be lysed by boiling, alkali, sodium dodecyl sulphate, SDS (ionic detergent), triton-X (non-ionic detergent), phenol, etc. In this lab, you use the QUAGEN plasmid kit which uses mild alkali (up to pH 12.5) treatment to break most of the hydrogen bonds in DNA and degrade chromosomal DNA. Closed circular plasmids regain their native configuration when returned to neutral pH while larger linear chromosomal DNA fragments remain in the denature state trapped in the cell debris. The cell debris is precipitated and the supernatant containing the plasmid applied to a silica gel membrane that binds the plasmid. The membrane bound plasmid is washed to remove contaminants then eluded as a pure plasmid prep. The yield of plasmid DNA is dependent on the plasmid copy number, plasmid type, bacterial strain, and growth conditions. See lab manual appendix for function of various QIAGEN plasmid solutions.

Transformation of Competent *E. coli* cells

Mandel and Higa⁷ (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 heat shock treatment at 43°C. A similar procedure was used by Cohen et al⁸(1972) to transform bacteria with plasmid. This simple method similar to what we use in the lab generates 10⁵ to 10⁶ transformed colonies/ μ g plasmid DNA⁹. Up to 50 ng plasmid per 100 μ l competent cells should be added to obtain efficient transformation but the volume of plasmid should not be greater than 5% of the competent cells. Both these rules may be broken to some degree and still obtain fairly good transformation.

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, electroporation, etc. All these techniques optimize the efficiency of transformation making it possible to obtain as many as 10⁹ transformants/ μ g plasmid DNA. Once the plasmid DNA is inside the bacteria,

⁷Mandel M, Higa A. 1970. Calcium-dependent bacteriophage DNA infection. J. Mol. Biol. 53: 159-162.

⁸Cohen SN, Chang ACY, Hsu L. 1972. Nonchromosomal antibiotic resistance in bacteria: Genetic transformation of *Escherichia coli* by R factor DNA. PNAS 69: 2110-2114.

⁹Sambrook J, & Russell D. 2001. *Molecular Cloning: A Laboratory Manual*. 3rd edition. New York: Cold Spring Harbor Laboratory Press p 1.109-1.111.

the plasmid DNA replicates and expresses the drug-resistance marker that allows the transformed cell to survive in the presence of antibiotic.

Xgal Detection system¹⁰

The chromogenic substrate, Xgal (5-bromo-4-chloro-3-indolyl- β -D-galactosidase) is converted to an insoluble blue compound by β -galactosidase. In addition, Xgal is non-toxic making it a valuable tool for molecular genetics.

pBluescript® 3 kb plasmid (see diagram) contains the promoter region and the first 146 amino acids of *E. coli* β -galactosidase *lacZ* gene (α -donor fragment). The *E. coli* host for pBluescript contains a deletion *lacZ* Δ M15, missing the α fragment (or portion), this includes the 5' start methionine. As a consequence *lacZ* fragment is generated starting at the next methionine, generating an 3' fragment (ω or α -acceptor fragment). The α -acceptor fragment of *lacZ* is usually carried on F plasmid present in the the host *E. coli*. Neither fragment is active. However, they associate to form a functional β -galactosidase by α -complementation.

When *E. coli* DH5 α /pBluescript is plated on media containing Xgal/IPTG the colonies are blue. IPTG (isopropyl β -D-thiogalactoside), a non-fermentable lactose analog removes the repressor, ie fully activates *lacZ* gene. However, in most plasmid host *E. coli* strains the level of repressor is low making the need for IPTG optional.

A multi-cloning site (MCS) is inserted in the coding sequence of *lacZ* gene of pBluescript. The opening reading frame is maintained and the few additional amino acids does not interfere with a functional β -galactosidase. The MSC consists of numerous restriction enzyme sites that permit insertion of desired gene. In this lab the *rhaK* is cloned into pBluescript via the BamHI/HindIII restriction sites, designated pMR106. When *E. coli* DH5 α /pMR106 is plated on media containing Xgal/ IPTG the colonies are white due to inactive β -galactosidase.

¹⁰ Sambrook, J, Russell, DW. 2001. Chapter 13 Mutagenesis. In Molecular Cloning A Laboratory Manual 3rd edition. Cold Spring Harbor: CSHL Press p. 1.116 - 1.150

E. coli STRAIN LIST

STRAIN NUMBER	GENOTYPE	IMPORTANT PROPERTIES
DH5 α	F'phi80 <i>lacZ</i> delta(<i>lacZYA-argF</i>)U169 <i>deoR</i> <i>recA1 endA1 hsdR17</i> (rk-, m k+) <i>phoA supE44</i> <i>lambda-thi-1 gyrA96 relA1/F' proAB+</i> <i>lacIqZdeltaM15 Tn10(Tcr)</i>	Lac ⁻ Amp ^s
DH5 α /pBluescript	as above for DH5 α with pBluescript plasmid contributing <i>bla</i> (TEM β -lactamase) and functional β - gal gene	Amp ^r Lac ⁺
DH5 α /pMR106	as above for DH5 α with pBluescript plasmid contributing <i>bla</i> (TEM β -lactamase) and <i>R.</i> <i>leguminosarum</i> <i>rhaK</i> gene insert via BamHI/ HindIII restriction enzymes in multi-cloning site	Amp ^r Lac ⁻

Rha = rhamose C-source

PROCEDURE

Week 3

Part I Plasmid DNA preparation (QIAGEN¹¹ kit method)

E. coli cultures were grown overnight with rotation at 37°C in 5 ml LB-AMP broth.

Each group carries out the following procedure for *E. coli* DH5 α / pBluescript and *E. coli* DH5 α /pMR106.

- Cell Harvesting:** Aseptically transfer 3 ml *E. coli* culture 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.
- Condensing two tubes to one tube and Cell Suspension:** Add 250 μ l 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 solution 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.
- Cell Lysis:** Add 250 μ l 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

¹¹QIAprep® Miniprep Handbook. QIAGEN March 2003.

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 a 1.5 ml Eppendorf tube. Then place the cartridge into the tube. Add 50 μl sterile 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.
 9. Clearly label tube with **plasmid name**, DNA, concentration, group # ON LID TOP, and your initials. Remove 5 μl plasmid from each plasmid prep and add to a new labelled eppendorf tube containing 1 ml ddH₂O for the spectrophotometer reading.
 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 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 0.020 this means that you have 0.20 $\mu\text{g}/\mu\text{l}$ plasmid DNA (ie. $0.020/1 \times 50 \times 201 = 200 \mu\text{g}/\text{ml}$ or $0.2 \mu\text{g}/\mu\text{l}$).
- Note:**
An absorbance reading of 1 corresponds to approximately 50 $\mu\text{g}/\text{ml}$ for double stranded DNA. Maximum yield expected is 30 $\mu\text{g}/\text{cartridge}$.
11. After completion of labelling including concentration store your plasmid samples at -20°C in student sample box .
 12. For your lab report you need to determine ng plasmid added to each transformation mixture using plasmid concentration. For example, if your concentration is 0.2 $\mu\text{g}/\mu\text{l}$ and you add 5 μl of 10^{-2} dilution to the competent cells, this is 10 ng.

Part II Preparation of competent *E. coli* DH5 α cells

1. 5 ml LB broth inoculated 1/50 with overnight culture of *E. coli* DH5 α . Rapid rotated at 37°C for 3 1/2 h. Abs_{550 nm} should be 0.4-0.5. It is important a good oxygen supply is present to ensure log phase growth of *E. coli* subsequently competence. Each group receives two 5 ml log phase *E. coli* DH5 α cultures.

