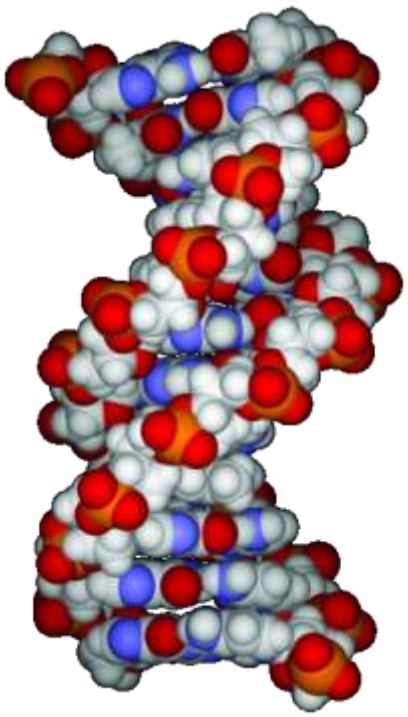
Molecular Biology 4XX3

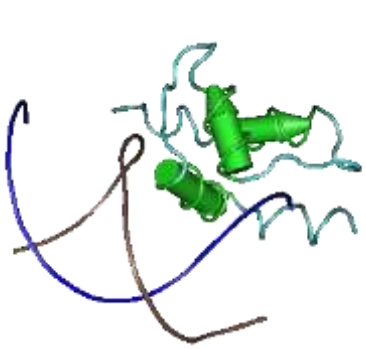
WORKSHOP IN MOLECULAR GENETICS



LABORATORY MANUAL

H.E. SCHELLHORN

SUMMER, 2013



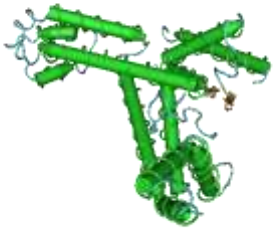


Table of Contents

1. Introduction

MolBio4XX3 is an intensive two-week laboratory and lecture course. The primary goal of the course is to give students hands-on experience with a variety of laboratory techniques commonly used in Molecular Biology and Biotechnology.

- media preparation and cultivation of bacteria
- overproduction of proteins in *E. coli*, protein purification (Taq polymerase and sigma factors) and enzyme assays
- spectrophotometry: measurement of bacterial growth and determination of protein concentration (Bradford assay)
- PCR amplification, primer design and optimization of conditions
- SDS-PAGE of proteins and Coomassie Blue staining
- Immunoblotting (Western) blotting and detection with chemoluminescence techniques
- mammalian tissue culture techniques
- Scanning electron microscopy

Students will work individually whenever possible. They will prepare reagents themselves and carry out experiments individually. Goal is to make this an experience as close to the real-life laboratory environment, including the repetition of experiments that (in reality) often do not work properly on a first trial. We will meet every morning at 8:45 am for instruction (LS-B130E) to discuss the experiments of the day followed by experiments or a lecture. The lectures will cover different areas of contemporary Molecular Biology that are necessary background for the course.

Genome	PCR, Mobix
Proteome	Protein production/purification/protein-enzyme function
Cell Biology	Immunology (background) & methods
	Basic tissue culture techniques
Intellectual property	M. Mongeon, Taq polymerase
Ethics / responsibility	To be determined

In accord with the requirements for laboratory workers, the students will be given the opportunity to attend **Fire Safety, Biosafety and WHIMS** sessions during the first week of the course

Grading Scheme

Quiz I	20%	(May 10th, content: days 1-4)
Quiz II	30%	(May 17th, content: days 1-9 = cumulative)
Overnight	10%	
Rotation Assignments	10%	
Course performance	10%	
Lab notebook	20%	

Literature

Immunology: Brock, Biology of Microorganisms, Chapters 22-24 and as posted on the LabWiki
(other intro micro texts are acceptable as well).

2. Lab Rules and General Tips/Organization:

Safety: The locations of fire extinguisher, eye wash station, and shower will be introduced and you should be aware of those in case of an accident.

Protection: **Lab coats** must be worn to protect you and your clothing from stains and corrosive reagents used. **Safety goggles** are available and must be worn during the handling of acids and bases. The **fume hood** should be used for handling of SDS powder and of concentrated solutions of HCl and β-mercaptoethanol. **Gloves** are available and should be worn for handling of corrosive (acids, bases) and toxic substances (e.g. acrylamide solutions and ethidium bromide). In case of doubt about any chemical used, ask the TAs about its proper handling. Safety information (Material Safety Data Sheets, **MSDS**) is also available on the internet: http://ccinfoweb.ccohs.ca/msds/search.html

Biohazards: Treat all organisms used as though they are dangerous, and be careful to avoid any direct contact with them. UNDER NO CIRCUMSTANCE WILL DRINKING OR EATING INSIDE THE COURSE ROOMS BE TOLERATED! Follow the recommended techniques when transferring or inoculating microorganisms. If in doubt about any method, consult one of the instructors or TAs before proceeding. Plates not used any more should be disposed in the **biohazard waste cartons** in the lab.

Spills/Sterilization: There are bottles of a disinfecting solution (80% Ethanol) at each table for wiping your work areas before the beginning and after the end of work. If a culture is spilled, disinfect the area with 80% Ethanol. Wash your hands with the soap provided before leaving the laboratory.

Glass waste and sharps: Dispose of slides and damaged glassware that has come in contact with microorganisms in the Biohazard sharps containers. Dispose other glass waste in the glass waste cartons. Other sharps, such as needles, should also be disposed in the yellow biohazard sharps containers.

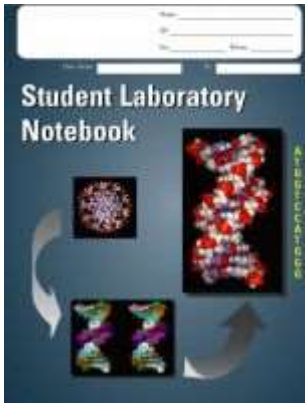
Microscopy: Microscopes will be used in this laboratory and they can be easily damaged. If you are not familiar with the use of the microscope, you should review your Cell Biology lab manual and get instruction before trying to operate. In case of doubt you should rather ask your TA than risk damage to the expensive equipment.

General tips/advice: -

Clean the balances after using them!!!!!!