STUDENT LAB STARTS HERE

2. Combine two 5 ml *E. coli* DH5 α cultures in one centrifuge tube. Centrifuge at 10,000 rpm for 5 min at 4°C. Aspirate off the supernatant or use P-1000 to remove the supernatant. Discard supernatant. Resuspend the cells in 4 ml* sterile 0.1 M MgCl₂. Centrifuge at 10,000 rpm for 5 min at 4°C. Discard supernatant. Resuspend cells in 4 ml* sterile 0.1 M CaCl₂.
3. Incubate on ice for 30 min.
4. Again centrifuge at 10,000 rpm for 5 min at 4°C
5. Resuspend the cells in 0.8 ml sterile CaCl₂ + glycerol. Label eight Eppendorf tubes with ONLY your group # on the lid, no initials or names. On the side of tube, label competent *E. coli* DH5 α . Check fridge or freezer door to make sure you have the correct group #. Dispense 100 μ l in each Eppendorf tube. Incubated on ice overnight in student cold box. A box of ice should already be in the cold box.
6. Your competent cells will be transferred to the -80°C freezer.

*The prep room has dispensed these solution in 4 ml aliquots, no need to measure just use.

When **removing supernatant** pour away from pellet. Pellet should be on the upper side such that it is always visible to you as you remove supernatant. Supernatant may be remove using a Pipetman or pour off away from pellet, keep tube slightly tilted removing the remainder of the liquid using aspirator or Pipetman. Never allow the remaining supernatant to run back into the pellet . When using the aspirator again keep the pellet on the upper side and tube tilted so that you can remove the complete supernatant without disturbing the pellet with the tip.

Resuspension of pellet: You should always use the gentlest method possible. Froth has the potential to kill cells. Resuspend cell by (1) minimum time vortexing, (2) controlled shaking, (3) or using Pipet or Pipetman tip bringing the liquid up and down on the pellet, remember not to create a froth. Check that the pellet is completely suspended.

Week 4**Part III Transformation: Xgal Detection System****Day 1**

1. Thaw 7 tubes 100 μ l competent *E. coli* DH5 α cells at room temperature and put on ice (if not used immediately).

Set up the following transformation reactions.

plasmid name/concentration	competent <i>E. coli</i> DH5 α (μ l)	volume of plasmid	ng of plasmid added - depends on concentration
pBluescript concentration (μ g/ μ l): _____	100	5 μ l (10^{-2} dilution ^a)	
	100	5 μ l (10^{-1} dilution ^a)	
	100	5 μ l	
pMR106 (rhaK insert) concentration (μ g/ μ l): _____	100	5 μ l (10^{-2} dilution ^a)	
	100	5 μ l (10^{-1} dilution ^a)	
	100	5 μ l	
Negative control - no plasmid	100	0	NA

NA = not applicable

^a prepare 10^{-1} of your plasmid prep by putting 45 μ l sterile ddH₂O in an Eppendorf tube, add 5 μ l plasmid and mix. Transfer 5 μ l of the 10^{-1} dilution to 45 μ l sterile ddH₂O (10^{-2} dilution) in another Eppendorf tube and mix.

2. Heat shock at 43°C for 1 min. Put on ice for 3 min.
3. Add 1 ml prewarmed LB broth (37°C) and incubate for 1 hour in a 37°C waterbath.
4. For each plasmid transformation tube prepare 10^{-1} dilution in saline by adding 100 μ l transformation mixture to 900 μ l saline in an Eppendorf tube. Mix. Repeat 10-fold dilution twice more until 10^{-3} . Spread plate 100 μ l of each dilution (10^{-1} , 10^{-2} and 10^{-3}) on LB-AMP agar plates. Do not plate in duplicate. Ideally plates should be plated in duplicate for accurate titer determination, but due to cost/size of class duplicate plating has been omitted.
5. For the negative control transformation tube plate only the undiluted on one LB-AMP agar plate.
6. Incubate plates at 37°C for 1 days.

Day 2

7. Count colonies of all transformations.
8. For each plasmid transformation, ie., plasmid pBluescript and pMR106 pick plate 6 colonies onto ONE LB-AMP-Xgal IPTG agar plate using pick plate pattern from lab 1. For each plasmid type select any one ng plasmid transformation, not all. Incubate at 37°C for 1-2 days. If expected color has not developed, transfer LB-AMP-Xgal IPTG agar plate to Student Cold Box - enhances color. Check color development after one or two

days.

9. Record pick plate results.

LAB 3 REPORT (report format available as a Word document on lab website)

Data Presentation and Analysis

- 1.5 1. In the following table record absorbance_{260 nm}, plasmid concentration, and plasmid amount added to transformation mixture for each plasmid, pBluescript and pMR106. Show sample calculation for plasmid concentration ($\mu\text{g}/\mu\text{l}$) calculation and amount of plasmid added to transformation mixture (state dilution).

Table 1. Determination of the amount of plasmid added to each transformation mixture.					
plasmid	Absorbance at 260 nm	Concentration ($\mu\text{g}/\mu\text{l}$)	ng plasmid added to transformation mixture		
			5 μl undiluted	5 μl 1/10 dilution	5 μl 1/100 dilution
pBluescript					
pMR106					

Sample Calculations:

a) plasmid concentration ($\mu\text{g}/\mu\text{l}$) for _____ plasmid:

b) amount of plasmid added to transformation mixture (ng) for _____ plasmid at dilution _____:

- 1 2. Record transformation plate count results in the following table. Indicate in table with an asterisk values used to calculate transformation/ml titre.

Table 2. Plate count results for transformation of <i>E. coli</i> DH5 α with pBluescript and pMR106.			
plasmid	μl plasmid added to 100 μl competent cells	LB-AMP plating.	
		Dilution plated (0.1 ml)	Plate count
pBluescript	5 μl (10^{-2} dilution)	10^{-1}	
		10^{-2}	
		10^{-3}	
	5 μl (10^{-1} dilution)	10^{-1}	
		10^{-2}	
		10^{-3}	
	5 μl	10^{-1}	
		10^{-2}	
		10^{-3}	
pMR106	5 μl (10^{-2} dilution)	10^{-1}	
		10^{-2}	
		10^{-3}	
	5 μl (10^{-1} dilution)	10^{-1}	
		10^{-2}	
		10^{-3}	
	5 μl	10^{-1}	
		10^{-2}	
		10^{-3}	
negative control	0	10^0	

TNTC = too numerous to count

- 2.5 3. a) Record bacteria/ml and efficiency of transformation in the following table. Include sample calculation for transformants/ml and # transformants/ μg . Indicate in table with an asterisk the best amount of plasmid to add to 100 μl competent cells.

Table 3. The effect of plasmid concentration on the efficiency of <i>E. coli</i> DH5 α transformation with plasmids pBluescript and pMR106.			
plasmid	ng plasmid ^a added to 100 μl competent cells.	transformants/ml of each transformation solution	# transformants/ μg ^b plasmid
pBluescript			
pMR106			

^atransfer from Table 1 of this report.

^bfor calculation must consider the total volume of the transformation solution plated

Sample calculations.

Transformants/ml for plasmid _____ at ng plasmid _____ :

transformants/ μg (same as above):

- 0.5 b) Does your results support the literature that ≤ 50 ng plasmid added per 100 μl competent gives optimum transformation efficiency? Just state yes or no for each plasmid.

for pBluescript:

for pMR106:

0.5 4. a) Record pick plate results in the following table.

Table 4. <i>E. coli</i> DH5 α transformation with plasmids pBluescript and pMR106 LB-AMP-Xgal/IPTG pick plate results.	
Plasmid	Number/Colony color ^b
pBluescript	
pMR106	

^a 6 transformants picked

^b record as number of colonies of a particular color, eg. for pBluescript, 6 blue colonies.

0.5 b) The expected result for plasmid pMR106 transformation of *E. coli* DH5 α is 6 white colonies. Give one genetic reason why a white colony may not contain an insert in plasmid pMR106.

0.5 c) What experiment should be performed to ensure that an insert has been inserted in the MCS of pBluescript prior to sending the plasmid for sequencing. Do not include experiment details.

Question

0.5 1. Frequently 'satellite colonies' appear around an ampicillin resistant colony. What causes this?

LAB 5 TRANSDUCTION: P1 GENERALIZED TRANSDUCTION

Introduction

Generalized transduction¹² of *E. coli* is mediated by phage P1. P1 phage contain a 110 kb, double stranded, linear, terminally redundant and circularly permuted DNA genome. During the lytic cycle, some P1 phage accidentally package the host genomic DNA, the resulting P1 particle is called a transducing particle. Although the transducing particle is defective (cannot form lysogen or enter lytic cycle), it has the ability to inject host *E. coli* DNA into another host *E. coli*. The injected DNA can undergo homologous recombination with host genome. Via transducing particles any gene on the *E. coli* chromosome may be introduced into another *E. coli* strain at a ratio of 1 in 10⁶ to 10⁸ infected cells. Due to the large size of DNA that can be packaged it is possible to transfer genes which are closely linked on the *E. coli* chromosome (within 2.2 min), i.e., cotransduced. For instance, *purB* (25.6 min)¹³ and *galU* (27.8 min)¹³ are 2% cotransducible. This means that if we transduce a PurB⁻ GalU⁻ strain with a P1 lysate from a wild-type strain and select for PurB⁺, then 2% of the PurB⁺ transductants will also be GalU⁺. In fact, the frequency of cotransduction often serves as a good genetic estimate of the distance between two markers, the higher the frequency the closer the genes.

In preparing strains by P1 transduction, it is desirable to prevent non-defective P1 phage particles (majority of particles in P lysate) from lysogenizing or lysing the recipient strain. This is because P1 lysogens restrict foreign DNA introduced during phage infection or conjugation and continue to interfere with future genetic crosses. By using low multiplicities of infection (only one phage/host cell) and by adding citrate to prevent adsorption (chelating agent that binds the divalent cation, calcium which is required for adsorption), we can virtually eliminate this problem. Also, we can use a virulent mutant of P1 (P1_{vir}) which is unable to form lysogens.

In this lab we will use P1 generalized transduction to construct a new *E. coli* strain. P1 phage lysate carrying transducing particles of *E. coli* UM122 is used to transduce *E. coli* JM96(*cysH*). First transduced *E. coli* JM96 is selected on LBTc for *katF::Tn10*. Then picked onto defined medium with and without cysteine to select for *CYSH* cotransduced with *katF::Tn10*. This results in a new *E. coli* strain construct which is resistant to tetracycline and no longer auxotrophic for cysteine. Since a defined M9 medium is used to selected for *CYSH* all auxotrophic nutrients required by *E. coli* JM96 are added.

¹²Madigan MT, Martinko, JM. 2006. Brock: Biology of Microorganisms. 11th edition. Pearson/Prentice Hall: Upper Saddle River. p. 272
Sambrook J, & Russell D. 2001. *Molecular Cloning: A Laboratory Manual*. 3rd edition. New York: Cold Spring Harbor Laboratory Press p. 4.35-4.4.36.

Table 1. Map position (min) of selected <i>E. coli</i> genes ¹³ .	
gene	map position (min)
<i>rplS</i>	59.1
<i>katF::Tn10</i>	61.9 (not on cited map)
<i>cysH</i>	62.2
<i>argH</i>	89.5

Phage Lysate

Prior to the start of this lab P1 phage lysate, was prepared by infecting host *E. coli* UM122 with P1 phage producing 100-200 progeny per host cell, with some of the phage being transducing particles with *E. coli* UM122 DNA packaged. After infection, cells are either plated in a layer of soft agar on nutrient plate, or else grown with aeration in liquid broth. In the former case enough phage, usually 10^5 , are applied to each plate to allow confluent lysis over the surface of the plate. This is a result of overlapping of plaques. The soft agar layer is scraped into a test tube or centrifuge tube. Chloroform is added to further disrupt the cell wall. Cell debris is then centrifuged, and the supernatant, which is the lysate, is stored in the cold. Lysates are stored at 4°C in the presence of chloroform without decrease in titre for several years.

E. coli strain list

Strain number	Genotype	Important properties
P1(UM122)		Tc ^r Thi ⁻
JM96	<i>cysH thr leu trp his argH rpsL thiI lac xyl gal mal supE44</i>	Cys ⁻
UM122	HfrH <i>thiI katF::Tn10</i>	Tc ^r

Notes:

(i) The transposon Tn10 (marker gene), which contains a tetracycline resistance marker, is inserted in the *KATF* gene (catalase).

(ii) Only defective genes are noted in genotype/important properties. It is assumed that any gene not noted is wild type or not of particular interest for this lab. Also it is assumed that *E. coli* strains are sensitive to antibiotic if resistant gene not present.

rpsL = streptomycin resistance

¹³Berlyn MKB. 1998. Linkage map of Escherichia coli K-12, Edition 10: The Traditional Map. Micro Mol. Biol. Rev. 62: 814-984. <http://mmbr.asm.org/cgi/reprint/62/3/814> or search ASM journals, UM NETDOC or Google Scholar.

PROCEDURE

CAUTION: When using a Pipetman to pipette phage you must wipe the Pipetman barrel at the tip end with alcohol when you are finished using a particular phage. Just pour a small amount of 70% alcohol onto a kleenex to wipe the tip. For the remainder of the labs you must always wipe the Pipetman barrel tip with alcohol before using to pipette bacteria or a different phage. If you neglect to do this you may ruin your experiment.

It is good practise to pipette slowly when pipetting phage to reduce aerosol.

Week 5

Day 1

1. An overnight cultures of *E. coli* JM96 has been subcultured (1/50 dilution) into 5 ml LB + 5 mM CaCl₂ and grown for 4 hours (mid-log phase - 1×10^9 cells/ml) at 37°C with rotation.
2. Transfer *E. coli* JM96 culture to sterile plastic capped centrifuge tube and centrifuge at 10000 rpm for 5 min at 4°C. Gently resuspend pellet in 5 ml MC buffer (0.1 M MgSO₄, 5 mM CaCl₂). Each tube of MC supplied by prep room contains 5 ml, no need to measure.
3. Dilute P 1 phage by mixing 0.1 ml phage with 0.9 ml MC buffer (10^{-1}) in a sterile 5" metal capped test tube. Transfer 0.1 ml dilution to another tube containing 0.9 ml MC buffer and mix (10^{-2}) dilution.
4. Set up transduction experiment as follows using 5 inch sterile metal capped test tubes: mix 0.1 ml of *E. coli* JM96 cells with 0.1 ml of 10^0 , 10^{-1} , and 10^{-2} dilutions of the P1 lysate **in duplicate**. Also prepare two control tubes containing (1) only 0.1 ml bacterial cells and (2) only 0.1 ml phage lysate. Incubate tubes in a 37°C waterbath for 20 min. During this time label your LBTc agar plates.
5. Add 0.2 ml 0.1 M Na citrate to each tube. Then working with one tube at a time, add entire tube of melted F-top agar (3.5 ml) from a 55°C waterbath and immediately* pour on room temperature LBTc agar plate, controlled swirl to spread. Repeat for remaining transductions. Any signs of agar solidifying, stop swirling, its too late.
6. Incubate plates 1 - 2 days at 37°C .

*No need to mix as this happens when you swirl the top agar on the plate.