- **Label** the storage box you receive with your group number and properly label all tubes so that YOU can identify its content.
- Label flasks with tape prior to autoclaving to make sure that the label does not wash off.
- When you make an agar, leave the **stir bars** in during autoclaving! It is necessary to mix the agar after autoclaving and this will enable you to cool down the solution faster.
- **Minimal media** – as a general rule, pay close attention to the instructions as preparation of such media often requires separate autoclaving of components.
- The **pH meters** need to be calibrated before use with the available solutions at pH 4 and 7.
- Tris buffer is used often and it changes its pH value in response to temperature. The solution heats when the pH-value of Tris buffers is adjusted with HCl. Keep this in mind when preparing solutions for use at a specific temperature and re-calibrate if necessary.

3. Guidelines for Laboratory Notebook Keeping



Maintaining a good laboratory notebook is essential in government, industrial and academic laboratories for many reasons. In government health laboratories, detailed records of procedures must be kept for later scrutiny (the Walkerton incident is an example improper documentation later revealed in court). In the biomedical industry (e.g. pharmaceutical manufacturing), properly recorded laboratory notebooks must be supplied for patent applications. Since such research is often years in scope, the amount of accumulated documentation can be considerable.

For this course, will be provided with a **Student Laboratory Notebook** which will be given out in lecture 1. The laboratory notebook is a diary of activities that are described in sufficient detail to allow another scientist to follow your steps. The notebook section will be worth 20% of your final grade.

An important overall criterion for the evaluation of your laboratory notebook will be that it was written in real time as you perform the experiments. You should write legibly, underline scientific names of microorganisms, draw tables with rulers (you may also create tables and graphs on the computer, print and paste them in) and clearly label the different subsections and figures of your writing so that others can easily comprehend what you did. Remember that the laboratory notebooks will be evaluated in comparison to each other, you are expected to keep the laboratory notebooks in an organized manner and you will lose grades if you do not submit a well-organized notebook. It will be necessary to plan ahead in the organization of your labbook.

The following format should be followed (see also first page of laboratory notebook):

1. Title: e.g. Lab #1 - Microscopy and Examination of Living and Stained Cultures

2. Introduction: Briefly state why this experiment was conducted in your own words – do not copy the lab manual.

3. Objective: Briefly state what you are attempting to determine (measure, weight, stain, identify, infect...)

4. Materials and Methods: write out all the protocols for all procedures, you can paste in the page from the lab manual but assume the person reading your lab book does NOT have access to the 4XX3 Lab Manual.

5. Results: If possible, use table(s) and/or figure(s) to present raw data. Provide brief descriptions of what the data mean.

6. Discussion: Briefly discuss what you can conclude from your results. Sometimes experiments fail either because of an unanticipated variable or because of experimental error. If your results deviate from expectations, identify possible sources of error, provide alternative hypotheses, and suggest improvements for future experiments.

7. Fill in the table of contents to permit easy orientation.

8. Sign and date each page. Signature of lab partners or advisors are waived here due to class size. However, this is an essential procedure in government and industry labs.

11. Answer the Lab Rotation questions in your lab book.

4. Statement on Academic Integrity

Academic dishonesty consists of misrepresentation by deception or by other fraudulent means and can result in serious consequences, e.g. the grade of zero on an assignment, loss of credit with a notation on the transcript (notation reads: "Grade of F assigned for academic dishonesty"), and/or suspension or expulsion from the university

It is your responsibility to understand what constitutes academic dishonesty. For information on the various kinds of academic dishonesty please refer to the Academic Integrity Policy, specifically Appendix 3, located at http://www.mcmaster.ca/senate/academic/ac_integrity.htm and the student information of the Office for Academic integrity http://www.mcmaster.ca/academicintegrity/

McMaster University considers academic dishonesty to be a very serious matter. Instructors will seek to identify instances of academic dishonesty, charge those responsible and impose the appropriate penalty as defined by Senate regulations. We have prepared the following guidelines to ensure that you are aware of what the McMaster University considers academic dishonesty. Study these examples with care so that you will never inadvertently commit such an offense.

A. Deliberate Cheating - Examples include:

- Use of unauthorized aids during an exam.
- Alteration of an exam after it has been returned and claiming that the altered sections were present in the original exam script.
- Alteration of the marks given on an exam and then claiming that the original addition was incorrect.
- Verbal communication of answers from one student to another during a test.
- Allowing someone else to write an exam in your place.

B. Presentation of Material That Is Not Your Own Work - Examples include:

- Copying an answer from another student during an exam
- Copying (either directly or after memorization) from texts, lab reports, essays, old exam scripts etc. written by others without appropriate reference citation.
- Submitting essays, drawings, micrographs, cultures or other laboratory results that were prepared or obtained by others as though they were your own.

C. Aiding Other Students to Commit an Act of Academic Dishonesty

This includes not only giving unacceptable aid to students taking a course at the same time you are taking the course, but also providing the means whereby students in future may cheat.

Examples include:

- Writing an exam or completing an assignment for someone else.
- Assisting another student to cheat by making it possible for that student to see your exam.
- Discussing an exam that you have just completed with students from other sections that have yet to write the exam.

Course Program Molecular Biology 4XX3 2013 - Schedule Week 1

Time	Mon May 6th	Tue May 7th	Wed May 8th	Thu May 9th	Fri May 10th
9:00	T: Introduction- (H.E. Schellhorn) Laboratory Notebook Graphs and Tables	T- Bacterial Culture and Sample Storage	T: Cell Fractionation Proteins (H. Schellhorn)	Prepare and Load SDS Gel	T: Protein/Enzyme Assays (H. Schellhorn)
9:30					
10:00		E: Store cultures and label boxes	E: His Tag Purification (Assigned Sigma factor)	SDS-Gel	E: Protein/Enzyme Assay:
10:30					
11:00	Pipetting-Co-efficient of Variation	T: SDS-PAGE	E: Load and Run SDS-Gels	T: Immunoblot (Western) Analysis	
11:30					
12:00	Lunch	Lunch	Lunch	Lunch	Lunch
12:30					
13:00	T: DNA Management- In Silico Management	E: Protein Assay	T: Spectrophotometry and enzyme kinetics	E: Prepare Blot for Immunoblotting	Quiz I
13:30					
14:00	E: Preparation of media and reagents		E: Stain Gel (30 min)	E: Run semi-dry gel	E: Load gel blot into film cassette and develop
14.30			E: Destain Gel (2 h)		
15:00		E: SDS-PAGE (setup)			
15:30				Setup overnight blocking for Immunoblotting	
16:00					T: Tissue Culture (B130E) (A. Cowie)
16:30	E: Streak Out/Inoculate Cultures	E: Innoculate O/N cultures	E: Photograph Gels		Organization of Week II (HES)
O/N	PCR Primer Design	Prepare a publication quality graph	Fill out an order form	Chemical Waste Labelling	Read a paper and write a summary

T-Theory E-Experimental

Course Program Molecular Biology 4XX3 2013 – Schedule Week 2

Time	Mon May 13th	Tue May 14th	Wed May 15th	Thu May 16th	Fri May 17th
9:00	T-Real time PCR theory and assignment Sean Taylor Biorad	Rotation (see schedule)	E: Harvest pTaq Culture Lysozyme/Heat	pTaq assay by PCR	Quiz II
9:30					
10:00					
10:30	Rotation (see schedule)			T-Science Methodology (R.A. Morton) (assigned paper)	
11:00			pTaq dialysis for O/N		T: Intellectual property (Marcel Mongeon)
11:30					
12:00	Lunch	Lunch	Lunch	Lunch	Lunch
12:30					
13:00	T: Electron Microscopy (B130E) (K. Schultes)	Rotation (see schedule)	T: PCR Theory (A. Cowie)	T: Fluorescence and Microfluidics	E-Cleanup
13:30					
14:00	Rotation (see schedule)			Agarose Gel of PCR product	
14.30			SDS Protein gel		
15:00					T-Discussion—The Future of Molecular Biology—Systems and Synthetic Biology
15:30					
16:00		Taq Polymerase (intro)			
16:30		Inoculate Taq Overexpression strain			
17:00					
O/N	Realtime PCR analysis	Read Assigned Paper	Fill out DNA sequencing order	Write an SOP	

T-Theory E-Experimental

6. Methods

Preparation of stock chemicals precedes any experiment. The amount of any chemical made should be balanced against cost and need (you should always make enough so that all compared samples are made from the same material). Sterile solutions should, preferably, be made just before use or at least stored separately. Once a bottle containing sterile solution has been opened, always view it with suspicion—although media will often show visible contamination because of high nutrient availability, other stock solutions may become contaminated but may not show obvious signs.

6.1. Media Preparation Days 1 and 2

Make first, each group:

Growth of *E. coli* : 1x 200 mL LB in 1,000 mL flask (Styrofoam plug and aluminum cover)
1x 100 mL LB in 100 ml bottle
1x 125 mL flask with Styrofoam plug and aluminum cover

For Taq production: 1x 200 mL LB in 1,000 ml flask (Styrofoam plug and aluminum cover)
1x 125 mL flask with Styrofoam plug and aluminum

To be made later:

Group 1: **Upper Tris Buffer (dissolve in 950 ml on day 1, adjust pH and re-adjust adjust on day 2!)**
0.5 M Tris/Cl pH 6.8 + 0.4 % SDS (aliquot 4x 0.25 L in 0.25 L bottles)

Lower Tris Buffer (dissolve in 950 ml on day 1, adjust pH and re-adjust adjust on day 2!)
1.5 M Tris/Cl pH 8.8 + 0.4 % SDS (aliquot 4x 0.25 L in 0.25 L bottles)

Group 2: **PBS buffer:** Make 500 mL: 4 g NaCl, 0.1 g KCl, 0.72 g Na_2HPO_4, 0.12 g KH_2PO_4 dissolve in 450 ml of distilled H_2O and adjust the pH to 7.4 with HCl. Add H_2O to 500 mL, autoclave and aliquot 45 mL each into 8x 50 mL sterile plastic tubes

Group 3: **10x Laemmli Electrophoresis buffer**
60.5 g Tris, 288 g Glycine, 20.0 g SDS, H_2O add 2.0 L

10% Ammonium persulfate (= APS): 10 mL (aliquot 8x 1 mL)

Sterile saline (0.85 % NaCl)
make 1600 mL, aliquot 200 mL each into 8x 500 mL bottles and autoclave (label!)

Group 4: **Laemmli Sample Buffer (SB)** 40 mL
5.0 mL 1.0 M Tris/Cl pH 6.8, 4 mL β-Mercaptoethanol, 1.84 g SDS, 8 mL Glycerol (heat in microwave), Bromophenol Blue (a few grains), 23.2 mL H_2O (aliquot 8x 5 mL in 14 mL culture tubes)

Sterile 5.0 M NaCl
make 500 mL, autoclave and aliquot 45 mL each into sterile 50 mL plastic tubes

Group 5: **Buffers for Taq purification (1M Tris-HCl pH 8 provided by TAs)**
500 mL Buffer A for Taq Polymerase (50 mM Tris-HCl pH 8, 50 mM Glucose, 1 mM EDTA), aliquot 45 mL each into 8x 50 mL sterile plastic tubes

2x 1 L Storage Buffer for Taq Polymerase (50 mM Tris-HCl pH 8, 50 mM Glucose, 1 mM EDTA, 50% glycerol) immediately before use add 1 mM DTT

500 mL lysis buffer for Taq Polymerase (10 mM Tris-HCl pH 8, 50 mM KCl, 1 mM EDTA, 0.5% Tween 20, 0.5% Nonidet P40), immediately before use add 1 mM PMSF and aliquot 45 mL each into 8x 50 mL sterile plastic tubes

Group 6: **Buffers for His-tag purification and Acid phosphatase (AP) activity assay**

Wash Buffer
20mM disodium phosphate, 500mM NaCl, 10mM imidazole, pH=7.5, adjust using HCl

Elution Buffer
20mM disodium phosphate, 500mM NaCl, 500mM imidazole, pH 7.5, adjust using HCl)
(make 500 ml each, aliquot 8x 45 ml in 50 ml tubes)

AP buffer: 250 mM glycine/HCl buffer (pH2.5), add 25 mM *p*-nitrophenylphosphate (PNPP) before use. (make 100 ml)

1.0N NaOH : make 200 ml

Group 7: **TBST buffer**
8.7 g NaCl, 10 ml 1 M Tris/Cl pH 8 (provided by TAs), 0.5 ml Tween 20 / L
(Aliquot 4x 1,000 mL)

Protein Staining solution:
1.00 g Coomassie Brilliant Blue R250,
300 mL methanol
100 mL acetic acid (glacial) store in plastic bottle. (Be especially careful with the staining solution as it will also readily stain proteins in your hands.)
` Make to 1.0 L with distilled water

De-staining solution:
200 mL methanol, 80 mL acetic acid (glacial)
730 mL distilled water to 1.0 L
store in plastic bottle

Sterile H_2O
aliquot 200 mL each into 8x 250 mL bottles, label and autoclave.