Day 2 or 3

7. Count plate data.
8. Pick between 100 and 120 isolated colonies^a growing on the LBTc agar plates (from any dilution) onto M9glucose plus cysteine and M9glucose minus cysteine. Use the grid supplied. Two plates of each type are available per group. If you do not have 100 colonies, pick as many single colonies as you have. All auxotrophic nutrients required by *E. coli* JM96 are added to the M9 glucose medium.
9. Incubate at 37°C for 2 or 3 days (over the weekend). Record data.
10. Hand in a **COPY of your P1 generalized transduction DATA SHEET** to the slotted filing cabinet in room 204. ONE PER GROUP. May be hand written. Include all requested information: group #, group names (in full), number of picked colonies that grew on M9glucose plus cysteine and M9glucose minus cysteine. **Data must be handed in by 2:30 pm Tuesday, Oct 17(both sections)**. The data will be compiled and POSTED ON THE WEBSITE in Excel format as soon as possible.

Molecular Genetics of Prokaryotes MBIO 4600**Lab 4 P1 Generalized transduction DATA SHEET - available as pdf file on lab website**

[only one copy/group required]

Group #: _____

Group names (in full): _____

picked colonies that grew on M9glucose plus cysteine: _____

picked colonies that grew on M9glucose minus cysteine: _____

Pick plate grid.

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

plate 1

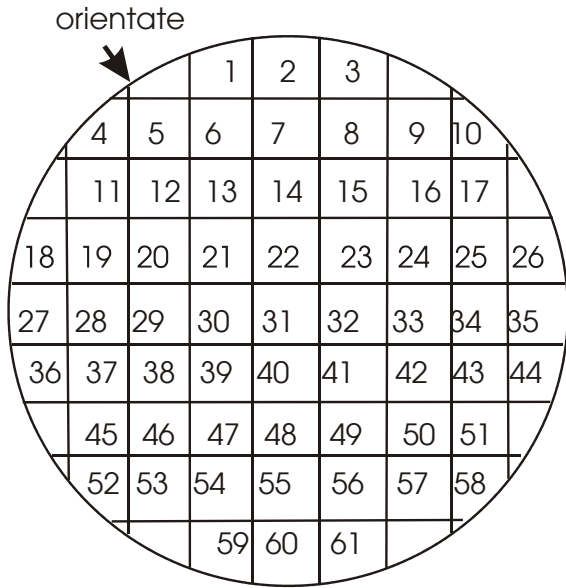


plate 2

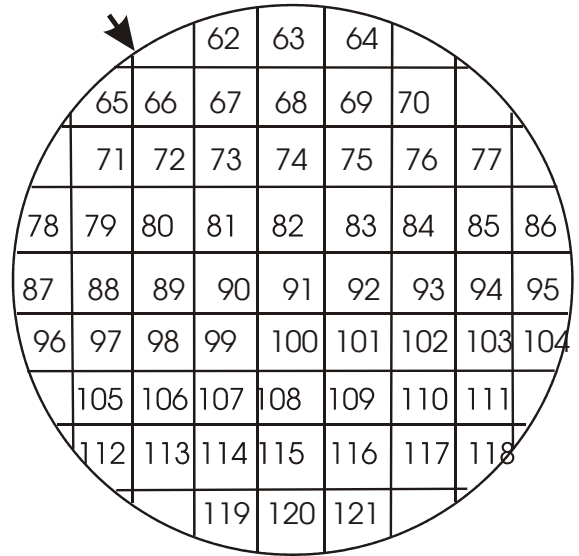


plate 3

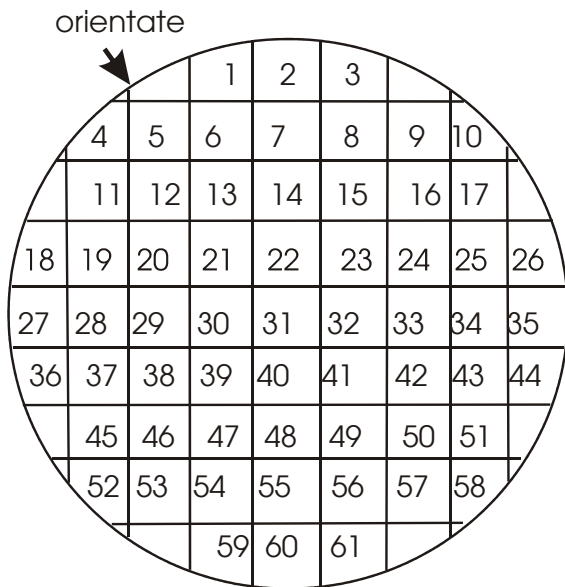
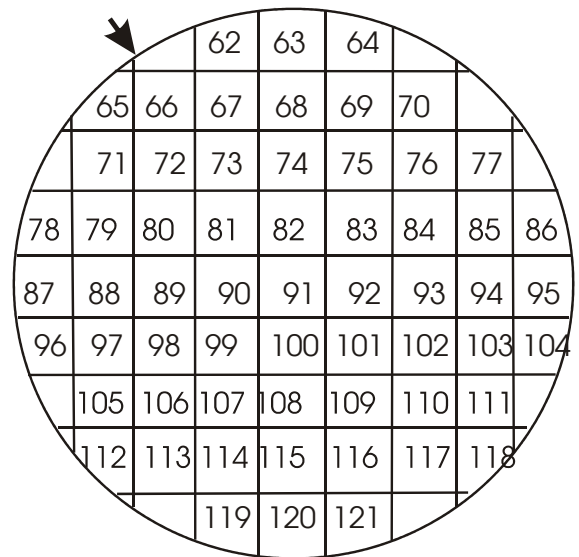


plate 4



LAB 4 REPORT

(Part 1. and 2. available as an Excel spreadsheet on lab website.)

Data Presentation and Analysis

- 2 1. Include a completed group data spreadsheet for tetracycline gene transduction (P1(UM122) into *E. coli* JM96 and sample calculations.
- 1 2. Include a completed class data spreadsheet including the frequency of cotransduction of *katF:: Tn10* and *CYS H*. Highlight or indicate your group's data with asterisk.

Question

- 0.5 3. Assume you have successfully constructed a new *E. coli* strain with genotype *thr leu trp his argH rpsL thiI lac xyl gal mal supE44 katF::Tn10* using P1(UM122) transduction. Is it possible to eliminate any other amino acid auxotroph besides cysteine using P1 transduced *E. coli* JM96 Tc resistant cells? *E. coli* linkage map.¹⁴
- 3.5 4. Using P1 transduction create a new strain, *E. coli hisG thiH xylA*, using only the following bacteria strains. Outline experiment. No details required. Must include media. State purpose of each medium used. **Experiment MUST BE presented as numbered steps or as a flow chart.** Keep experiment as uncomplicated as possible. Marks subtracted for unnecessary steps.
E. coli linkage map.¹

<i>E. coli</i> Strain	Genotype	Phenotype
AB12	<i>argH hisG xylA</i>	Arg ⁻ His ⁻ Xyl ⁻
CD24	<i>thiH galE</i>	Thi ⁻ Gal ⁻

Thi = thiamine, Arg = arginine, His = histidine, Xyl = xylose, Gal=galactose

¹⁴Berlyn MKB. 1998. Linkage map of Escherichia coli K-12, Edition 10: The Traditional Map. Micro Mol. Biol. Rev. 62: 814-984. <http://mmbbr.asm.org/cgi/reprint/62/3/814> or search ASM journals, UM NETDOC or Google Scholar.