Group 8: **1x TBE Electrophoresis Buffer**
Tris Base 41.2 g, Boric Acid 22.0 g, 0.5 M EDTA (pH 8) 4.0 mL, H_2O to 4.0 L, (Aliquot 4x 1.0 L)

Western blot transfer buffer 4 L
57.6 g Glycine, 12.1 g Tris, 800 mL Methanol, ad 4 L store in plastic bottle

Ampicillin stock solution
make 4.5 mL stock of 100 mg/mL, sterile-filter into sterile 14 mL tube, aliquot 500 µl each into 8 sterile 1.5 mL microfuge tubes (label and store at -20°C)

IPTG stock solution
make 4.5 mL stock of 500 mM, sterile filter into sterile 14 mL tube, aliquot 500 µl each into 8 sterile 1.5 mL microfuge tubes (label and store at -20°C)

6.2. Overproduction and Purification of Proteins—*E. coli* sigma factors.

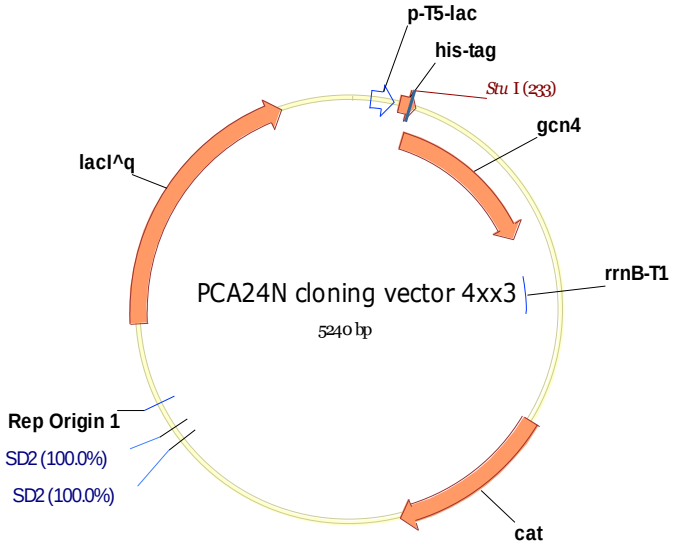
Bacterial RNA polymerase consists of several subunits, including a sigma factor that confers specificity for promoter recognition. During the two weeks of the lab, we will use *E. coli* strains that overexpress a bacterial sigma factor to demonstrate principles important in current molecular biology projects. It should be appreciated that the choice of materials is one of convenience. Protein overexpression, purification and testing using electrophoresis and Western analysis techniques are relevant to a wide range of biomedical and environmental research projects. We will try to emphasize wider application of technology whenever possible.

The basic goals will be to examine over expression of RpoS, a non-essential sigma factor, purify the protein using His-tags and examine expression in several sigma factor deletion mutants. Later in the week, in keeping with the theme, we will perform an enzyme assay on prepared extracts to quantify expression of acid phosphatase, and RpoS-dependent enzyme. During the second week, we will purify Taq polymerase and may repeat electrophoresis from the first week, if time permits.

Strain list

Strain	Genotype	Reference
AG1	recA1 endA1 gyrA96 thi-1 hsdR17(rK–mK+) supE44 relA1	(Kitagawa et al, 2005)
AG1 *rpoS*	as AG1, but Δ*rpoS::kan*	(Kitagawa et al, 2005)
AG1 *rpoN*	as AG1, but Δ*rpoN::kan*	(Kitagawa et al, 2005)
AG1 *rpoF*	as AG1, but Δ*rpoF::kan*	(Kitagawa et al, 2005)
AG1 *rpoH*	as AG1, but Δ*rpoH::kan*	(Kitagawa et al, 2005)
Plasmid		
pCA24N	cloning vector	(Kitagawa et al, 2005)
p*rpoS*	*rpoS* gene on pCA24N	(Kitagawa et al, 2005)

Plasmid map



6.2.1. Protein overproduction

Day 1

- Innoculate overnight cultures - inoculate colonies of *E. coli* Ag1 strain carrying sigma factor genes into 10 mL of LB with chloramphenicol at 30 μg/ml (LB cm 30). Incubate the culture at 37°C O/N

Day 2

1. Measure optical density (OD_{600}) of the night culture (take 100 μL of overnight culture and dilute with
2. 900 uL of water). This is a dilution factor of 10. Thus, you need to multiply the measured OD_{600} by 10 to determine the OD_{600} of your overnight culture.
3. Inoculate your overnight culture into 10ml LB cm30 contained in a 50 mL flask to obtain a final
4. OD_{600} of 0.05.
5. Incubate at 37°C and measure the OD_{600} periodically. About 2-3 hours after inoculation, the OD_{600} should be in the exponential range (0.3-0.6). Split 10ml LB culture into two 50 mL sterile flask (5 ml each).
6. Add IPTG (final concentration 1mM) to one flask for induction. Keep incubating both flasks at 37°C for 2h.
7. Transfer cultures to 15ml Red cap centrifuge tubes (polypropylene, SARSTEDT, cat#: No.62.554.205). Collect cells by centrifugation at 4,000g for 10 min at 4 °C.
8. Discard supernatants.

6.2.2 Preparation of cell lysates

1. Resuspend the pellets using vortex mixer. Add 10 ml chilled PBS buffer (4°C), and centrifuge.
2. Repeat step 6 two times.
3. Resuspend pellets in 500 ul PBS buffer, and transfer to 15 ml blue cap tubes (polystyrene, BD Falcon, cat#: REF 352095)—this will yield an OD600~ 15 corresponding to 10^{12} cells per mL
4. Sonicate the cell suspension until it becomes translucent or transparent (about 5 min).
5. Transfer cell crude extracts to 1.5 ml Eppendorf centrifuge tubes and centrifuge at 13,000xg for 10 min at 4°C.
6. Quantify protein using Bradford assay (see 6.5)

6.2.3 Purification of His-tagged sigma factor proteins

see 6.5

Kitagawa M, Ara T, Arifuzzaman M, Ioka-Nakamichi T, Inamoto E, Toyonaga H, and Mori H. 2005. Complete set of ORF clones of *Escherichia coli* ASKA library (a complete set of E. coli K-12 ORF archive): unique resources for biological research. DNA Res.12(5):291-9.

6.3. SDS-PAGE (Laemmli)

Solutions for 2 Laemmli gels – mix solutions for separating gel in 50 ml plastic tubes and solutions for stacking gel in 15 ml plastic tubes

		7 %	10 %	12.5 %
Separating gel	4x Lower Tris buffer	3.7 ml	3.7 ml	3.7 ml
	40% Acrylamide Solution	2.6 ml	3.8 ml	4.7 ml
	H_2O	8.7 ml	7.5 ml	6.6 ml
	APS	75 µl	75 µl	75 µl
	TEMED	7.5 µl	7.5 µl	7.5 µl
Stacking gel	4x Upper Tris	1.25 ml	1.25 ml	1.25 ml
	40% Acrylamide Solution	0.5 ml	0.5 ml	0.5 ml
	H_2O	3.25 ml	3.25 ml	3.25 ml
	APS	75 µl	75 µl	75 µl
	TEMED	7.5 µl	7.5 µl	7.5 µl

Solutions for SDS-PAGE:

Ammonium persulfate *(= APS):* 10%
4x Lower Tris (buffer for separating gel): 1.5 M Tris/Cl pH 8.8 + 0.4% SDS
4x Upper Tris (buffer for upper gel): 0.5 M Tris/Cl pH 6.8 + 0.4% SDS

10x Laemmli running buffer *(1 l):*

Tris	30.25 g
Glycine	144 g
SDS	10 g

2x sample buffer (=SB) *(40 ml):*

1 M Tris/Cl pH 6.8	5 ml
β-Mercaptoethanol	4 ml
SDS	1.84 g
Glycerol	8 ml (heat in Microwave)
Bromophenol blue	(a few grains until colored)
H_2O	23.2 ml

Staining solution: 30% (methanol) MeOH, 10% Acetic Acid, 0.1% Coomassie Blue R250

Destaining solution**:** 20% (methanol) MeOH, 8% Acetic Acid

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227** (5259): 680–685.

6.4. Immunoblotting (Western) Blotting
(Note this year we will employ semi-dry transfer units which are a little easier to use)

6.4.1 Prepare and load gel (See 6.3.1-6.3.3)

6.4.2 Protein transfer
-Materials:

1.]semi-dry transfer chamber
2. 8 Whatman blot papers 7 x 10 cm (per membrane)
3. 1 PVDF membrane 7 x 10 cm, wet with methanol and then in transfer buffer

Procedure:

1. Cut 7x9 cm PVDF membranes using pencil and being careful not to touch the membrane.
2. Pour Western Blot transfer buffer (keep cool at 4°C) into a large container and put in filter paper to soak.
3. Let membrane soak in methanol for 1 min, then transfer to semi-dry transfer apparatus immediately.
4. Onto the semi-dry transfer apparatus load:
    a. 4 Whatman blotting papers (wet)
    b. PVDF membrane (wet)
    c. gel
    d. 4 Whatman blotting papers (wet)
5. (*keep air bubbles minimal by "rolling" each layer on semi-dry transfer apparatus*)
6. Transfer at 50 mA for 75 min.

Transfer Buffer: 57.6 g Glycine, 12.1 g Tris, 800 mL Methanol, add dH2O to 4 Liter

←Old

New→

6.5. Bradford Assay for Protein Quantification

The Bradford Assay is the most commonly used assay for measuring soluble proteins. It is based on a simple dye-protein interaction that produces a blue colour that can be measured a spectrophotometer. To determine the amount of protein in an unknown sample, we must prepare a standard curve using known amounts of protein. Although the assay is sensitive, care must be taken to ensure valid protein determination. Many compounds interfere with colour development and the effect of this upon the standard curve can sometimes (but not always...) be quantified by including an appropriate concentration of the interfering compound in the standard curve. In addition, the assay is non-linear above 10 ug protein (microassay)—use only the linear portion of the standard curve.

STEPS

1. Prepare standard curve samples
2. Prepare unknown samples
3. Measure optical density using a spectrophotometer. Tabulate results in a chart.
4. Calculate regression estimates (slope and intercept). If intercept is different from zero, recalculate forcing the best fit line through the intercept. Why?
5. Use regression estimates (standard curve data) to calculate the amount of protein (µg) in unknown samples
6. Calculate concentration of unknown samples. In some cases, the total amount of protein is also of interest (particularly for purifications)

Reagents provided:

1. 100 µL of 1.0 mg/ml Bovine Serum Albumin (BSA) for standard curve (BSA is everyone's favourite standard)
2. 5 mL Bio-Rad Bradford dye reagent

One standard curve per student. Prepare in triplicate.

Sample #	Volume of H_2O (µl))	Bio-Rad dye reagent (µl)	BSA (µl)
1	800	200	0.0
2	798	200	2.0
3	796	200	4.0
4	794	200	6.0
5	792	200	8.0
6	790	200	10.0

For an unknown protein sample, analyze as follows:

1. 800 – X µl H_2O + 200 µl Bio-Rad dye reagent + X µl protein sample (e.g. 20 µl).
2. Gently mix components in the cuvettes using 1 mL plastic tips (new tip for every cuvette).
3. Measure OD_{595} for all samples.
4. All BSA samples are used to draw a standard curve by plotting the average (OD_{595} readings as Y-axis vs. µg BSA as X-axis using Excel—**you will be given a spreadsheet to do this**).
5. Based on the curve, calculate the slope and use the average OD_{595} readings of the unknown protein sample to calculate its concentration.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal Biochem, 72, 248–254.

6.6 Enzyme assay for acid phosphatase activity

(Modified from (Dassa et al, 1982) : http://www.jbc.org/cgi/reprint/257/12/6669)

Determining enzyme activity is a common laboratory procedure use to examine gene expression or the conditions that affect production of the protein of interest. Although enzyme activity can be expressed a number of ways, the most common form is umole of substrate converted per minute. Specific Activity is Enzyme Activity normalized for protein concentration and can thus be expressed as umol per minute per mg protein. Determination of protein concentration is described in the previous section.