EXCEL INFORMATION

- When taking the SUM of more than one column just hold down the CTRL key when selecting data. Best to use a combination of SHIFT block and CTRL key.
- Each spreadsheet should be printed to fit ONE PAGE. Select on Page Setup via File pull down menu or Toolbar Button.
- Make sure you select all information before printing. If you have used a text box for sample calculation make sure it is entirely in selected area to print before printing. PROOF READ page to ensure all information is included.
- RIGHT CLICK is extremely helpful in EXCEL. Especially for formatting.
- If you want to do your numerical calculations in word prior to copying to EXCEL spreadsheet that is fine but calculations must be on EXCEL spreadsheet. Just copy to an inserted Text Box. Good idea to have the Drawing Toolbar open which allows you to easily insert a Text Box. Just RIGHT CLICK on top Toolbar and select the DRAWING Toolbar, opens at bottom of worksheet. Put the cursor where you want the text box (easily moved) and then select insert Text Box button.
- RIGHT CLICK on appropriate cell and select FORMAT to change Number, Alignment, Font, etc.

LAB 5 TRANSPOSITION

INTRODUCTION

Transposons are important bacterial genetic tools that create non-leaky mutations, antibiotic resistance to facilitate genetic mapping and strain construction, create gene fusions, facilitate cloning of genes onto plasmids, etc. Transposon movement is easily detected due to the presence of antibiotic resistant gene. There are two distinct mechanisms of transposon movement in prokaryotes. One mechanism involves replication of a copy of the transposable element into the target DNA, for example Tn3. The other mechanism is conservative transposition, for example Tn10 excises from the donor DNA and integrates into the target DNA at staggered IS10 sites (cut by transposase) resulting in duplicate repeats at each end of the inserted transposon.

To obtain transpositions, a carrier vehicle is required to introduce the transposon into the cell. This is commonly a plasmid or a phage. In this lab, the transposon is delivered to the bacterial chromosome via an infecting λ derivative, λ 1098. The λ derivatives have a number of alterations that allow transposition to occur with increased efficiency and stability; (1) nonsense mutations in the replication genes (*O* and *P*), therefore they cannot synthesize DNA in a Su^- host, (2) carry a mutation in the λ *cI* gene, which prevents repression at temperatures above 39°C and (3) mutations that inhibit λ integration. λ 1098 phage¹⁵ carries the mini- Tn10*Tc* instead of Tn10. This also increases transposon efficiency and stability by (1) increasing the activity of transposase, (2) positioning the transposase outside of the segment to be transposed; once the antibiotic resistance gene is transposed it cannot be transposed again, (3) removing segments of the IS10 elements thereby eliminating inversions and deletions, and (4) allowing placement of different antibiotic markers (Kan^r, Cam^r, Amp^r, and original Tc^r) between shortened Tn10 ends.

In this lab you prepare λ 1098 phage stock, titre the phage using both the standard and rapid methods and then infect the host bacterium with λ 1098. The resulting transposed bacteria are plated for antibiotic resistance marker and then scored on indicator plates for transpositions in specific genes, ie lactose inactivation.

E. coli Strain List

Strain Number	Genotype	Important Properties/Phenotype
CSH110	<i>ara</i> Δ (<i>gpt-lac5 supE gyrA argE_{am} metB rpoB</i>)	Su ⁺
CSH140	<i>F'</i> lac ⁺ <i>proA</i> ⁺ , <i>B</i> ⁺ (<i>carries I</i> mutation in the <i>lac</i> region) <i>ara</i> Δ (<i>gpt-lac5</i>)	Su ⁻ I ⁻ Z ⁺

λ 1098 phage make clear plaques at 39.5°C on *supE* strains.

¹⁵Way JC, Davis MA, Morisato D, Roberts, DE, Kleckner, N. 1984. New Tn10 derivatives for transposon mutagenesis and construction of lacZ operon fusions by transposition. *Gene*. 32: 369-379.

PROCEDURE

CAUTION REMINDER: When using a Pipetman to pipette phage you must wipe the Pipetman barrel at the tip end with alcohol when you are finished using a particular phage. Just pour a small amount of 70% alcohol onto a kleenex to wipe the tip. For the remainder of the labs you must always wipe the Pipetman barrel tip with alcohol before using to pipette bacteria or a different phage. If you neglect to do this you may ruin your experiment.

It is good practise to pipette slowly when pipetting phage to reduce aerosol.

Week 6

Part I: λ 1098 Lysate Preparation

1. *E. coli* CSH110 was grown overnight in 5 ml LB with rotation at 37°C.
2. Add 0.2 ml *E. coli* CSH110 to four 5 inch sterile metal capped test tubes. Stock λ 1098 lysate titre is approximately 10^8 phage/ml. Add 50 μ l λ 1098 phage stock to 3 of the bacteria tubes. Add 50 μ l 0.1 M $MgSO_4$ to each tube. Do not add phage to the fourth test tube as this is a control. Incubate all tubes at 37°C waterbath for 15 min.
3. The prep room has already melted the LBCas SOFT top agar (melted) and placed the tubes in a 55°C waterbath. The LBCas (LB + casamino acids) agar plates are available in the 39.5 °C incubator (transferred from cold box to incubator 2 hours prior to start of lab). Prewarmed plates allow slightly more time to spread soft agar over surface of plate before hardening. Use pour plate technique procedure.
4. Label and set up 4 prewarmed LBCas agar plates (39.5°C). Remove a tube of melted LBCas-SOFT top agar from the 55°C waterbath (make sure that the agar is completely dissolved before using) and quickly pour into *E. coli* CSH110 - λ 1098 incubation tube. Then quickly pour the mixture on a LBCas agar plate and tilt plate to spread evenly over the surface of the plate. If the top agar starts to harden, stop swirling and allow to set. Incubate face up overnight at 39.5°C.
5. **Next day**, confirm that phage lysis has occurred by comparing the 3 experimental plates with negative control. The lysate plates will appear relatively clear compared to negative control (lawn of bacteria). Often resistant colonies appear but they do not interfere with lysate preparation. Using a sterile spreader scrape the soft top agar layer from the 3 plates containing phage-bacteria mixture into one large sterile opaque plastic screw capped centrifuge tube. Wash the surface of each plate with 2 ml LB medium containing 0.01 M $MgSO_4$ and transfer the wash to the centrifuge tube by titling the plate and using a P1000. Add 3 drops of chloroform (use long tipped Pasteur pipet) and vortex vigorously for 30 seconds. Centrifuge at 10000 rpm for 10 min at 4°C. Using pipetman, transfer the supernatant (phage stock) to Eppendorf tube(s). Normally additional chloroform is added for prolonged storage. However you will be using your phage stock next week - do not add chloroform at this stage. Label top of Eppendorf tube(s) with your group #, no initials. Label side with λ 1098 stock. Store at 4°C (student cold boxes) in λ 1098 TRAY, do not freeze. The centrifuge tube must be cleaned before placing on the discard trolley. First remove the debris/bacteria (not the chloroform)

-If you do not know how to use the floor model centrifuges, get help. Find TA to help you -TA should be available in their labs. See appendix for instructions.

-Chloroform is located in the fumehood in room 204. Do not remove from fumehood, add to your tube in the fumehood. Discard Pasteur pipet in beaker supplied in fumehood. Do not use a pipetman to add chloroform.

-Must use opaque plastic tubes as chloroform will melt clear plastic tubes.

pellet using a metal spatula to a paper towel. Discard debris/bacteria paper towel in Petri plate container. Most likely there is a chloroform bubble below the pellet. After remove pellet debris pour chloroform into specified organic container in the fumehood (room 204). Rinse the tube with lots of water before placing on discard trolley. All hazardous organics must be disposed of through the UM Safety Office and cannot be autoclaved.