Protocols

A. **Prepare sample extract**

1. Inoculate overnight cultures of WT and *rpoS* mutant in 10 ml LB and growth at 37^{o}C
2. Collect cells by centrifugation at 4,000 rpm for 10 min
3. Discard supernatant and resuspend pellet in 10 ml LB 20mM Phosphate buffer (pH7.0) (Pi buffer), ice cold.
4. Centrifuge the cells at 4,000 rpm for 10 min
5. Repeat steps 3 and 4 two more times
6. Discard supernatant and resuspend pellet in 1.0 ml Pi buffer
7. Transfer 0.5 ml to 15 ml Falcon blue cap tube, and save the other 0.5 ml on ice
8. Sonicate until cultures turn clear or translucent (less than 5 min)
9. Transfer crude extracts to 1.5 ml Eppendorf tubes and spin down at 13,000 rpm for 10 min
10. Carefully transfer the supernatant to clean 1.5 ml tubes
11. Quantify protein concentration using Bradford assay (see section 6.5)

B. **Enzyme assay**

1. Prepare reaction buffer containing : 250 mM glycine/HCl buffer with 25 mM *p*-nitrophenylphosphate (PNPP)
2. Prepare 3 tubes, labelled with (**1. Control 2. Wildtype 3. *rpoS* mutant**)
3. Add 0.5 ml reaction buffer into each tube
4. Incubate all three tubes in water bath at 37^{o}C for 2 min to equilibrate
5. Start the reaction by adding 20 µl cell extract (if the protein concentration is higher than 1 mg/ml) of WT and rpoS extract to tube 2 and 3, respectively. Add 20.0 µl of Pi buffer to control tube 1
6. Incubate at 37^{o}C for 15 min and stop the reaction by adding 1 mL of 1.0N NaOH
7. Transfer 1.0 ml reaction mixture to a semi-micro cuvette to measure absorbance at 410 nm

C. **Calculation of activity**

1. One unit of enzyme is defined as the amount of acid phosphatase required to release 1.0 nmol of PNPP/min under the above defined conditions (At 25 mM, the no enzyme rate of PNPP hydrolysis is 0.49 nmol/min/mL)
2. Alternatively, one unit of enzyme can be defined as 1000 x A410 per min per mL (Atlung et al. 1989)

Atlung T, Nielsen A, and Hansen FG. 1989. Isolation, characterization, and nucleotide sequence of *appY*, a regulatory gene for growth-phase-dependent gene expression in *Escherichia coli*. J Bacteriol. 171(3):1683-91.

Dassa E, Cahu M, Desjoyaux-Cherel B, and Boquet PL. 1982. The acid phosphatase with optimum pH of 2.5 of *Escherichia coli*. Physiological and Biochemical study.
J Biol Chem. 257(12):6669-76.

6.7. Culture and Propagation of Chicken Embryo Fibroblast (CEF) cells

Tissue culturing is a commonly-used technique that must be mastered for many basic molecular biology studies. Commonly problems include bacterial contamination (usually mycobacteria) for fungal contamination. Contamination by other cells types or lineages is, perhaps surprisingly, also a well-documented problem even in culture collections. It is important to routinely change media ("split cells") or viability will be compromised. Cells need some CO_2 and must be grown in high humidity to prevent desiccation. Cultures should be routinely examined by microscopy check for contamination (may be difficult or impossible to conclusively identify all forms). We will propagate CEF cells for safety reasons (CEF are Level I) but the procedures used to transfer humans cells (that you may employ in some research labs…) are identical.

- incubator 41.5°C, 95% humidity, 5% CO_2
- microscopy to assess confluency of cells
- wipe down biosafety hood with 80% Ethanol before beginning work
- organize all equipment and supplies close to the hood, wear lab coat and gloves

Materials

Complete DMEM medium: To one 500 mL bottle of DMEM (Dulbecco's modified Eagle medium) add:

- 5 mL penicillin/streptomycin (stored in –20°C freezer)
- 5 ml L-glutamine (if not in the medium already, stored in –20°C freezer)
- 25 mL CCS (Cosmic calf serum; Hyclone, stored in –20°C freezer)
- 25 ml Tryptose Phosphate Broth (TPB)

These will be provided for you.

Splitting/passing CEF cells

On the lab bench:

1. 60 min prior to passing cells, heat media and trypsin/EDTA solution to 41.5°C
2. spray all bottles with 80% ethanol prior to putting it under the hood

In the sterile cabinet:

1. aspirate/remove old medium from a plate of confluent CEF
2. wash cells with 2 mL trypsin/EDTA per 100 mm plate and aspirate i**mmediately**
3. add 2 mL trypsin/EDTA one more time and aspirate immediately. Dissociate cells by gently tapping the dish - watch under microscope.
4. As soon as the cells are dissociated, add a few ml of complete DMEM medium to dilute the trypsin (why is this important?).
5. Seed appropriate volume of cell suspension to obtain a 1 to 3 dilution
6. Complete volume of medium to a total of 8 ml of medium per 100 mm plate
7. NB Be quick! Primary cells, such as CEF, are sensitive!

When finished using the Biosafety hood spray down hood with 80% Ethanol, wipe the surface of the cabinet dry.

6.8. Purification of *Taq* DNA Polymerase

(modified from: Nucl. Acids Res. 1993, 21:4850-4851)

Purifying proteins allows us to study enzymes in isolation from other potential interfering effects. One particularly useful enzyme is Taq polymerase which catalyzes the high temperature synthesis of DNA used in PCR. We will isolate Taq from a culture of *E. coli* that overexpresses the protein. You should understand how each step contributes to the purification procedure.
IMPORTANT: Do not discard any samples (extracts, different fractions) until the final analysis is finished. We will need these for the determination of protein concentrations and SDS-PAGE analysis.

1. **Day 1:** 10 mL precultures of pTaq-carrying strain were grown in LB medium with 100 ug/ml ampicillin. Inoculate 200 ml of LB with 100 ug/ml ampicillin in a 1 liter flask to OD_{600}=0.1 and grow at 37°C. After approximately 3 to 4 hours (OD_{600} of 0.5-0.8), add IPTG to a final concentration of 1 mM (from 0.5 M Stock solution), take **Sample 0** and grow at 37°C over night.

2. **Day 2:** Take **Sample 1**, collect cells from 200 mL culture by centrifugation, wash one time in 20 mL buffer A (50 mM Tris-HCl pH 7.9, 50 mM glucose, 1 mM EDTA) in an SS-34 tube, resuspend in 10 mL of buffer A and add 40 mg of lysozyme.