Week 7

Part II: λ 1098 Phage Titration on CSH110

A. Standard Method

1. *E. coli* CSH110 was grown overnight in 5 ml LB with rotation at 37°C.
2. Prepare 10-fold serial dilutions of phage lysate to 10^{-10} . Prepare dilutions in saline using a total dilution volume of 1 ml (sterile 5 inch metal capped test tubes).
3. Add 0.1 ml of 10^{-6} to 10^{-10} dilutions in duplicate for λ 1098 to sterile 5" metal capped test tubes. Next add 0.2 ml *E. coli* CSH110 to each tube plus one additional sterile tube that does not contain phage (negative control). Add 50 μ l 0.1 M MgSO_4 to each tube. Mix by hand. Incubate these tubes in a 37°C waterbath for 15 min (no shaking). Note: After preparing dilutions return λ 1098 phage lysate to 4°C. DO NOT FREEZE.
4. Meanwhile make sure there are enough tubes containing 3.5 ml melted LBCas top agar at 55°C (check to make sure agar is completely dissolved) and label prewarmed (39.5°C) LBCas agar plates with group #, names, phage type, and dilution).
5. Transfer 3.5 ml melted LBCas top agar (entire tube), quickly but smoothly, to incubation test tubes (there is no need to mix). Then immediately transfer to middle of LBCas plate and quickly tilt plate to evenly distribute the top agar over the entire surface of the LBCas plate before it hardens. The surface should be smooth. If it starts to harden, stop swirling. If a plate has uneven, hardened agar, it is almost impossible to count the number of plaques. Allow plates to stand upright at room temperature until top agar hardens, and incubate upside down overnight at 39.5°C.
6. **Next day**, record plaque count data.
7. Determine λ 1098 volume to add to transposition mixture and moi or vice versa before coming to lab. The TA needs to check your calculations before you proceed. See transposition experiment protocol to help you with this calculation.

B. Rapid method

1. Prepare λ 1098 phage dilutions - use the same dilutions prepared for standard method.
2. Label bottom of ONE LBCas agar plate with six \sim 1 cm circles. Label each circle with dilution* to be plated, 10^{-4} to 10^{-9} . There is no need for duplicate plating as this is only an estimate of titre.

If a rapid estimation of titre of one or many phage is required, there are several methods to decrease time, labour, and supplies required. The rapid method may or may not work depending on strain of phage and bacterium used. To check for viability of phage stock just spot 5 μ l of the stock on a lawn of host bacterium.
* Dilutions plated differ from standard method as only 5 μ l of each dilution plated instead of the usual 100 μ l.
3. Spread plate 150 μ l *E. coli* CSH110 on LBCas plate labelled with circles. Put in 39.5°C incubator for 10 min to dry.
4. Using a P20, drop 5 μ l of each λ 1098 dilution on designated circle. You may need to gently touch the agar to release the droplet. Do not puncture the agar plate. Use a different pipet tip for each dilution. Allow to dry before moving or keep plate level.
5. Incubate upright to allow drop to absorb. Incubate 20-24 h upside down at 39.5°C. Count the number of plaques for each dilution. It may be difficult to see plaques, holding plate at various angles in good light helps you see the plaques. Often it is easier to see plaques after 24 h.

Week 8

Part III: Tn10 Transposition with λ 1098

1. An overnight culture of *E. coli* CSH140 was subcultured (1/50 dilution) into 10 ml LB and rotated for 4 h at 37°C. The culture **cell density is 3×10^8 cells/ml.**

STUDENT LAB STARTS HERE

2. Centrifuge culture at 10000 rpm for 5 min at 4°C. **Resuspend the pellet in 1 ml LB** containing 10 mM MgSO₄.
 - a) First, **remove 0.1 ml** *E. coli* CSH140 and spread plate on one LB + Tc agar plate (negative control).
 - b) Then, add appropriate amount of phage to remaining *E. coli* CSH140 to give a multiplicity of infection (moi) of 0.3 to 10. If your λ 1098 phage titer is too low, $<9 \times 10^9$ or too high, $>10^{11}$ (incorrect titration), use supplied λ 1098. The volume of λ 1098 should be 1/10 of the bacteria added (smaller volume is acceptable). The easiest method is to add 1/10 volume and make sure the moi is within the acceptable range, 0.3 to 10. You need to check you calculations with the TA before proceeding.
3. Incubate in a 37°C waterbath for 15 min to allow phage adsorption. Add 1 ml LB and continue incubating in the 37°C waterbath for 90 min to allow expression of antibiotic resistance gene.
4. Plate 0.1 ml of 10^0 , 10^{-1} , 10^{-2} , and 10^{-3} dilutions (prepared in total volume 1 ml saline) in duplicate on LB Tc agar plates.
5. Incubate overnight at 39.5°C. λ 1098 has a temperature sensitive repressor. At temperatures between 39°C and 42°C the repressor is inactivated preventing the formation of lysogens.
6. **Next Day:** Record plate count data of tetracycline resistant colonies. These colonies are mini-Tn10 carriers.
7. Pick plate 200 colonies onto M9glucoseXgalTc agar plates. If you have less, pick as many colonies as you have. **Incubate 1 day at 37°C.**
8. Count the total number of white colonies and total number of blue colonies.
9. Hand in a **COPY** of your group's completed Transposition DATA SHEET to the slotted filing cabinet in room 204. May be hand written. Include all requested information. **Hand in data by 2:30 pm Monday (Nov 6).** The data will be compiled and class data posted on the website (Excel format) as soon as possible.

Molecular Genetics of Prokaryotes MBIO 4600**Lab 5 Transposition DATA SHEET - available as pdf file on lab website**

(only one copy per group is required)

Group #: _____

Group names (in full): _____

picked colonies that are BLUE on M9glucoseXgalTc: _____

picked colonies that are WHITE on M9glucoseXgalTc: _____

LAB 6 REPORT (Report available as a Word document online at lab website, just save and open in Word before entering requested information. Also include requested Excel spreadsheet).

Data Presentation and Analysis

- 3 1. Record requested information in the following table. In table indicate data with an asterisk used to calculate titer (pfu/ml) using the standard method. Include pfu/ml calculation for both methods.

Table 1. λ 1098 plaque plate count data on <i>E. coli</i> CSH110 using the standard method .			
Dilution plated	Standard Method plaque plate count data ^a		Rapid Method plaque plate count data ^c
	plate 1	plate 2	
10 ⁻⁴	NA	NA	
10 ⁻⁵	NA	NA	
10 ⁻⁶			
10 ⁻⁷			
10 ⁻⁸			
10 ⁻⁹			
10 ⁻¹⁰			NA
negative control ^b		NA	NA
Titer (pfu/ml)			

^a 0.1 ml of 10⁻⁴ to 10⁻¹⁰ dilutions in duplicate on *E. coli* CSH110; ^b only *E. coli* CSH110

^c 2 μ l dilutions plated on *E. coli* CSH110; TNTC = too numerous to count; NA = not applicable

λ 1098 pfu/ml calculations:

Standard Method:

Rapid Method:

Minimum number of plaques does not apply for calculation. Use all countable plaques, most likely 1-30 plaques that are countable.

- 1 2. State the multiplicity of infection (moi) used in your group's transposition experiment. State volume of $\lambda 1098$ phage required to give this moi. Show volume calculation.

OR

If you opted to add 1/10 volume of bacteria, state volume and resulting moi. Show moi calculation.

Comment: You require information given in Part III **Transposition with $\lambda 1098$ procedure** for total number of *E. coli* CSH140 cells and volume calculations.

- 1 3. Record requested information in the following table. In table indicate with as asterisk data used to calculate titer (Tc^r bacteria /ml). Sample titre calculation not required here.

Table 2. $\lambda 1098$ mini-Tn10Tc transposition plate count data into <i>E. coli</i> CSH140.		
Dilution plated	Plate counts ^a on LB+tetracycline at dilutions	
	plate 1	plate 2
10^0		
10^{-1}		
10^{-2}		
negative control ^b		NA
Titer <i>E. coli</i> CSH140 Tc^r (bacteria/ml)		

^a 0.1 ml of 10^0 to 10^{-2} dilutions in duplicate on *E. coli* CSH140; ^b only *E. coli* CSH140

TNTC = too numerous to count; NA = not applicable

- 1.5 4. Calculate the transposon insertion rate into *E. coli* CSH140.

Transposon insertion rate = $\frac{\text{total number of tetracycline resistant bacteria in the reaction mixture}^a}{\text{total number of phage added to the reaction mixture}}$

^a when calculating the total number of tetracycline resistant bacteria in the reaction mixture you need to know the total volume of the solution used to determine *E. coli* CSH140Tc^r titre. This allows you to correlate to total number of phage.

Must restate the following information in answer:

Titre *E. coli* CSH140 Tc^r (bacteria/ml): _____

Titre λ1098 (pfu/ml): _____

moi: _____

- 1 5. Include a completed class data Excel spreadsheet (available on lab website, save and open in Excel to enter requested information) for mini-Tn10 Tc^r transposition into the *lac* gene (Xgal glucose minimal medium agar plates) set up as a table. Record all requested information. Highlight, footnote or indicate your group's data. Fit all requested information on one page.

Frequency of insertion = $\frac{\text{total Lac}^- \text{ colonies}}{\text{total viable colonies picked}}$

Question

- 1.5 1. Explain the function of the *SupE* gene in *E. coli* CSH110. In your explanation include the molecular bases of the *supE* gene.

APPENDIX

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 medium is a complex media that supplies all essential nutrients, C-source, N-source, vitamins, minerals and trace metals. Yeast extract supplies all essential nutrients while tryptone is mainly a N-source and to a lesser degree a C-source.

M9 Medium Agar plates: All plates are prepared with the final concentration per liter. Autoclave agar and salts separately. Salts; 10.5 g K_2HPO_4 , 4.5 g KH_2PO_4 , 1.0 g $(NH_4)_2SO_4$, and sodium citrate.2H₂O. 15 g/l agar. After autoclaving add cooled $MgSO_4 \cdot 7H_2O$ (add 1 ml from a stock solution of 20g/100 ml) and approximately 1 μ g/ml B1 (thiamine hydrochloride - add 0.5 ml from a 1% stock solution). Add carbon source (10 ml from a 20% stock solution). Some type of carbon source must be added, for example, the most common carbon source added is glucose (assume if not stated). If the medium requires additional nutrients, add 20 μ g/ml L-amino acids or 40 μ g/ml D,L-amino acids (add 10 ml from a 4 mg/ml stock solution). Amino acid is not essential unless the bacteria is an auxotroph for a particular amino acid. If the medium requires, add nucleotide, ie if bacteria is a nucleotide auxotroph.

Xgal M9Glucose IPTG agar plates: Prepare M9 glucose agar. After autoclaving, add Xgal (5 bromo - 4- chloro - 3 indolyl b D galactoside) in *N,N*-dimethylformamide at a final concentration of 40 μ g/ml and IPTG (Isopropyl β -DThiogalactoside) at a final concentration of 24 μ g/ml.

M9 medium is a defined medium. All components known. This allows you to select for desired bacteria by adding or removing nutrient of interest.

F-top agar: per liter, 8 g Difco agar, 8 g NaCl.

MacConkey Agar plates: Prepared medium purchased from Difco Laboratories. Components: peptone, poly peptone, lactose, bile salts, sodium chloride, agar, neutral red, crystal violet, distilled water. pH 7.1

Stock solutions of Antibiotics:

- Streptomycin (Sm): prepare 10 mg/ml stock solution in distilled water, filter sterilize. Add to cooling agar at a final concentration of 100 µg/ml.
- Tetracycline (Tc): prepare a 1 mg/ml stock solution in distilled water, filter sterilize. Add to cooling agar at a final concentration of 10 µg/ml.
- Ampicillin (Amp): prepare 10 mg/ml stock solution in distilled water, filter sterilize. Add to cooling agar at a final concentration of 100 µg/ml.

SOLUTION COMPONENTS AND FUNCTION

Saline: dissolve 8.5 g NaCl in a total volume of 1 liter distilled water 0.85% saline is physiological concentration, ie., isotonic. Maintains the stability of bacteria, ie. do not lyse.

Addition of Mg²⁺ or Ca²⁺ during phage lysate, titration or infection of host bacterium: enhance adsorption of phage on host bacteria.

λ phage adsorb to trimeric maltoporin receptors¹⁶. Magnesium facilitates the reversible attachment of the phage tail to the maltoporin receptor. Once attached the λ DNA is injected into the host bacterium. Of interest neither injection or lytic phase occur at room temperature, must be greater than 28°C.

P1 similar to λ phage require divalent cations for optimum adsorption. However, P1 adsorption is facilitated by calcium and to a lesser degree, magnesium.

Magnesium or calcium are often added to LB medium during growth of host bacterium to increase subsequent adsorption of phage. It is common practise to add vitamin free casamino acids to medium to enhance phage growth.

Chloroform during preparation and storage of phage lysate: Chloroform lyses bacteria. During lysate preparation host *E. coli* cells not yet lysed by infecting bacteriophage are lysed by chloroform releasing remaining phage to give the maximum number of phage. Chloroform is added during storage (as at 4°C) to prevent bacteria growth (lyse) especially resistant bacteria.

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.

¹⁶Sambrook, J, Russell, DW. 2001. Chapter 13 Mutagenesis. In Molecular Cloning A Laboratory Manual 3rd edition. Cold Spring Harbor: CSHL Press p. 2.4

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 enhances 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 - stability 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

ddH₂O

-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. ddH₂O is used to ensure that no component are present that may interfere with subsequent reaction, eg. PCR, restriction digest, DNA sequencing.

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 diagram

OPERATION OF FLOOR MODEL CENTRIFUGES

Note: If procedure varies depending on centrifuge manufacturer a step by step operation procedure is usually located on or nearby the centrifuge or the teaching assistant will help you.

HITACHI HIGH SPEED HIMAC REFRIGERATED CENTRIFUGE

- to select or change settings the CHECK button must first be pressed (light on). The light stays on for 16 sec. When the light is off you can no longer select, change setting or carry out any operation, just press check button again and continue.
- When the centrifuge is turned on and the CHECK button is not pressed. The centrifuge displays real time parameters.

OPERATION

Centrifuge tubes should be balanced by scale by adding or removing appropriate solution from one of the tubes.

1. Turn power switch on. The indicators on the control panel are illuminated. The door lock is released.
2. Open door. If required set the rotor gently in position and close door. Turn the rotor lightly by hand to check that the rotor is correctly set. Remove the rotor lid and place balanced tubes opposite each other in rotor. You cannot run the centrifuge with an odd number of tubes.
SCREW ON LID.
3. Call up memory code number or enter parameters.

Call up pre-programmed memory code number: Press CHECK button, MEMORY button, memory code number, and CALL button. Each memory code number consists of a specified set of operation parameter (see sheet on centrifuge cover). See below for a list of operation parameters and how to set and store operation parameters.

OR

Real time operation (enter original parameters): see setting of operation parameters below.

4. After the parameters are set make sure the check light is still on. If not, press the CHECK button.
5. Press the START button. The rotor starts running. The start lamp begins flashing. The timer starts to count down.
6. The timer counts down to zero or press the STOP button. The rotor begins to decelerate. The stop light begins flashing.
7. The rotor stops. The stop light stops flashing. A buzzer sound occurs. The door lock is released.
8. Unscrew rotor lid and remove tubes. If required, use tweezers to help remove tubes. Wipe out rotor if spills occur. DO NOT SCREW ON THE LID just place on top of the rotor.
9. Close centrifuge lid and turn off power.

AUTOMATIC COLONY COUNTER

There are several makes of automatic colony counters in this department. All automatic colony counters work on the same principle. The counter registers a count every time you touch the colony with the counter probe as long as the L-shaped probe is inserted into the agar at the edge of the plate. This completes the electrical circuit through the agar from the L-shaped probe to the counter probe (needle shaped probe) touching the colony.