3. Incubate cells with occasional swirling at room temperature for 15 min. Add an equal volume of lysis buffer (10 mM Tris-HCl pH 7.9, 50 mM KCl, 1 mM EDTA, 1 mM PMSF, 0.5% Tween 20, 0.5% Nonidet P40) and incubate the solution at 75°C with gentle shaking for 1 hour.

4. Remove precipitated protein by centrifugation in an SS-34 rotor at 12,000 rpm for 15 min (tare carefully!). Transfer the clarified crude extract (**Sample C**) to a clean centrifuge tube and keep 50 ul at –20°C.

5. Add solid streptomycin to the crude extract to a final concentration of 2.5%, and mix slowly. After 15 min at room temperature centrifuge solution at 12,000 rpm for 15 min and transfer the supernatant (S**ample S**) to a clean SS-34 tube, resuspend pellet (**Sample SP**) in 100 ul Laemmli sample buffer for SDS-PAGE.

6. Add solid $(NH_4)_2SO_4$ (grind 1x 100 g in with mortar and pistil) SLOWLY to a final concentration of 15% of saturation (8.2 g/100 mL) to the supernatant, and mix slowly but constantly. After 15 minutes at room temperature, centrifuge the solution at 12,000 rpm for 15 min and transfer the supernatant to a clean SS-34 tube (**sample N1**).

7. Add additional $(NH_4)_2SO_4$ SLOWLY to bring the final concentration to 50% of saturation (20.4 g/ 100 mL), and slowly mix constantly. After 15 min at room temperature, centrifuge the solution at 12,000 rpm for 10 min. Decant the supernatant (**Sample N2**) carefully leaving the layer of protein in the centrifuge tube.

8. Resuspend the protein precipitate in 1 mL per centrifuge tube of buffer A and dialyze against two changes of 1 L of storage buffer (50 mM Tris-HCl pH 7.9, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol) at 4°C. Store solution (**Sample F**) at –20°C (short term) or at –70°C (long term).

9. **Day 3:** Determine the protein concentration of the crude extract (**sample C**) and of the final product (**sample F**) with the Bradford assay (do not forget standard curve, dilutions may be necessary). Monitor the purification of the protein by

10. **SDS-PAGE analysis:** 10 ul each: Sample 0, Sample 1, Sample C, Sample S, Sample SP, Sample N1, Sample N2, Sample F, reference proteins

Pluthero FG. 1993. Rapid purification of high-activity Taq DNA polymerase. Nucleic Acids Res. 11;21(20):4850-1.

6.9. Polymerase Chain Reaction (PCR)

We use PCR as a routine procedure for many purposes (e.g. amplifying fragments to be cloned, diagnostic analysis etc.). Several parameters can be altered in the design of an amplifications strategy and these will be discussed.

Example: PCR amplification of *E. coli* sigma factor genes to assess the quality and quantity of Taq DNA Polymerase produced.

1. Design Primers using Vector NTI software (to be demonstrated)

2. 200 µl PCR Master Mix

(pipet these very carefully! Check pipettors for leaks before pipetting these small amounts)

10x Buffer	20.0 µl (with $MgCl_2$)
10 mM dNTPs	4.0 µl
Primer A	4.0 µl
Primer B	4.0 µl
H_2O	160.0 µl
Template	4.0 µl
Taq Polymerase	none

3. Preparation of Samples

Each group should run **7 reactions and 1 control without Taq**, aliquot 20 ul into 8 PCR tubes, add 1 ul of the following Taq or 1 ul dialysis buffer as control. control: without Taq, commercial Taq (undiluted), lysate C (undiluted), final product F (undiluted, 1:10, 1:100, 1:1000 and 1:10,000; perform dilutions in dialysis buffer). Prepare a table like the following for PCR reactions in your lab book.

Sample	**Master Mix (µl)**	**Buffer (µl)**	**Comm. Taq (µl)**	**Cell Ex. (ul)**	**Lysate C dilutions (µl)**				
					undil	**1:10**	**1:100**	**1:1,000**	**1:10,000**
1	20	1.0		-	-	-	-	-	-
2	20	-	1.0	-	-	-	-	-	-
3	20	-	-	1.0	-	-	-	-	-
4	20	-	-	-	1.0	-	-	-	-
5	20	-	-	-	-	1.0	-	-	-
6	20	-	-	-	-	-	1.0	-	-
7	20	-	-	-	-	-	-	1.0	-
8	20	-	-	-	-	-	-	-	1.0

4. Amplification reaction

(pre-programmed in Eppendorf or Biorad Gradient PCR)

denaturation:	95°C	5 min
cycle (30x):	60°C	30 s
	72°C	2 min
	95°C	30 s
strand completion:	72°C	5 min
stop:	4°C	open end

General Considerations for optimization of PCR conditions:

- annealing temperature (gradient)
- Mg^{2+} concentration
- annealing time
- solvents such as DMSO
- use of PCR for mutagenesis, proofreading enzymes

6.10 Affinity His-tag purification
Chelate agarose beads (Agarose Bead Tech., Inc.)

Proteins tagged with an amino-terminal or carboxy-terminal poly Histidine tag can be purified using Metal-complexed beads (usually Ni). The following describes the procedure that you will use to purify over-expressed sigma factor proteins.
Throughout the procedure, mild centrifugations (1,500rpm, ~ 600g) are sufficient.

1. **Elimination of the preservative.**
   a. Twist off the lower cap of the Mini-column.
   b. Place the Mini-column in a 1.5ml microcentrifuge tube.
   c. Shake the metal chelate resin container (to disperse the agarose beads) and add 200 ul of the resin to the Mini-column.
2. **Centrifuge and discard flow-through.**
   a. Place Mini-column back into 1.5ml microcentrifuge tube and add 0.5ml distilled water.
   b. Centrifuge and discard flow-through.
   c. Repeat steps 1e-1h 4 times.
   d. Prior to step 2, centrifuge Mini-column without further addition of distilled water to remove excess water and discard flow-through.
3. **Equilibration of the Mini-column.**
   a. Place Mini-column back into 1.5ml microcentrifuge tube.
   b. Add 1ml binding buffer (20mM disodium phosphate, 500mM NaCl, 10mM imidazole, pH=7.5) to the Mini-column.
   c. Centrifuge and discard flow-through.
   d. Repeat steps 2a-2c four times.
   e. Prior to step 3, centrifuge Mini-column without further addition of binding buffer to remove excess buffer and discard flow-through.
4. **Application of the sample.**
   a. Place the Mini-column back into 1.5ml microcentrifuge tube.
   b. Apply 100-500 ul of the protein sample (One of the following: RpoS, RpoN, RpoH, RpoE, or FliA) with the histidine-tagged protein to the Mini-column.
   c. Centrifuge and discard flow-through.
5. **Washing of the Mini-column.**
   a. Place the Mini-column back into 1.5ml microcentrifuge tube.
   b. Add 0.5ml binding buffer to the Mini-column.
   c. Centrifuge and discard flow-through.
   d. Repeat steps 4a-4c four times.
   e. Prior to step 5, centrifuge Mini-column without further addition of binding buffer to remove excess buffer and discard flow-through.
6. **Elution of the pure protein.**
   a. Place the Mini-column into a **new** 1.5ml microcentrifuge tube.
   b. Add 100-500 ul (same amount as protein sample added in step 3b) of elution buffer (20mM disodium phosphate, 500mM NaCl, 500mM imidazole, pH 7.5) to the Mini-column and centrifuge (DO NOT DISCARD).
   c. Pure protein with histidine-tag can now be used in further experiments.