Operation

1. Push or flip the power switch to turn on counter.
2. Press the button on the counter that resets the counter to zero.
3. Place agar culture plate on counter and remove cover.
4. Insert L-shaped probe into the agar at the edge of the plate.
5. Count colonies by touching each colony with the counter probe tip (needle shaped probe).
6. Remove plate, replace lid.
7. Remember to turn off power switch when you are finished counting.

Notes:

- (i) Use a marker to divide the plate into sections or use the grid on the automatic colony counter to facilitate counting.
- (ii) The counter also comes with a magnifying glass but it is not required unless you are counting very small closely spaced colonies.

SAMPLE CALCULATION of the number of bacteria per ml

Data for example calculations using the following sample data

Dilution plated	Number of colonies	
	Plate 1	Plate 2
10 ⁻²	TNTC	TNTC
10 ⁻³	320 ^a	316
10 ⁻⁴	34	27
10 ⁻⁵	2	3

TNTC = too numerous to count

^a counts greater than 300 are acceptable as long as accurately counted. All counts recorded in your tables must be accurate counts or record as TNTC.

Terms

Plating factor = reciprocal of volume plated

Dilution factor = reciprocal of dilution for significant counts

Significant plate counts = the sum of the plate counts at significant dilution divided by number of plates.

Often more than one dilution has significant plate counts. It is important to use all significant plate count data; number of colonies per plate are between 30 and as high as bacteria can accurately be counted (colonies not overlapping). There are several ways to deal with data that has more than one significant plate count dilution.

Number of plates = number of significant plates

Calculation

Do not average an average value as it incorporates error in your calculation (not statistically accurate). Use one of the following methods to calculate bacteria titer.

Bring all significant counts to the same dilution:

$$\text{Bacteria/ml} = \frac{\text{significant plate counts}}{\text{number of plates}} \times \text{dilution factor} \times \text{plating factor}$$

$(320 + 316 + 340)/3 \times 1/10^{-3} \times 1/10^{-1} = 3.25 \times 10^6$ bacteria/ml, since the smallest number of significant figures for plate counts is two, the answer is 3.3×10^6 bacteria/ml

Or calculate the titer for each significant plate count and average.

$$\text{Bacteria/ml} = \text{significant plate count} \times \text{dilution factor} \times \text{plating factor}$$

$$320 \times 1/10^{-3} \times 1/10^{-1} = 3.20 \times 10^6 \text{ bacteria/ml}$$

$$316 \times 1/10^{-3} \times 1/10^{-1} = 3.16 \times 10^6 \text{ bacteria/ml}$$

$$34 \times 1/10^{-4} \times 1/10^{-1} = 3.4 \times 10^6 \text{ bacteria/ml}$$

Average all values: $(3.20 \times 10^6 + 3.16 \times 10^6 + 3.4 \times 10^6)/3 = 3.25 \times 10^6$ bacteria/ml, since the smallest number of significant figures for plate counts is two, the answer is 3.3×10^6 bacteria/ml

Calculation of phage titre is identical to bacteria titre. Express in units of cfu/ml or phage/ml.

cfu = colony forming units

DATE: sample PAGE: _____ TIME: 1.2 h
 INSTRUCTOR: Dr. L. Cameron

WRITE IN PEN ONLY.

CONCISELY ANSWER ALL QUESTIONS in spaces provided on exam paper.

Exam is longer than actual to demonstrate a wide selection of question types.

(spaces have been removed for sample exam)

- 2 1. Explain the following *E. coli* strain list and state phenotype.

<i>E. coli</i> Strain	Genotype
CSH143	$F'lacproA^+, B^+$; $ara, \Delta(gpt-lac)5, gyrA$

pro = proline; *ara* = arabinose; *gyrA* = naladixic acid

- 1 2. a) State the phenotype and most likely I and Z genotype of an *E. coli* strain that has pale blue colonies on M9glucose Xgal and red colonies on MacConkey.
- 1 b) *E. coli* CSH100 genotype includes $I^R P^c$ mutations in the lac region. Explain genotype at the molecular level and state expected phenotypic expression on M9glucose Xgal.
- 5 3. State the chemical that corresponds to the following functions as relates to your MBIO 4600 lab.
- promotes the adsorption of λ phage _____
 - prevents the adsorption of P1 phage _____
 - maintains physiological ionic conditions _____
 - ensures β -galactosidase is produced at maximum levels _____
 - preparation of competent *E. coli* _____
- 2 4. a) In your MBIO 4600 lab F factor transfer allowed lac reversion of *E. coli* CSH104 $F'lacproA^+, B^+$ (carries IZ mutations in the lac region) $ara \Delta(gpt-lac)5$ at high frequency. Explain why at the molecular level.
- 2 b) Design an Hfr conjugation experiment such that the recipient *E. coli* strain is $Met^-Tc^rSm^f$. Do not include any experiment details except donor and recipient genotype and selection medium. The only condition is that the recipient *E. coli* strain must be wild type and only one antibiotic resistance gene.
- 1 c) Present a labelled papillae colony for a Lac⁻ reversion. In your diagram make it obvious what is the original color of the colony and selection medium.

- 1 5. The X-gal detection system permits detection of recombinant plasmids. Explain why it is important that the F factor is present in the plasmid host *E. coli*.
- 1 6. a) Determine pBluescript DNA concentration ($\mu\text{g}/\mu\text{l}$) and amount of plasmid (ng) added to transformation mixture (5 μl of 1/10 dilution). Absorbance reading 0.035 for 1 ml ddH₂O + 5 μl pBluescript DNA.
- 1 b) Determine # transformants/ μg plasmid for the following data. 50 ng pBluescript added to transformation mixture. Ampicillin colony titer 6.2×10^4 bacteria/ml. Transformation mixture total volume plated = 1.2 ml.
- 5 7. Explain the function of each of the following procedure steps/chemicals used in your molecular genetics lab.
- a) Plasmid DNA preparation solution containing 0.2 M NaOH, 1% SDS
 - b) incubation of $\lambda 1098$ and CSH140 transposition mixture at 39.5°C
 - c) centrifugation of all molecular genetic lab *E. coli* cultures at 4°C
 - d) Plasmid DNA preparation solution containing high concentration potassium acetate, pH 4.8 and guanidine hydrochloride
 - e) neutral red in MacConkey agar
- 3 8. a) What is the advantage of P1 transduction when creating new *E. coli* strains.
- b) What improved features does mini-Tn10 have compared to Tn10?
- c) State how the procedure for phage titre determination differs from bacteria. State only the procedure that differs. State why.
- 3 9. Experimental Data: Ten ml log phase culture of *E. coli* CSH140 (4×10^8 bacterial/ml) was centrifuged and resuspended in 1 ml LB.Mg broth. $\lambda 1098$ (stock titre 6.8×10^{10} phage/ml) added at a multiplicity of infection of 5. Mixture incubated at 37°C for 15 min. One ml LB broth added and incubated a further 90 min at 37°C. Dilutions of incubation mixture prepared and 0.1 ml dilutions plated on LB agar plates containing tetracycline. The following plate count data was obtained for duplicate plating at each dilution: 10^{-1} : 250/245, 10^{-2} : 25/24, and 10^{-3} : 1/0.
- 300 colonies pick plated onto glucose minimal medium containing Xgal and tetracycline resulting in 5 white colonies and 295 blue colonies.
- Determine:
- (a) State bacteria/ml for titration data.
 - (b) Calculate volume of $\lambda 1105$ phage stock added to *E. coli* CSH140 to give a multiplicity of 5.
 - (c) Calculate the transposon insertion rate into *E. coli* using phage $\lambda 1105$.

(d) Calculate the lac mutation rate.

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