7.1 Laboratory Skills Instruction Rotation

To give students the opportunity to receive personal instruction in the use of key techniques and equipment, we will have an equipment rotation schedule so that small groups can be accommodated. Each rotation will be 30 min in duration. The final schedule will be given out on Friday May 7th. At each station, you will be given 5 short questions about the technique—these will be answered in your lab book on the following pages**.**

Technique	**Questions to be answered on Page…**
Tissue culture	**80-81**
Electron Microscopy	**82-83**
Microtiter Plate Reader	**84-85**
HPLC	**86-87**
PCR	**88-89**

Appendices

8.1 Appendix Chemicals used in the course

Chemical	Company	Catalogue Number
Tris (Base)	Bioshop	TRS001.1
SDS	BDH Chemicals	30176
EDTA	Sigma	E-5134
APS	Sigma	A3678-100G
$NaH_2PO_4.H_2O$ (monobasic, monohydrate)	EMD	SX0710-1
$K.H_2PO_4$ (monobasic)	J.T.Baker	1-3246
β-mercaptoethanol	Sigma	M-6250-1L
Bromophenol blue	BDH Chemicals	20015
Imidazole	Boehringer Mannheim	R34-22
IPTG	Roche	10 724 815 001

8.2 Appendix Sample spreadsheet for calculations

General Enzyme Calculation Template Date 4/28/2010 19:00

-determine protein concentration of samples using ProteinMicroassay or ProteinMicrotiterPlateassay sheet
-determine activity of samples using standard curve calibration assay below to obtain Total Units per mL
-divide Total Units per mL by Protein (mg/ml) to obtain specific activity

A. Enzyme assay
1. Prepare reaction buffer containing : 250 mM glycine/HCl buffer with 25 mM *p*-nitrophenylphosphate (PNPP)
2. Prepare 3 tubes, labelled with (1. Control 2. WT 3. rpoS)
3. Add 0.5 ml reaction buffer into each tube
4. Incubate all three tubes in water bath at 37oC for 2 min to equilibrate
5. Start the reaction by adding **20 µl** cell extract (if the protein concentration is higher than 1 mg/ml) of WT and rpoS to tube 2 and 3, respectively. Add 20 µl of Pi buffer to control tube 1
6. Incubate at 37oC for **15 min** and stop the reaction by adding 1 ml of 1N NaOH **(TOTAL Volume = 1.52 mL)**
7. Transfer 1 ml reaction mixture to cuvette to measure absorbance at 410 nm

Assay Notes : **(1) make sure that you perform linearity checks**
(2) use only determine unknown values with in the standard curve (do not extrapolate..dilute and re-assay instead)
(3) biological replicates usually yields standard errors of less than 10% (5% is quite good). If yours is higher check your technique..

ACID PHOSPHATASE ASSAY DATA

Sample	Sample ID	Protein concentration (mg/mL)				Volume of Sample (ul)	Time of Incubation(min)	OD410 READINGS						Total Activity (umol/min/mL)				SPECIFIC ACTIVITY	Standard Error	Error Flags	
		Rep1	Rep2	Rep3	Average			Rep1		Rep2		Rep3		Rep 1	Rep 2	Rep 3	Average OD410			10%	20%
								Sample1	Sample2	Sample1	Sample2	Sample1	Sample2								
1	a																				
2																					
3																					
4																					
5																					
6																					
7																					
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CALCULATION NOTES
Protein Concentration (from Protein Assay Table)
Rep - Biological Replicate of Experimental unit
Sample - Technical Replicate
TOTAL ACTIVITY = (AverageOD410/1770 M-1) x (1000 nM/M) x (1.0 l/1000mL) x 1.52mL (total assay volume) x (1/sample volume(mL)) x ((1/Incubation time) (min))
SPECIFIC ACTIVITY = TOTAL ACTIVITY/PROTEIN CONC. (e.g. P2S/D2S)
STANDARD ERROR = STANDARD DEVIATION of Biological Replicate Specific Activities (e.g. Standard Deviation (P2S/D2S, Q2S/E2S, R2S/F2S) / SQRT(3))
ERROR FLAGS - these will indicate if variation is high. If high, check to make sure numbers are typed correctly--it is easy to mistype a decimal)
-this is not operational
MOLAR EXTINCTION COEFFICIENT (ε) OF pPNP = 17,700 M-1 cm-1

= user inputed data
= spreadsheet calculated data
= negative number (this occur if blank values are higher than measured values)
error flag - excess variation
error flag - very high variation that will likely affect ability to employ statistical tests of significance

8.3 Appendix Acronyms

Acronym List:

APS	ammonium persulfate
BSA	bovine serum albumin
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EDTA	diaminoethane-tetraacetic acid
FBS	fetal bovine serum
IPTG	isopropyl-β -D-thio-galactoside
LB	Luria-Bertani
MSDS	Material Safety Data Sheet
PAGE	polyacrylamide gel electrophoresis
PBS:	phosphate buffered saline
PCR:	polymerase chain reaction
PMSF	phenylmethanesulfonyl fluoride
PNPP	p-nitrophenyl phosphate
PVDF	polyvinylidene fluoride
SB	Sample Buffer
SDS	sodium dodecyl sulfate
SOP	Standard Operating Procedure
TAE	Tris-Acetate-EDTA
TBE	Tris-borate-EDTA
TBST	Tris buffered saline with tween
WHMIS	Workplace Hazardous Materials Information System
XGAL:	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside