## LAB - EXPERIMENT

## MOLECULAR BIOLOGY LAB MANUAL 2022

### **PATH547**

Prepared by David Ng

With contributions from

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## WEEK 1 - 11

SCHEDULE

#### SCHEDULE OF LABS

WEEK 1:	(Jan 13)	am:	Course introduction + lecture (virtual)
WEEK 2:	(Jan 20)	am/pm:	LAB A: DNA fingerprint assay (polymerase chain reaction)
WEEK 3:	(Jan 27) (Jan 28)*	am/pm: am/pm:	LAB B1/B2: Ligations, transformation procedures. LAB B3: Plate analysis and culture start for plasmid preps.
WEEK 4:	(Feb 3)	am/pm:	LAB B4: Mini Plasmid preps.
WEEK 5:	(Feb 10)	am/pm:	LAB NGS1: Sampling and 16s Library prep. <i>(lab book PART I due at end of class)</i>
WEEK 6:	(Feb 17)	am:	NGS2: Visual check and MinION prep/sequencing (Concept journal club of 1 <sup>st</sup> paper).
MIDTERN	I BREAK (Feb	o 21 to 2	5)
WEEK 7:	(Mar 3)	am: pm:	LAB C1: Total RNA prep. LAB C2: Reverse Transcriptase + real time PCR. <i>(paper 1 due by Mar 4, 2022 via email)</i>
WEEK 8:	(Mar 10)	am: pm:	LAB D1/D2: PAGE pouring and running protein samples. LAB D2: Transfer of proteins.
LAB CLO	SED (Mar 14	to 18)	
WEEK 9:	(Mar 23)* (Mar 24)	am/pm: am: pm:	LAB E1: 2D gel start. Hydration of IPG strips. LAB E2: 2D gel: isoelectric focusing. LAB E3: Western blot analysis
WEEK 10	9: (Mar 31)	am: pm:	LAB E3/E4/E5: 2D gel – staining 1 <sup>st</sup> dimension. Equilibriation of IPG strips. Prepping 2 <sup>nd</sup> dimension. LAB E6: 2D gel staining.
WEEK 11	: (Apr 7)	am:	Pick up 2D gel data. Lab Book Part II and paper 2 due by April 14, 2022 (Dave's Mailbox)

\* Note that Jan 28, and Mar 23 are additional dates. On these days, the lab will be open from 10am to noon, and 1pm to 3pm for the required procedures.

## WEEK 1 - 11

## **STREAMS**

#### CONTENTS BY STREAM:

- LAB A: POLYMERASE CHAIN REACTION.
- LAB B: CLONING TECHNIQUES (INCLUDING RESTRICTION DIGESTS, PURIFICATION KITS, CIP ASSAYS, LIGATIONS, TRANSFORMATION, PLASMID PREPS)
- LAB C: RNA WORK: ISOLATION/PURIFICATION AND REVERSE TRANSCRIPTASE ASSAY. REAL TIME PCR.
- LAB D: DETECTION OF PROTEIN EXPRESSION FROM CLONED GENES BY WESTERN BLOTTING.
- LAB E: 2D PROTEIN GEL ELECTROPHORESIS
- LAB NGS: NEXT GEN SEQUENCING

## **EVALUATION**

### **CREDITS (3) LABORATORY IN MOLECULAR BIOLOGY**

The use of molecular biology techniques to explore biological problems.

#### Instructor:

Dr. David Ng (AMBL) db@mail.ubc.ca 604-822-6264

#### Time Commitment:

(JAN - PR) Michael Smith Building Rm 105: Thurs 10am - variable, SOME additional TIME also required sometimes the day before or the day after.

#### **Enrollment:**

Limited to 24 students. Prerequisite: none required.

#### Mark Breakdown:

1. **40% LabBook:** Lab books will be marked twice (sometime after the 1<sup>st</sup> third of class and sometime at the end) in the following format.

We will attempt to teach you to keep a lab book under the context of an industrial or company format. In other words, attention to legibility and detail are important for this area since the business aspect of your employment holds you accountable for ANY results you obtain. Therefore, it is simply in your best interest to make your lab notes as clear as possible.

- 1 you MUST provide a table of contents
- 2 you MUST write in pen
- 3 you MUST used a bound note book (provided)
- 4 you must very clearly show the DATE on every page.
- 5 you must very clearly show the AIMS for a particular day (a quick general blurb of what you are doing).
- 6 All experimental details MUST be clearly laid out or at least referred to.
- 7 You must have all data displayed on the particular date you obtained it.
- 8 No catch up, you have to record your information and notes on the SAME day as when the experiments are performed.
- 9 Your lab book must be unequivocally LEGIBLE.
- 10 Since the intent of your experiments must appear logical, you have to provide commentary on your rationale for what you are doing, why you are using this technique. In addition, some basic interpretation on your data is required. (i.e "S\*&t! It didn't work!! Maybe, the ... bla bla " or "Hooray, it worked, that means that ... bla bla .... and I can finally graduate")

## **EVALUATION**

In essence, your lab book should be clear enough so that ANYBODY (i.e. especially a lawyer) can follow it and know what you did on that particular day without any discrepancies. This means that it is permitted to simply refer to the lab manual, but you will find that we may routinely alter the technique to compensate for some unforeseen event. It is crucial that these changes are recorded, but for ease of clarity, it is probably worth paraphrasing the entire protocol anyway (i.e. flowcharts work well). Timekeeping is also handy, especially in instances where one veers away from the lab manual suggestions.

Since, the book is kept in a temporal fashion (i.e. the organization is by time NOT by exercise), it is important to make sure that you cross reference sections, especially if they are broken up over several sections of your lab-book due to the experiment taking several sessions to complete.

Your book will be marked on the basis on how easy it is for me to follow your progress and on your interpretive skills in dealing with the data. A book that is descriptively clear will be awarded approximately 75%. The other 25% is largely based on the level of interpretation. You will NOT have to write lab reports. At this level, I consider the lab report to be a redundant issue (you don't write lab reports in your thesis work!)

40% Papers: There will be two during the term. See below.

**20% Quizzes**: Quite frequent (> 6 of them) Will occur in the first 10 minutes of class, and primarily designed to get students to read manual beforehand, as well as show up on time. They will be very easy if you have taken the time to go through the day's procedure.

**Up to minus 5% penalty mark for general technique:** to be fair this is not where I expect you to always produce perfect data; and I also realize that there are varying levels of experience associated with any class. But, at the very least I hope to turn you into efficient researchers. This is essentially a potential 5% penalty towards your grade should the following happens: nothing ever works and there doesn't seem to be any constructive troubleshooting going on, you are almost always the last to finish the experiment, are unreliable to other classmates, and/or almost never prepared.

### Lecture Material and Lab Material will be presented on first day:

Students will be asked to pay \$20 fee for hardcopy versions of lab manual and lab lecture notes on first day, as well as a bound notebook.

### LITERATURE ASSIGNMENTS WORTH 40% OF YOUR FINAL GRADE:

## FIRST PAPER: (Concept due Feb 17th, Final due Mar 4th) – 20% of your Grade.

For this assignment, you can work solo or as a pair. Essentially, you will be asked to author a fake scientific paper that presents molecular biology data – this should also include high through-put -omic/next gen examples. Not only that, the paper will present credible looking data on an otherwise incredible phenomenon or pop culture topic (in other words, it will tread the fine line of being believable yet obviously unbelievable). Examples of such themes include working within Star Wars, Harry Potter, Star Trek, Superhero, Anime, Pokemon, type scenarios, but you are also welcome to suggest others.

A working 3 to 4 slide presentation with figures is expected on Feb 17th where you will be asked to present your paper to the class (journal club style!) Here is an opportunity to go over possible issues, so that your final paper is good to go.

Marks will be based on: 50% (scientific content – i.e. how convincing are the conclusions, and most importantly, the experimental narrative in the paper), 25% (how good/authentic it looks – i.e. does it look like a real paper, as well as writing and grammar all checking out), and 25% (creativity in the repercussions of the science – i.e. some scientific twist that makes the paper all the more interesting).

Note this project should be handed in as a word document, in formatting as described in a handout that will be given in class. Length is flexible, but should have approximately 4 to 5 pages (single spaced) of text per person involved (not including figures and references). Full formatting details are provided in the appendix.

### SECOND PAPER (Due Apr 14th) – 20% of your grade.

You will be required to author **one** paper for the course that will allow you the opportunity to discuss a science topic within a layman setting. In other words, the piece in question should tackle some element of science (technical or creative or opinion piece) that (say) someone in the first year sciences can comprehend.

This, I feel, is an important skill, especially in the context of the sciences, and in molecular biology in particular. It is becoming necessary and a responsible need for scientists to step back and look at their work from a bigger picture perspective, and ideally, I want these papers to give you an opportunity to explore that theme. But, for those who like details – as in "what exactly do you want me to write?" Use the following as a guideline.

## **EVALUATION**

**Topic:** Pretty much completely open as long as there is a link to the sciences. This means that you can choose something you are comfortable with, choose something that you want to learn because you feel it will be beneficial to your career, or choose something because you don't know much about it, but want a reason to learn more (I strongly advise this one, since it often leads to the most rewarding experience).

If it helps, examples of previous pieces have often appeared at *The Science Creative Quarterly* (http://scq.ubc.ca). This is a retired publication, hosted by the lab, that aimed to take science writing of any connotation. This includes shortish science review papers (category "textbook"), written explicitly for ease of reading and comprehension. Do not be afraid to examine fairly advanced topics, but understand that the primary audience is the educated layman (say upper highschool, or 1<sup>st</sup> year undergrad). Also topics that tackle a science theme with a global, societal or ethical angle is encouraged if you prefer to not write on a technical subject. This is also a platform where you can be as creative, journalistic, humourous, educational, progressive as you like, tackling any science topic of your whim. This includes pictorials, video, audio, etc.

**Length of paper**: The paper should hover around 1500 to 2000 words (about 3 - 4 pages single spaced). That's not really very much, but please make sure you realize that this paper is worth 20% of your final grade, whereby approximately half is determined by the amount of content covered, a quarter by how accessible is the writing, and another quarter determined solely by your writing chops. In other words, I'm expecting *high quality* stuff. Get your classmates to review and edit your piece, really make sure it is something you are proud of.

For those that want to tackle a creative piece, I'm expecting a similar word count, meaning that you may have to provide multiple pieces (i.e. 3 x 500 word humour pieces) to fulfill the assignment's requirements. Also, if you want to take a stab at a creative piece, I would first like to check in with you, just to make sure that your idea has merit and requires an equivalent amount of effort (you can email me at <u>db@mail.ubc.ca</u> to set up a time to meet).

**Marking Rubric:** 50% Content (amount of material, research put in, factual mistakes, etc); 25% Writing (grammar, diction, transitions, etc); 25% Accessibility (i.e. too technical for the Grade 12, 1<sup>st</sup> year undergraduate student level).

**Notes on Due Dates:** Assignments can be handed in either as hard copy or electronic copy (pdf) via email (at the above address). Students will lose 10% of their paper grade each day it is late, without a UBC policy condoned excuse.

### SAFETY REGULATIONS:

- 1 No eating, drinking, or smoking in the laboratory.
- 2 All accidents must be reported to the instructor.
- 3 Lab coats must be worn while in the lab. No open-toe sandals and bare feet are allowed.
- 4 EXTREME CAUTION: must be taken when handling the following hazardous chemicals/materials in the lab:
- 5. **Phenol and chloroform (Trizol reagent)**. Leakproof gloves must be worn! Phenol-chloroform extractions wastes must be disposed of in designated containers only.
- 6. Live cultures: Contaminated wastes must be disposed of into biohazard waste containers only. Non-disposable wares used for cultures must be placed in designated trays for autoclaving.
- 7. Sharps (needles, broken glass, Pasteur pipets, etc). Disposal must be into designated containers only. Do not recap needles.
- 5 Make sure all gas burners and hot plates are turned off after use. Do not spray alcohol to disinfect the biosafety hood while the gas burner is on.
- 6 All nondisposable nonbiohazard glasswares must be rinsed at the sink before leaving in soaking trays.
- 7 Take gloves off from hands before touching any fixtures in the lab (i.e. telephones, door knobs, common equipment unless designated otherwise, books, etc) whether or not you think the gloves are clean.
- 8 Wash hands thoroughly before leaving the laboratory.

### UBC COVID-19 POLICY FOR IN-PERSON CLASSES.

Here's what you need to do to help ensure a safe environment for yourself and those around you. Please make sure you adhere to these guidelines:

- Know the symptoms of COVID-19 and complete a daily health assessment. If you are sick, stay at home. Learn about the selfassessment tool at <u>https://bc.thrive.health</u>.
- Make sure you have a non-medical mask, as they are required in public indoor spaces on campus including classrooms.
- Wash your hands regularly.
- If you are not yet vaccinated, make a plan to receive your COVID-19 vaccination. You can book your first and second doses via <u>https://immunizebc.ca</u>. Drop-ins are welcome at all Vancouver Coastal Health clinics. For details visit <u>http://www.vch.ca/covid-19/covid-19-vaccine#vaccination</u>. In the Okanagan, information about immunization clinics can be found at <u>https://news.interiorhealth.ca/news/covid-19-immunization-clinics/</u>.
- As a reminder, in BC, free vaccines are available to students arriving from international destinations and other provinces.

The BC Centre for Disease Control and BC Ministry of Health have issued return to campus public health guidance. You can find details at <a href="http://www.bccdc.ca/Health-Info-Otherwork">http://www.bccdc.ca/Health-Info-Otherwork</a>

Site/Documents/COVID\_public\_guidance/Public\_Health\_Guidance\_Campus.pdf.

LAB A - PCR

## LAB A: POLYMERASE CHAIN REACTION

### A SIMPLE FINGERPRINT ASSAY USING POLYMERASE CHAIN REACTION

We'll start the course with a simplified polymerase chain reaction (PCR) experiment. Specifics of our experiment as well as theoretical information will be provided throughout the course.

Briefly, PCR is a powerful genetic technique that allows researchers to amplify DNA sequences of interest. This amplification allows better and easier genetic characterization and can be used for a variety of tasks including those in the context of genetic disease diagnosis, maternity/paternity tests, DNA forensics, or simply in the production of large amounts of DNA fragments for further study.

As well, PCR is often the first step in a generalized workflow of experiments, where a researcher wishes to obtained a DNA sequence of interest, which is then further characterized by placement in a carefully chosen vector.

\*In our lab A, we'll be using primers designed to seek out a specific Alu insertion that resides in the Chromosome 8, TPA-25 locus. More detail will be shown in class.

#### PROCEDURE:

1. Obtain a 5ml saline solution (saline pod) and squeeze/pour into your mouth. Swish around your cheek area for 30 seconds. Do not discard the empty tube that contained the saline solution.

2. Spit out the sample solution into a disposable paper cup.

3. Pour the sample solution, from the paper cup, back into a 10ml tube and close cap tightly (label tube with your initials).

4. Spin sample at medium speed (1500rpm) for 10 minutes in the swinging bucket BECKMAN centrifuge.

5A. Assuming you have a tight (and not loose) pellet, you can carefully pour off supernatant (liquid on top) into your paper cup. Then, you can discard this fluid down one of the sinks in the back of the room – the empty cup can then go in the biohazard discard. Note that you will now have a tube with only your pellet and probably a little bit of fluid as well (that's o.k.)

5B. If you do have a loose pellet, you will have to transfer it over to a new microcentrifuge tube by using a plastic pipette and sucking up as little sample as





possible whilst trying your best to obtain the majority of your pellet. Then, using your TE buffer (found in either a glass bottle or plastic tube on your bench), you will prepare an appropriate balance tube, and spin your two samples at maximum speed (14K RPM) in the microcentrifuge for 5 minutes. Once finished you will now have a small pellet at the bottom of the small microcentrifuge. Carefully use a p1000 pipetteman to ciphen off supernatent.

6. Using the plastic pipette, transfer as much of your cheek cell pellet as you can to a tube that contains about 500ul of chelex beads. *(if the beads are given in a tube with a sticker on top, make sure you remove the sticker and label your chelex beads tube directly on the plastic lid – the tubes will be boiled for several minutes which can often smear the ink left on stickers)* 

7. Mix cells and Chelex by vortexing for about 3 or so seconds.

8. Add a lid lock to your tube, and place your tube in a boiling water bath for about 8 minutes.

9. Carefully remove your tube from the boiling water bath and place on ice for one minute.

10. After boiling, centrifuge your sample at maximum speed for 3 minutes.

11. Use a p20 and a fresh pipette tip to transfer 10  $\mu$ I of supernatant (this clear solution on the top contains your solubilized DNA) to your PCR reaction tube. Note that the PCR reaction contains a premix with polymerase, primer and nucleotides already present (see table below). You will also prepare a negative control sample, by adding 10ul of nuclease free water to another PCR reaction tube.

```
PREMIX RECIPE (40ul total: you add 10ul of DNA sample)
```

```
4.5ul 10x PCR buffer
1.8ul 25mM MgCl2
3.6ul 2.5mM dNTP mix
1.0ul Alu F primer (@ 25pmol/ul) 5'-GTAAGAGTTCCGTAACAGGACAGCT-3'
1.0ul Alu R primer (@ 25pmol/ul) 5'-CCCCACCCTAGGAGAACTTCTCTTT-3'
0.5ul Taq polymerase (2.5units)
27.6ul ddH20
```

12. Both samples will then be placed in a PCR thermal cycler which has been programmed to cycle 30 times ( $2^{30}$  amplification). The reagents in the PCR tube are there to seek out the area containing the *Alu* insertion of interest in your cheek cell DNA.



#### PCR PROGRAM

STEP	1:	TEMP=	=94.0C	TIME=03:00	
STEP	2:		94.0C	01:00	
STEP	3:		58.0C	00:30	
STEP	4:		72.0C	00:30	
STEP	5:	GOTO	2 REPE	AT 30X	
STEP	6:		72.0C	05:00	
STEP	7		4.0C	HOLD	

13. After the PCR reaction is completed (~2.5 hours), the samples will then have 10ul of DNA LOADING BUFFER added, and then loaded on a pre-prepared 2.0% agarose gel for visualization.

# WEEK 3/4 LAB B - CLONING

### LAB B: CLONING TECHNIQUES

#### CLONING OF GENOMIC DNA RESTRICTION FRAGMENTS: PREPARATION OF FRAGMENTS, DEPHOSPHORYLATION OF VECTOR, AND LIGATION STEPS.

Cloning in molecular biology is a loosely used colloquism implying techniques that generate specific recombinant DNA molecules intended for a variety of uses including things like:

a) To obtain large quantities of specific DNA sequences for use in studying gene structure and gene regulation, DNA/protein sequence determination

b) To produce large quantities of proteins like growth hormones, cell surface receptors, enzymes, etc, for research or commercial use.

c) To modify the host cell's genotype or phenotype.

In Lab B, we will start with Hind III digested lambda genomic DNA (our insert sample); and a Hind III digested pUC18 (our vector sample). With these two samples, we will attempt to ligate our "inserts" into our "cut vector." The presence of successful ligations will be detected by transforming bacteria with our ligation mixture. Essentially only successful ligations (re-circularized plasmid, or re-circularized plasmid plus insert) will allow colonies to grow on media plates upon transformation. From here, we will characterize successful transformants by doing mini plasmid preps on colonies chosen from our plates.

# WEEK 3 LAB B1 – DIGESTS/GELS

### LAB B1: LIGATION AND AGAROSE GEL RUN.

### PROCEDURE – T4 LIGATION (WORKING IN PAIRS)

You will receive three different DNA samples to work with, which include the aforementioned pUC18 and Lambda samples digested with Hind III. Furthermore, the cut pUC18 sample will actually come in two versions – one that is dephosphorylated (CIP's pUC18), and one that is not (nonCIP'd pUC18). Explanation for the difference between these two pUC18 samples will be explained in class.

1. Label five tubes A to E. To each tube, add the various ligation components as follows (numbers are in ul units). **NOTE: make sure all components are mixed at the bottom of the tube or "buzz spinned"** <u>before adding the ligase.</u>

	А	В	С	D	Е
nonCIP'd pUC18	1	1	0	0	0
CIP'd pUC18	0	0	1	1	1
lambda DNA	0	3	1	3	6
5x ligase buffer	2	2	2	2	2
dH2O	6	3	5	3	0
T4 DNA ligase*	1	1	1	1	1

NOTE: ligations C, D, and E represent varying amounts of insert to vector ratios. \* add last.

2. Incubate a minimum of 30 minutes at room temperature.

As we wait for the ligation reaction to finish, we'll pour an agarose gel and will start a gel run that will allow us to visualize what cut and uncut versions of Lambda DNA and pUC18 look like.

3. As described in the demonstration, prepare one 0.8% agarose gel (per 2 pairs), which you will use to run and visualize your digests.

\*While the DNA samples are ligating, prepare the agarose gel and electrophoresis setup (There will be a quick run through of the equipment used). You will pour a 0.8% agarose gel – this is a weight per volume measurement. We'll have pre-weighed 0.8g of agarose in a 250ml Erlenmeyer flask. You will need to add 100mls of 1x TBE buffer and swirl gently to re-suspend the agarose. Microwave the mixture on high power until it boils and the agarose is completely dissolved (see o/h slide for specific microwave times). Look for the occurence of "chunkies" and "wisps" in your mixture. The dissolving step is a fine line between boiling your sample enough to dissolve all of your material, but not boiling it too much so that liquid starts to evaporate and significantly

## WEEK 3 LAB B1 – DIGESTS/GELS

change volume. Allow the solution to cool to 55/60°C by incubating in a 55 or 65°C waterbath for about 10 minutes. Then add the appropriate amount (10ul) of SYBR stain. **WARNING: Do not handle SYBR without wearing gloves and avoid spills**.

\*While the agarose is cooling off, prepare the gel casting setup. (This will be demonstrated)

\*Pour the cooled agarose into the apparatus – don't forget the comb! Watch for leaks! If there is a leak, a quick trick is to put ice in the offending buffer chamber. The gel will take approximately 20 minutes to set. We will be using it before leaving today.

Note that SYBR is especially light sensitive. Therefore, we may be covering the gel with foil containers, etc to protect it from too much light exposure.

4. In your ice bucket, you will have 5 tubes containing 10ul of samples for the gel loading. The samples are as follows, as well as the amount of loading dye buffer (the blue solution) that needs to be added:

pUC18 undigested	10ul +2ul loading dye
pUC18 Hind III digested	10ul +2ul loading dye
Lambda undigested	10ul +2ul loading dye
Lambda Hind III digested	10ul +2ul loading dye
Standard (Lambda HindIII)	10ul loading dye already added.

5. Once your 5 samples have been prepped as appropriate with the loading dye buffer, you can prep the agarose gel for running, and load 10ul of each sample into separate wells in your gel (5 wells in a row). There will be a brief demonstration on how you can best do this.

6. Plug the gel, and run at 100V. Look at the gel every now and then, as we want to stop the gel when the dye has reached the bottom (~1 hour). At that point in time, we will dismantle the gel and take a look.

# WEEK 3 LAB B2/TRANSFORMATION

### LAB B2 – TRANSFORMATION PROCEDURES

### TRANSFORMATION OF COMPETENT CELLS

Here, we will take a look at two methods of introducing DNA into bacterial cells (Heat shock and Electroporation). Both are in routine use with various advantages and disadvantages between them.

\*Heat Shock competent cells are very delicate. Be VERY gentle when handling them, and always ALWAYS keep them on ice unless instructed otherwise.

\*The two transformation procedures have different types of competent cells. It is absolutely crucial that you do NOT mix them up. Heat shock competent cells are labeled "M," and electroporation competent cells are labeled "E."

### PROCEDURE (WORKING IN PAIRS)

1. Prepare 9 fresh microcentrifuge tubes and label them from A(t), B(t), C(t) to I(t) (the "t" is short for transformation) – these will be the tubes where the heat shock transformation reactions will take place. Keep them on ice.

2. Heat Shock competent cells (labeled "M") will be brought out, placed in your ice buckets, and allowed to thaw slowly. When thawed, carefully transfer 20ul of competent cells to each of your 9 transformation tubes. Be *very* gentle – as mentioned above, heat shock competent cells are notoriously delicate.

To tubes A(t) to E(t), add 5ul of the A to E ligation mixes (from step 1, p14); To tube F(t), add 5ul (0.05ng total) of **BRL pUC19 control** DNA; To tube G(t), add 5ul (0.5ng total) of **undigested pUC18 control**; To tube H(t), add 1ul **nonCIP'd pUC18** (same as the nonCIP'd sample used in the ligation reactions earlier) To tube I(t), add 5ul sterile distilled H2O.

3 Leave the cell/DNA mixtures on ice for 30 minutes. Check to see that there is SOC media in the 37°C waterbath (being prewarmed). When first group reaches this stage, there will be a demonstration on how to use the electroporator/set up cuvettes.

The following procedure is as recommended for use with our BIORAD electroporator. Electroporation will be done using only the DNA from our tube D ligation. This way, we can ensure the presence of white colonies in future steps.

# WEEK 3 LAB B2/TRANSFORMATION

- Thaw the "E" cells on ice. Place a microcentrifuge tube and a 0.1 cm cuvette on ice.

- In the cold microfuge tube mix 40 ul of the "E" cell suspension with 2ul of DNA (from ligation mix D). Pipette up and down gently and incubate on ice for 1 minute.

- Set the MicroPulser to "Ecl" (short for e.coli).

- Transfer the mixture of cells and DNA to a cold electroporation cuvette and tap the suspension to the bottom. Place the cuvette in the chamber slide. Push the slide into the chamber until the cuvette is seated between the contacts in the base of the chamber. Pulse once.

Remove the cuvette from the chamber and immediately add 1 ml of prewarmed SOC medium to the cuvette. Quickly but gently resuspend the cells. (Delaying this transfer by 1 minute causes a 3 fold drop in transformation. This decline continues to a 20- fold drop by 10 minutes).
Transfer the cell suspension to a microfuge tube and incubate in a shaking incubator (in prep room) at 37°C for 1 hour.

4. (NOW back to your 9 heat shock tubes) After the ice incubation step, you will need to heat shock your 9 samples for 60 seconds in the 42°C waterbath. **Be careful not go significantly over this 60 seconds, and do not agitate cells!** 

5. <u>Immediately</u> transfer your heat shocked tubes to ice. Then add 480ul of the prewarmed SOC media to each tube. Do not mix further.

6. Incubate in a shaking 37°C incubator for about 1hr (this will roughly coincide with your electroporated samples).

7. At the end of the incubation, you will now have a total of 10 tubes to take care of (nine of which were heat shocked, and one of which was electroporated).

8. For your heat-shocked samples, Vortex each sample, and aliquot 100ul of the contents of each tube onto correspondingly labelled LB agar plates containing 100ug/ml ampicillin and 40ug/ml X-gal. You will spread plate your sample using disposable "hockey sticks" (This will be quickly demonstrated).

9. For your electroporated sample: vortex, and aliquot 5ul onto an appropriately labeled LB plate containing ampicillin and X-gal.

10. Incubate all 10 plates overnight at 37°C in an inverted position (agar side up).

# WEEK 3 LAB B3 – CULTURES LAB B2 – LOOK AT PLATES AND SET UP CULTURES

### PROCEDURE (done out of usual class time)

1. Look at your plates and note the colonies. Data will be discussed in class.

2. Some of these colonies will be picked for plasmid characterization. Note 6 whites (from any plate of your choice) and one blue colony. Use these colonies to each inoculate 3mls of LB broth + 50ug/ml ampicillin with a single colony of transformed bacteria. **NOTE: this is a total of 7 cultures.** You may use the sterile toothpicks offered, by dipping the end of the toothpick into the colony and then throwing the entire toothpick into the broth.

3. Grow the culture overnight on the roller drum inside the 37°C incubator. Remember to label the tubes and balance them properly in the apparatus (*we will remove cultures the following day and store at 4*°C *for the following week*)

# WEEK 4 LAB B4 – MINIPREPS

### LAB B4 – MINI PLASMID PREPARATIONS

### MINI PLASMID PREPARATIONS USING THE QIAprep SPIN MINIPREP KIT

Here, we will isolate plasmids obtained from our colonies to see what plasmid/insert molecules we have. This particular procedure is one of several methodologies to differentiate plasmid DNA from other nucleic acid species.

\*This kit is essentially a revamp version of a common plasmid prep procedure is known as the alkaline lysis procedure. Many kit based assays rely on this particular chemistry which will be discussed in class. \*In our case, this kit also incorporates a silica bead based affinity chromatography methodology.

#### PROCEDURE

0. At some point during this procedure, several of your group members can work together to prepare a 0.8% Agarose gel. (See page 16 for instructions - don't forget the stain)

1. Remove cultures from the 37C incubator and vortex each culture thoroughly. Transfer 1ml of each mixed culture into a clean microcentrifuge tube. The rest of the culture can be left on the bench or discarded.

2. Spin the cells down for 30 seconds at maximum speed in the microcentrifuge (don't forget to prepare a balance). Remove all of the supernatent by using your pipetteman. Do a quick vortex of your dry bacterial pellet (this will loosen it for easier resuspension in the next step)

3. Resuspend the pelleted bacterial cells in 250ul Buffer P1 (make sure this buffer is well mixed before use as some of its components may precipitate and settle to the bottom of the tube). The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.

4. Add 250ul Buffer P2 (make sure this buffer is also well mixed before use) and then invert the tubes 4 to 6 times. Do not vortex, as this will result in shearing of genomic DNA. If necessary, continue inverting the tubes until the solutions become viscous and slightly clear. Do not allow the lysis reaction to proceed for more than 5 min. Mixing should result in a homogeneously blue colored suspension, although the colour change can vary in intensity.

# WEEK 4 LAB B4 – MINIPREPS

5. Add 350ul Buffer N3 and mix immediately and thoroughly by inverting the tubes another 4-6 times. To avoid localized precipitation, mix the solution thoroughly and immediately after addition of Buffer N3. The solutions will become cloudy and all traces of blue in the suspension should disappear.

6. Centrifuge your samples for 10 minutes at maximum speed (14K RPM) in a microcentrifuge. Compact white pellets will form, with clear supernatents on top – **NOTE that you want the supernatent, so do not accidently discard!** 

7. Apply the supernatants from step 6 onto a QIAprep spin columns using your pipetteman. Make sure, you place your spin column inside a flowthrough collector (will be shown in class). Centrifuge at maximum speed for 1 minute. Discard the flow-through liquid (but will keep using the container).

8. Wash QIAprep spin column by adding 0.75 ml Buffer PE and centrifuge for 1 minute with flow through container.

9. Discard the flow-through (but not the container), and centrifuge (w/ empty flowthrough) for additional 1 min to remove residual wash buffer. Residual ethanol from Buffer PE may inhibit subsequent enzymatic reactions.

10. Place the QIA prep columns in clean 1.5 ml microcentrifuge tubes. To elute the DNA off the column, add 50ul Buffer EB to the center of each QIAprep spin column, let stand for 1 min, and centrifuge for 1 min (lids should be all "in" or all "out" – "out" usually ensures no breakage of lids). Your collected eluent is your purified "plasmid prep."

11. You will now set up 7 restriction digests with the following recipe for each digest:

*ul	dH <sub>2</sub> O
2ul	10X FastDigest Buffer
*ul	plasmid prep
1ul	HindIII (10U/ul) Fermentas FastDigest brand

\* We'll be doing nanodrop readings of our plasmid preps and will want to use enough of our prep to have about 50 to 150ng of plasmid per digest (this is ballpark and just to ensure we don't accidently overload, etc). Instructions on using the nanodrop is on the next page.

12. Incubate for 20 minutes in a 37°C waterbath.

**NOTE:** a common procedure for conducting large numbers of restriction digests is the preparation of a "cocktail" mixture. In our case, you would prepare one main solution

# WEEK 4 LAB B4 – MINIPREPS

containing 8 times (since we have 7 samples) the amount of everything except the DNA. (i.e. 120ul of water, 16ul of the 10x Buffer, 8.0ul of HindIII). This way, to your 2.0ul of DNA you can just add 18ul of this "cocktail" mix. If you are performing digests where you have 18 or 36 samples to deal with, this method can save you a lot of pipetting time.

13. After the digest incubation, add 4ul of DNA loading buffer to each tube and load all 7 samples - along with a ambda HindIII marker - to your agarose gel. Run gel at 100V for approximately 1 - 2 hours. Visualize and photograph gel.

#### How to use the Nanodrop Lite:

FIRST: blank the machine with a buffer alone sample. (This may be done for you by Li-Juan)

- i. Select assay type from **Home** screen (dsDNA, ssDNA, RNA, or Protein).
- ii. Establish a blank (2 ul nuclease free water or EB) onto the bottom pedestal, lower the arm and press **Blank.**
- iii. When measurement is complete, raise the arm and wipe the water from both upper and lower pedestals using a dry Kimwipes.
- iv. Redo step 3 to confirm blank measurement.

SECOND: Measuring a Sample (this, you will do). Note: depending on how busy the line up is for the nanodrop, we may take three representative reading for each pair, instead of assessing all 7 samples.

- i. Pipette 2 ul of sample onto the lower pedestal and press **Measure**.
- ii. For dsDNA, on screen, you will get a reading for A260, A260/A280 and DNA concentration.
- iii. Press **Print** to print your data.
- iv. Always wipe the upper and lower pedestals after each measurement.

## LAB NGS: NEXT GEN SEQUENCING

### MICROBIOME 16s SEQUENCING OF SOIL SAMPLE USING NANOPORE NEXT GEN SEQUENCING SET-UP

This procedure will involve the preparation of a soil sample for 16s microbiome sequencing. We'll be using materials and equipment from Oxford Nanopore's MinION system - specifically, usage of the flongle chips and their 16s barcoded sequencing kit. Note that each student will prepare an amplified 16s library and , but we have a maximum of 4 sequencing devices so samples may be doubled or tripled up depending on success rates.

### **STEP 1: Prepare the sample**

- 1. Add 600 µL of S1—Lysis Buffer to the Bead Tube
- 2. Add ~0.2g of soil to the tube and cap securely, then vortex.
- 3. Add 100 µL of S2—Lysis Enhancer, cap securely, and vortex briefly.
- 4. Incubate at 65°C for 10 minutes.
- 5. Homogenize by bead beating for 10 to 15 minutes at maximum speed on the vortex mixer. Use the hands-free adapter and horizontal agitation.
- 6. Centrifuge at 14,000 × g for 5 minutes.
- Transfer up to 400 μL of the supernatant to a clean microcentrifuge tube. IMPORTANT! A layer of debris may be present on top of the bead pellet. Avoid transfer of this debris with the supernatant. *If your sample is especially messy/dark/etc, you can play it safe by doing an additional 1 min spin and removing ~390ul from this prep.*
- Add 250 µL of S3—Cleanup Buffer, and vortex immediately. Vortex immediately to ensure even dispersion of S3 to ensure uniform precipitation of inhibitors
- 9. Incubate on ice for 10 minutes.
- 10. Centrifuge at 14,000 × g for 1 minute.

11. Transfer up to 500  $\mu$ L of the supernatant to a clean microcentrifuge tube, avoiding the pellet.

### STEP 2: Bind DNA sample to the column

- 1. Add 900 µL of S4—Binding Buffer, and vortex briefly.
- 2. Load 700  $\mu$ L of the sample mixture onto a spin column-tube assembly, cap the column, and centrifuge at 14,000 × g for 1 minute.
- 3. Discard the flow-through, and repeat step 2.2 with the remaining sample mixture. Ensure that the entire sample mixture has passed into the collection tube by inspecting the column. If sample remains in the column, re-centrifuge again (with no additional liquid) at 14,000 × g for 30 seconds.

### STEP 3: Wash and elute the DNA

- Place the spin column in a clean collection tube, add 500 μL of S5—Wash Buffer, then centrifuge the spin column-tube assembly at 14,000 × g for 1 minute.
- Discard the flow-through, then re-centrifuge the spin column-tube assembly (with no additional liquid) at 14,000 × g for 30 seconds. This second "empty" centrifugation step optimizes removal of S5—Wash Buffer, which could interfere with downstream applications.
- 3. Place the spin column in a **clean low bind tube**, add 100 μL of S6— Elution Buffer, then incubate at room temperature for 1 minute.
- 4. Centrifuge the spin column-tube assembly at 14,000 × g for 1 minute, then discard the column. The purified DNA is in the tube. This purified genomic can be stored at 4C for a week and -20C for long-term storage.

## STEP 4: Library AMPure preparation (2<sup>nd</sup> purification step – recommended for environmental samples).

1. Transfer 45ul of your bacterial genomic prep to a clean 1.5ml low bind tube (this is your sample tube). Note that you should have ~55ul of sample left over – you can put this in the 4C fridge.

- 2. Resuspend the AMPure XP beads by vortexing until resuspended.
- 3. Add 30ul resuspended AMPure beads to your sample tube and mix gently by pipetting up and down a few times.
- 4. Incubate on a hula mixer for 5 minutes at RT. During this time, prepare 500ul of a 70% ethanol wash solution (350ul of 100% ethanol plus 150ul nuclease-free water).
- 5. Remove sample from hula mixer, and pulse spin down sample (bubble centrifuge to pull liquid down) and place tube on a magnetic rack. This will pellet the AMPure beads to the side of the tube (~2min). Keep tube in magnet and carefully pipette off the supernatant.
- 6. With the tube still in the magnet, add 200ul 70% ethanol without disturbing pellet. Carefully remove the 70% ethanol without disturbing the pellet. This is a wash step.
- 7. Repeat wash and removal steps once more.
- 8. Try to pipette off residual ethanol using a p2 or p10 pipettemen, and then (with the and beads still in the magnetic rack) air dry for at least 30 secs (but not too long!)
- Remove the tube from magnet and add 10ul 10mM Tris-HCl pH8.0
   50mMNaCl. Mix by pipetting up and down a few times. This is the elution buffer. Incubate beads in this buffer for 2 minutes at RT on the hula mixer.
- 10. Place the tube back back in the magnetic rack to pellet the beads (~2min).
- 11. Carefully remove 10ul of elute into a new low bind tube. This is our PCR ready prep!

#### STEP 5: Quality control via qubit reading.

Sample Quality Control Check: Prep a 2.0ul sample for Qubit check. Record yield values.



### STEP 6: Set up 16s PCR run (for SQK-RAB204 kit)

We want to be able to have about **50ng** of cleaned genomic DNA for the PCR. We've also noted that having a genomic yield of >100ng/ul is useful to ensure minimal transfer of original sample liquid (and potential ride-along inhibitors). Note that the original procedure suggested a 10ng starting amount, so there may be value in starting with this lower amount (and bumping up cycle numbers) to ensure lower original liquid volume.

1. In a 0.2ml PCR tube, mix the following:

х	ul	genomic DNA
У	ul	nuclease free water (note that $x + y = 24ul$ )
1	ul	MinION barcoded primer set (01 to 12)
25	ul	LongAmp Taq (2x master mix)*

2. Mix gently by flicking the tube and spin down using the bubble centrifuge.

3. Set the thermal cycler to the following program:

STEP	1	1 min	95C
STEP	2	20sec	95C
STEP	3	30sec	55C
STEP	4	2 min	65C
STEP	5	GOTO STEP 2	(REPEAT 34x)**
STEP	6	5 min	65C
STEP	7	HOLD	4C

\* With problematic samples (i.e. soil), literature has suggested using the Q5 high fidelity polymerase which works with an elongation temperature of 72C and is apparently better suited for high GC sequences.

\*\* Use 40 cycles if yield appears negligable.

4. After the PCR is finished, the samples will be stored at -20C to be used in next week's class.

### STEP 7: Agarose Gel check of 16s Bands

- 1. Weigh out 1.0g of agarose and place in a 250ml Erlenmeyer flask. To this, add 100mls of 1x TBE buffer and swirl gently to re-suspend the agarose.
- Microwave the mixture on high power until it boils (~60 seconds). Remove from microwave, swirl and place back in. Microwave again until solution starts boiling, stop, and remove flask. Swirl once more.
- 3. Check to see that the agarose has completely dissolved. Look for the occurance of "chunkies" and "wisps" in your mixture. The dissolving step is a fine line between boiling your sample enough to dissolve all of your material, but not boiling it too much so that liquid starts to evaporate and significantly change volume.
- 4. Allow the solution to cool to about 55/65°C by incubating in a 55 or 65°C waterbath for ~10 minutes; or leaving it on the bench for ~7 minutes.
- 5. Add 10ul of SYBR SAFE stain to your gel solution, and gently swirl to mix. Note: Do not handle SYBR without wearing gloves.
- 6. While the agarose is cooling off, prepare the gel casting setup.
- 7. Pour the cooled agarose into the apparatus. Don't forget the comb and also watch out for leaks. *If there is a leak, a quick trick is to put ice in the offending buffer chamber.*
- 8. The gel will take approximately 20 minutes to set. Since the SYBR stain is light sensitive, it's a good idea to cover the gel to shield from light (we use these foil baking sheets)
- You will be loading some of your PCR sample (5ul PCR sample, 1ul DNA loading buffer) – load all 6.0ul into gel and run at 80 to 100V. The gel should take approximately 45 minutes to run.

**STEP 8: Library AMPure preparation** (*if you're pretty confident that things will be ok, you can do this as gel is running*)

- 1. Transfer PCR sample to a clean 1.5ml low bind tube (this is your sample tube). Note that you should have ~45ul of sample left over.
- 2. Resuspend the AMPure XP beads by vortexing until resuspended.
- 3. Add 30ul resuspended AMPure beads to your sample tube and mix gently by pipetting up and down a few times.
- 4. Incubate on a hula mixer for 5 minutes at RT. During this time, prepare 500ul of a 70% ethanol wash solution (350ul of 100% ethanol plus 150ul nuclease-free water).
- 5. Remove sample from hula mizer, and pulse spin down sample (bubble centrifuge to pull liquid down) and place tube on a magnetic rack. This will pellet the AMPure beads to the side of the tube. Keep tube in magnet and carefully pipette off the supernatant.
- 6. With the tube still in the magnet, add 200ul 70% ethanol without disturbing pellet. Carefully remove the 70% ethanol without disturbing the pellet. This is a wash step.
- 7. Repeat wash and removal steps once more.
- Try to pipette off residual ethanol using a p2 or p10 pipettemen, and then (with the and beads still in the magnetic rack) air dry for ~30 secs (do not overdry!)
- Remove the tube from magnet and add 10ul 10mM Tris-HCl pH8.0 50mMNaCl. This is the elution buffer. Incubate beads in this buffer for 2 minutes at RT
- 10. Place the tube back back in the magnetic rack to pellet the beads.
- 11. Carefully remove 10ul of elute into a new low bind tube. This is our sequencing ready prep!

Sample Quality Control Check: Prep a 1.0ul sample for Qubit check (see page 28). Record yield values

### STEP 9: MinION Sequencing.

We are using a flongle setup. This is a smaller flow cell, with possible yields of up to 3Gbp (note that we have a number of discounted older versions of the flongle cells, which tend to get <1Gb).

We wish to make a 5.0ul sample prep. In this, we ideally want about ~25 to 50ng of each barcoded library prep, and also ideally want similar amounts for each barcoded library.

- 1. Add 0.5 μl of RAP to the tube. Mix gently by flicking the tube, and then spin down.
- 2. Incubate the reaction for 5 minutes at room temperature. This is the prepared DNA library (used for loading into the flow cell). You may store this library on ice until you are ready to load.
- 3. Premix the Sequencing Buffer (SQB), Flush Buffer (FB or FLB), and Flush Tether (FLT) tubes by vortexing, spinning down, and then returning to ice.
- 4. Place the Flongle adaptor into the MinION device. Place the flow cell into the Flongle adaptor, and press the flow cell down until you hear a click.
- 5. Now you are ready to flush the flow cell. Note that there is a video of this setup halfway down the page at <u>https://tinyurl.com/flongle</u> (you may need to create a free Oxford nanopore account to see this), but a text description continues below.
- 6. In a fresh 1.5 ml Eppendorf DNA LoBind tube, mix 117  $\mu$ l of FB with 3  $\mu$ l of FLT and mix by pipetting.
- 7. Peel back the seal tab from the Flongle flow cell, up to a point where the sample port is exposed. (1) Lift up the seal tab. (2) Pull the seal tab to open access to the sample port. (3) Hold the seal tab open by using adhesive on the tab to stick to the MinION lid.



8. To prime your flow cell with the mix of FB and FLT that was prepared earlier, ensure that there is no air gap in the sample port or the pipette tip. Place the P200 pipette tip inside the sample port and slowly dispense the priming fluid into the Flongle flow cell. To avoid flushing the flow cell too vigorously, make sure you go slow and steady. You can also load the priming mix by twisting the pipette plunger clockwise (this slowly brings the plunger down). This is a little tricky!



9. Vortex the vial of Loading Beads (LB). Please note that the beads settle quickly, so immediately prepare the library in a fresh 1.5 ml DNA-LoBind Eppendorf for loading the Flongle, as follows:

Sequencing Buffer (SQB)	13.5ul
Loading Beads (LB) mixed immediately before use	11.0ul
DNA library	5.5ul
TOTAL	30.0ul

10. Just like the previous flush step (see figure), to add the 30ul Sequencing Mix to the flow cell, ensure that there is no air gap in the sample port or the pipette tip. Place the P200 tip inside the sample port and slowly dispense the Sequencing Mix into the flow cell by going slow and steady or by twisting the pipette plunger clockwise.

11. Now Seal the Flongle flow cell using the adhesive on the seal tab. (1) Stick the transparent adhesive tape to the sample port. (2) Replace the top (Wheel icon section) of the seal tab to its original position.



12. Replace the sequencing platform lid.

### STEP 8: Run MinKNOW and EPI2ME software.

- 1. The sequencing device control, data acquisition and real-time basecalling are carried out by the MinKNOW software. It is assumed you have already installed MinKNOW on your computer, or that you are using the MinION Mk1C device for data acquisition and basecalling.
- 2. Setting parameters on the MinKNOW run:

Sample/Run name: whatever you type, this will create a folder of the same name where the data will be collected. Kit: SQK-RAB204 kit Barcode: On Run Time: 16hrs (recommended flongle cell run time) Base Calling: real time (high accuracy) Data: FastQ (Fast5 is optional but contains raw/meta data).

- 3. The data analysis is done via EPI2ME software. You'll need an account for this (ask Dave to log in). We will be using the FASTQ 16S workflow. For this to work, you'll need to direct the analysis to the folder where data is collected (folder is in MinION data folder, under a unique folder named after the "run name".
- 4. Set parameters (if not already to the following):

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Note that the nanopore sequencing run will take 16 hours, but base calling tends to take an additional 24 hours. Results will be discussed at a later class.

# WEEK 7 LAB C1 – RNA PREP

### LAB C – RNA WORK.

In this procedure, you will obtain a purified total RNA prep (from your cheek cells) using the commercially available Trizol reagent. We will then reverse transcribed an actin mRNA for PCR quantitation using BioRad's chemistry and MyiQ real time thermal cycler (RT-PCR/qPCR).

\*As will be highlighted in class, RNA is extremely sensitive to degradation. Make a special effort to be as aseptic as possible. This will involve a number of different measures including: wearing gloves all the time and change them regularly; using special nuclease free solutions; using appropriate nuclease free plastic wear (tubes and filtered tips)

\* Note that the TRIZOL procedure is derived from phenol/chloroform type methodologies (another very common nucleic acid purification technique).

## LAB C1 – TOTAL RNA PREP

### PROCEDURE:

1. You will perform a cheek cell rinse using the saline solution, cup, and 1500rpm centrifugation spin (the same as the one done on the first PCR class, p12).

2. Carefully decant the supernatent without disturbing the pellet. You want the pellet and you want a tight pellet – our aim is to have at least one tight pellet per pair. After the supernatent has been removed, flick the bottom of the tube gently to loosen up the pellet – this will help in the resuspension of the pellet in the next step.

3. Add 1ml of Trizol reagent to your pellet and mix by carefully pipetting up and down for a minimum of 20 times with your p1000 – do this slowly to avoid uptake of TRIZOL into the barrel of the pipetteman. **NOTE: Trizol contains phenol so take care!** Incubate at room temperature for 5 minutes. Transfer your sample to a microcentrifuge tube, and then add 200ul of chloroform. Vortex for 15seconds and incubate again at room temperature for about 3 minutes. NOTE that your pellet may not "dissolve" into TRIZOL – we are just hoping for resuspension.

4. Centrifuge at 12000rpm at 4°C for 15 minutes. NOTE: after the centrifugation step, you will notice two phases of liquid. The upper phase is aqueous and contains your RNA prep. The lower phase is the organic phase – you don't want this.

# WEEK 7 LAB C1+C2 – RT PCR

5. Transfer the upper phase to a fresh microcentrifuge tube. Take care not to disturb the interface when retrieving the aqueous phase. Note that it is at this step and thereafter that special care regarding RNAse free methodologies is utilized (including handling, and use of RNAse free materials, as discussed and demonstrated in class).

6. Precipitate the RNA by adding 500ul of ice cold isopropanol (isopropyl alcohol); mixing by inversion; and incubation on ice for 3 minutes.

7. Centrifuge at 12000rpm for 10 minutes at 4°C. Carefully discard the supernatent. Wash the pellet with 1ml of DEPC treated (RNAse free) 75% ethanol and spin again at 12000rpm for 5 minutes. NOTE that it is common to not be able to see the pellet at this step. Regardless, make a mental note of where the pellet "would" be, by remembering how you load your tube in the centrifuges.

8. Discard the supernatent and air dry the pellet for approximately 10 minutes (this step is actually to evaporate excess ethanol). Dissolve the pellet in 100ul of DEPC treated (RNAse/nuclease free) distilled water.

### LAB C2 – REVERSE TRANSCRIPTASE AND RT-PCR

9. Using BioRad's iScript cDNA synthesis kit, each group will set up a reverse transcriptase reaction using the following table. Note that the iScript reaction mix contains oligo dT primers.

TABLE:	
iScript Reaction/Reverse Transcriptase 1	Mix 10ul
RNA template (from step 8)	10ul

10. Each sample will then be loaded into the MyiQ thermal cycler, where the assay will be performed under the following parameters:

5 minutes at 25C
30 minutes at 42C
5 minutes at 85C
(hold at 4C)

11. Once the reverse transcriptase portion is finished, we can then set up our real time PCR reactions. Roughly, we will use the below reaction set up but also utilizing a two fold serial dilution - specific details will be announced in class.

## LAB C2 – RT PCR

**Note:** There will be a demo on setting up the software on the MyiQ system, as well as an opportunity to analyze our data at a later date.

Component	Volume per reaction
iQ SYBR Green Supermix +	
Primer Mix (100 nM-500 nM)	13.0 ul
Reverse Transcriptase sample	10.0 µl
Total Volume	23.0 µl

NOTE: we are using a final concentration for each primer of 250 nM. The recommended amount of primer based on the MyiQ Supermix is 100 - 500 nM of each primer.

NOTE: We have chosen a beta-actin primer set, that should amplify out a 61bp fragment. We have also selected a primer set that is prone to amplifying out a larger genomic contaminant sequence. Sequence of primers are as follows: F-Primer: 5'- cccagcacaatgaagatcaa -3' R-Primer: 5'- cgatccacacggagtacttg -3'

12. After the reverse transcriptase assay is finished (you now have a cDNA sample), you will then set up a strip tube of 8 samples, running a two fold serial dilution of your newly made cDNA sample (this will be explained more fully in class).

13. Once, set up, load your strip tube samples into the MyiQ cycler programmed in the following manner:

STEP	1	95C	3 minutes	"hot start load"
STEP	2	95C	20 seconds	Denaturation
STEP	3	55C	30 seconds	Annealing
STEP	4	72C	20 seconds	Elongation
STEP	5	repeat step	os 2 - 4 (x	50)
STEP	6	72C	1 minutes	Final Elongation
STEP	7	50C	30 seconds	
		MELT FUNCTI	$10N + 1^{\circ}C/30$	seconds for 45°C

WEEK 8 LAB D – PAGE/BLOT

### LAB D – SDS PAGE AND WESTERN BLOT

## DETECTION OF PROTEIN EXPRESSION FROM CLONED GENES BY WESTERN BLOTTING

Proteins will be fractionated by electrophoresis in denaturing discontinuous polyacrylamide gels (Laemmli, 1970, Nature. 227: 680-685), blotted onto a membrane by an electrotransfer procedure, and probed with a specific antibody to the protein of interest. The detection procedure in this case is a two-step process using a primary antibody which is unlabeled and specific to the protein of interest, and a labeled secondary antibody which binds to the constant regions of the primary antibody. The secondary antibody that will be used for this exercise is enzyme-conjugated and will be detected by incubation with a chromogenic substrate.

\*Essentially, your bench will be running two polyacrylamide gels (each pair will be responsible for one gel) with the intent of performing a western blot on one of them. The samples that you will be running are four different bacterial cultures, three of which contain a variant of the pGEX-2T vector. The vector is designed for the expression of recombinant fusion proteins. Here, one is able to express a protein of interest that includes an additional protein domain at its N-terminal side. In this case, the protein domain is called glutathione-S-transferase (or GST for short). The purpose of this additional domain is to allow your fusion protein to be purified easily by affinity chromatography. Presently, the GST system is one of the most popular fusion systems.

\*The three bacterial cultures that you will be working with are as follows: i. pGEX-2T LCK. This will express a GST / wildtype LCK fusion protein (~85kDa) ii. pGEX-2T N32. This will express a GST-LCK construct that contains amino acids –8 to 234 of p56lck (actually contains the N-terminal, the SH3 and SH2 domain of lck). p56lck is a tyrosine kinase by the way. iii. A negative control (no pGEX vector, labeled NEG)

\*You will be using a relatively nasty neurotoxin, acrylamide. Please be careful and make special care to wear safety goggles, gloves and your labcoat.

## WEEK 8 LAB D1 – POUR SDS PAGE

## LAB D1 – POURING SDS PAGE GELS

### PROCEDURE:

#### Gel Part:

1. You will first pour a polyacrylamide gel using the BioRad Mini Protean system. There will be a quick demo on how to set up the apparatus.

2. When you have set up the apparatus and are ready to pour the resolving gel section, you will need to prepare a 10ml solution of the resolving gel using the following recipe (good for one 12% gel):

5.0mls resolving buffer
4.0mls 30% acrylamide/0.8% bis-acrylamide
1.0ml water
100ul 10% ammonium persulfate
6.5ul TEMED

## Don't forget to add the TEMED and ammonium persulfate last, and just prior to gel pouring.

3. The resolving gel will be poured approximately 1cm below the well line (*this will make more sense after viewing the demonstration*). Immediately after this step, you will need to pour an overlay using the water saturated butanol.

4. After approximately 10minutes, the resolving gel should be polymerized enough to allow you to continue pouring the stacking gel. Prepare a 5ml solution of the stacking gel using the following recipe (good for one gel):

4.5ml stack buffer
0.5ml 30% acrylamide/0.8% bis-acyrlamide
25ul 10% ammonium persulfate
5.0ul TEMED

#### Again, don't forget to add the TEMED and ammonium persulfate last!

5. Pour the stacking gel to the brim of the gel cassette and carefully place the comb into the cassette. Do not worry about the slight overflow of acrylamide. The stack will need about 1hr to polymerize fully.

# WEEK 8 LAB D2 – RUN SDS PAGE

### LAB D2 – RUNNING AND TRANSFER OF PROTEINS

### PROCEDURE:

1. Check to see your gels have polymerized. We will be running bacterial samples in them today. Use the below instructions for sample preparation.

a) You will have three microcentrifuge tubes labeled "NEG", "N32" and "LCK." In each tube is 50ul of the aforementioned bacterial culture.
b) To each tube, add 25ul of a 3x sample buffer (blue stuff) to each tube. Now, take your four samples and your prestain standards ("STD" tube, already coloured), and boil them for a minimum of 8 minutes.

2. Whilst your samples are boiling, prepare the gel running set up (this will be demonstrated), such that (with the electrophoresis buffer) the upper buffer chamber is full, and the lower buffer chamber is filled up at least an inch over the bottom of the gel.

3. You are now ready to load your samples. You can load using your p20 pipetteman and yellow tips (you can also use the thin drawn-out tips provided). In general, place the tip directly into the well and slowly push the liquid out, taking care not to introduce bubbles. You can even use the same tip throughout the loading procedure if you rinse the tip out in the upper buffer chamber between samples. Essentially the following lane order is a guideline and applies to both gels (remember that <u>per bench</u> one will be stained for total protein, and the other will be used for western analysis).

		(both gels	good)*	(only one of	gel good)*
lane	1	standard	(10ul)	standard	(10ul)
	2	NEG	(10ul)	NEG	(20ul)
	3	NEG	(20ul)	GST	(20ul)
	4	GST	(10ul)	N32	(20ul)
	5	GST	(20ul)	LCK	(20ul)
	6	N32	(10ul)	standard	(10ul)
	7	N32	(20ul)	NEG	(20ul)
	8	LCK	(10ul)	GST	(20ul)
	9	LCK	(20ul)	N32	(20ul)
	10	blank		LCK	(20ul)

\*lanes are dependant on the number of good gels produced per pair of students. If both gels are in good order, then use the first column for each gel. If only one gel is good, then use the second column, and the gel will be cut into two after the run.

## WEEK 8 LAB D2 – STAIN/TRANSFER

4. Put the lid on the gel apparatus (stay colour coordinated) and set voltage to 100V. The gel will probably take about 1 1/2 hours to run. You want to stop it when the dye has reached (but not past) the bottom of the gel.

5. Whilst the gel is running, prepare your transfer buffer by simply taking the supplied "transfer buffer" and adding methanol until it is  $\sim 20\% - 25\%$  methanol (this may be already done for you). About 10 minutes before the gel is ready, you will also need to prepare your PVDF membrane (immobulon P) by prewetting in 100% methanol for a few seconds in a small plastic container. Dump out the methanol (down the sink), and add a small volume of transfer buffer (+20% methanol) to cover the membrane. Let the membrane soak until transfer procedure is ready.

At this point, one of your gels will be used to begin the transfer procedure, while the other will be stained in the following manner.

- carefully place the acrylamide gel into a plastic container filled with dH2O. Let the gel incubate, rocking for 5 minutes. Remove the water, and repeat two more times. After the last water discard, use a paper towel to dab away excess water from the container (do not touch the gel as it may rip!). Add the **BioSafe Coomassie Blue stain** to immerse the gel and leave to stain on the rocker overnight.

### TRANSFER PART

6. When you set up your transfer you need to think a little. Basically, you want the proteins in your gel to migrate onto the PVDF membrane. Sounds simple, but inevitably you may one day accidently mix things up, and your proteins will run away into the buffer.

7. Remember, your proteins are coated with SDS so they are essentially negatively charged. Therefore, they will move towards the positive electrode, away from the negative electrode. *We will be using Biorad's Midi Transfer Pack* (Single Midi Gel or Two Mini Gels) Setup. There will be a DEMO on how to do this, as the system has been designed so that you should not make placement mistakes.

8. Below is the text from the Biorad instructional booklet (which for the record is very confusing to read!)

"The packaging for the midi transfer pack has a tray containing the two ion reservoir stacks. These are placed on top of each other in the tray.

## WEEK 8 LAB D2 – STAIN/TRANSFER

The ion reservoir stack on the top has a tab on the right side and is for the anode (+'ve charge). This stack is layered with the transfer membrane. The ion reservoir stack for the cathode (-'ve charge) is located below the membrane/anode stack and has a tab on the left side (Figure 1). Use the finger wells and tabs to access the appropriate stack and lift it from the tray.

The text above the finger wells signifies the stack location in the cassette. The top stack and membrane, with the tab on the right, is labeled Bottom (+) and is placed on the anode in the cassette base. The stack below the anode stack, with the tab on the left, is labeled Top (-) and is placed on top of the transfer gel, closest to the cathode.

The transfer membranes and the stacks of the transfer packs are prewetted with transfer buffer and do not require any further pretreatment. Be quick to avoid drying the membrane."



Fig. 1. The anode stack with the membrane is on the top while the cathode stack lies beneath the anode stack and membrane.

9. Once gels are loaded for transfer, we will run the machine according to the prescribed program for PDVF. This will take about 7 minutes, and we are able to transfer 4 gels each time. During transfer time, it is appreciated if you wash the plates and gel apparatus with tap water and then rinse with distilled water.

10. When 7 minutes has passed, turn off the power supply, and carefully remove the membrane with a pair of tweezers. Place membrane, protein side up on a clean paper towel to dry for a minimum of 1 hour (we will store until next class for *lab D3*, *p39*).

## WEEK 9 LAB E1 STRIP HYDRATION

## LAB E - 2D POLYACRYLAMIDE GEL ELECTROPHORESIS

In this lab, we will be performing a 2-D PAGE analysis using BioRad's ReadyPrep kit. The ReadyPrep 2-D starter kit was designed as a single-use kit to familiarize first-time users with the utilization of the Bio-Rad PROTEAN IEF cell and ReadyStrip IPG strips, a very common smaller scale 2D system that provides steps to enhance reproducibility, one of the most important aspects of 2 dimensional data. We will be generating a gel that looks at *e.coli* lysates.

\*Essentially, each bench of four will be responsible for running 2 IPG strips, one for use to separate in the second dimension, and the other to stain immediately after the isoelectric focusing step. Due to time considerations, and various steps involved, this procedure will span over three days.

## LAB E1 - "IPG STRIP REHYDRATION"

### PROCEDURE (done outside of usual class time)

1. You will pipette 125ul of the reconstituted *E. coli* protein sample as a line along the back edge of channel #1. This is equivalent to 169ug of total protein loaded. The line of sample should extend along the whole length of the channel except for about 1 cm at each end. Take care not to introduce any bubbles which may interfere with the even distribution of sample in the strip (See Figure 1. Sample loading of rehydration/equilibration trays).



**Fig. 1.** Sample loading of rehydration/equilibration trays. Pipet the sample along the back edge of the tray channel except for about 1 cm at each end. Note the even distribution of the sample along the edge of the channel. The figure shows the last of six samples being pipeted in the tray. You will be loading two samples.

# WEEK 9 LAB E1 STRIP HYDRATION

2. Repeat this process for another sample by pipetting the same volume of sample into a channel at the opposite end of the tray. During the IEF step when strips are placed on both sides of the focusing tray, the weight of the tray lid is evenly distributed over all the samples so contact between the IPGs and the electrode remains uniform.

3. When all the protein samples have been loaded into the rehydration/equilibration tray as pictured in Figure 1, using forceps, peel the coversheet from one of the pH 4-7 ReadyStrip IPG strips (this is tricky and will be demonstrated).

4. Gently place the strip gel side down onto the sample as illustrated in the figure below. The "+" and "pH 4-7" should be legible (not in mirror image) and positioned at the left side of the tray. Take care not to get the sample onto the plastic backing of the strips as this portion of the sample will not be absorbed by the gel material. Also take care not to trap air bubbles beneath the strip. If this happens, carefully use the forceps to lift the strip up and down from one end until the air bubbles move to the end and out from under the strip.



5. Overlay each of the strips with 2 to 3 ml of mineral oil to prevent evaporation during the rehydration process. Add the mineral oil slowly, by carefully dripping the oil onto the plastic backing of the strips while moving the pipet along the length of the strip.

6. Cover the rehydration/equilibration tray with the plastic lid provided and leave the tray sitting on a level bench overnight (11-16 hr) to rehydrate the IPG strips and load the protein sample.



LAB E2 - IEF

## LAB E2 - ISOELECTRIC FOCUSING

### PROCEDURE

1. Place a clean, dry PROTEAN IEF focusing tray the same size as the rehydrating IPG strips onto the lab bench.

2. Using forceps, place a paper wick at both ends of the channels covering the wire electrodes. Use channels with the same numbers as those used during rehydration. As shown in Figure, channel numbers 1 and 12 contain electrode wicks.

3 . Pipette 8.0  $\mu$ I of nanopure water (provided) onto each wick to wet them. Readjust their position if necessary. **Electrode wicks are important as** receptacles for salts and other non-amphoteric constituents of the sample, and they improve the quality of the results.



4. Remove the cover from the rehydration/equilibration tray containing the IPG strips. Using forceps, carefully hold the strip vertically for about 7 to 8 seconds to allow the mineral oil to drain (See figure), then transfer the IPG strip to the



## LAB E2 – IEF

corresponding channel in the focusing tray (maintain the gel side down) (See Figure Below). Remember to observe the correct polarity during the transfer of strips into the focusing tray. The convenient "+" and "pH4-7" printed on the strips (acidic end) should be positioned at the end of the tray marked with the "+" Repeat for the other strip.



Draining the oil

Placing ReadyStrip Gel down

5. Cover each IPG strip with 2 to 3 ml of fresh mineral oil. Check for, and if applicable remove, any trapped air bubbles beneath the strips. Place the lid onto the tray.

6. Place the focusing tray into the PROTEAN IEF cell and close the cover.

7. Program the PROTEAN IEF cell using the appropriate 3-step protocol in Table below . For all strip lengths, use the default cell temperature of 20°C, with a maximum current of 50  $\mu$ A/strip and No Rehydration.

8. Press START to initiate the electrophoresis run.

		Voltage	Time	Volt-Hours	Ramp
Step	1	250	20 min		Linear
Step	2	4,000	2 hr		Linear
Step	3	4,000		10,000	V-hr Rapid
Total			5 hr	14,000 V-hr	

9. This run will take approximately 5 - 6 hours. We will shut down the apparatus, remove the strip cassette, and place in a -70°C freezer until the following week.

# WEEK 9 LAB D3 – WESTERN BLOT

## LAB D3 – WESTERN BLOT ANALYSIS

### PROCEDURE

### (Quick Method devised by Millipore for use with Immobulon P membrane).

1. After drying your membrane, add your primary antibody solution (10mls of anti-SH3 domain rabbit antisera at 1/2500 dilution in TBS+Tween20 and 5% BSA – *Note that we may be using a different antibody: if so, we will announce details in class*) to your membrane in a clean plastic container. Mix solution around so that the membrane is completely immersed. Place on shaker for about 1 hour. You may find that your membrane looks like its half wet and half dry – this is normal so don't fret.

2. Pour back the antibody solution into the 15ml FALCON tube. This antibody solution can probably be used 3 more times. Wash your membrane by addition of TBS+Tween to just cover the membrane. Shake by hand for about 10 seconds and dump the solution into the sink. Do this a total of 3 times.

3. Add your secondary antibody solution to your membrane (10ml Goat antirabbit IgG heavy and light chains w/ alkaline phosphatase conjugate @ 1/5000 dilution in TBS+Tween20 + 5% BSA). Incubate on shaker for about 30minutes.

4. Pour back secondary antibody solution back into 15ml tube. Wash membrane as above for at least 8 washes. Wash once in buffer that does not have Tween20 detergent (we will use TBS). Your membrane is now ready for substrate detection protocol which will be outlined in class (we will be using the ASBI-Fast Red system).

# WEEK 10 LAB E3 – IPG STAIN + $2^{D}$

### LAB E3 – IPG STAINING & 2ND DIMENSION

### **IPG STAINING**

1. (From last week, LAB E2, p38). Transfer one of the IPG strips to a clean, dry piece of blotting filter paper with the gel side facing up.

2. Thoroughly wet a second filter paper of the same size with nanopure water. Carefully lay the wet filter paper onto the IPG strip. Press firmly over the entire length of the strips. Do NOT squish the gels. When finished, carefully "peel" back the top filter paper. This blotting step removes mineral oil on the surface of the IPG, thereby reducing background staining and generally improving the staining of the IPG strips.

3. Transfer the IPG strip to a staining tray containing approximately 50 ml of Bio-Safe Coomassie stain or Bio-Rad's IEF stain.

4. Place the tray onto a rocking platform or orbital shaker for >1 hour.

### EQUILIBRATIONS OF IPG STRIP

5. (From last week, LAB E2). Transfer one of the IPG strips to a clean channel of a loading tray.

6. Add 2.5ml of **Equilibration Buffer I** to the channel containing your IPG strip. NOTE that this buffer will be placed on the center cart, and will be shared by the class.

7. Place the tray on an orbital shaker and gently shake for 10 minutes. Select a slow shaker speed to prevent the buffer from sloshing out of the tray.

8. During the incubation in Equilibration Buffer I, Li-Juan will complete the preparation of **Equilibration Buffer II**. Here, she will add the contents of one bottle of iodoacetamide to each bottle of equilibration buffer II, and stir until the iodoacetamide is fully dissolved.

9. At the end of the 10 minute incubation, discard the used equilibration buffer I by carefully decanting the liquid from the tray. Decanting is best carried out by positioning the tray vertically, so that the liquid pours out. Take care not to pour out the liquid too quickly at first, as the strips may slide out of the tray. When most of the liquid has been decanted, "flick" the tray a couple times to remove the last few drops of equilibration buffer I.

# WEEK 10 LAB E3 – 2<sup>ND</sup> DIMENSION

10. Add 2.5ml of complete **Equilibration Buffer II** (containing iodoacetamide) to each strip.

11. Return the tray to the orbital shaker for 10 minutes.

12. During the incubation, we will melt the overlay agarose solution in a microwave oven using the following method.

a. Loosen the cap of the bottle of overlay agarose and place the bottle in the center of a microwave oven.

b Microwave on high 45 – 60 seconds until the agarose liquifies. Times will vary depending upon the power of the microwave. It is best to stop the microwave after 30 seconds and swirl the bottle to mix the solution. Then, return the bottle and microwave for 15 additional seconds, swirl the liquid again and repeat this again if needed. CAUTION should be taken as the overlay agarose contains SDS, which when heated can cause bubbling over of the agarose solution. Set the overlay agarose solution aside. If desired a stirbar can be added and the bottle set to stir slowly.

13. Discard the equilibration buffer II by decanting at the end of the incubation period as described in step 9 above.

### PREPARING THE PRE-CAST SDS-PAGE

14. Remove from the refrigerator the same number of 8-16% precast polyacrylamide gels as the number of IPG strips to be run in the second dimension.

15. Open the packaging for each gel and remove the gels.

16. Remove the IPG comb from each gel and rinse the well briefly with nanopure water using a water bottle. Place the rinsed gels on bench.

17. Fill a 100 ml graduated cylinder or a tube that is the same length or longer than the IPG strip length with 1X Tris-glycine-SDS running buffer. Use a Pasteur pipette to remove any bubbles on the surface of the buffer.

18. Finish preparing the SDS-PAGE gels by blotting away any excess water remaining inside the IPG well using Whatman 3MM or similar blotting paper. Lay the gels onto the bench with the top of the gel facing you and the back (tall) plate on the bottom; see Figure below.

## WEEK 10 LAB E3 – 2<sup>ND</sup> DIMENSION



19. Remove an IPG strip from the disposable rehydration/equilibration tray and dip briefly into the graduated cylinder containing the 1X Tris/glycine/SDS running buffer, as shown in Figure 10. Lay the strip gel side up and onto the back plate of the SDS-PAGE gel above the IPG well (see Figure below).



20. Take the first SDS-PAGE gel with the IPG strip resting on the back plate and hold or stand vertically with the short plate facing towards you as in figure below. Use a pasteur pipette or and pipet overlay agarose solution into the IPG well of the gel.

## WEEK 10 LAB E3 – 2<sup>ND</sup> DIMENSION



21. Using the forceps, carefully push the strip into the well as shown in Figure below, taking care not to trap any air bubbles beneath the strip. When pushing the IPG strips with the forceps be certain the forceps are pushing on the plastic backing to the strip and not the gel matrix.



22. Stand the gel(s) vertically by placing them in the gel box or in a test tube rack. Allow the agarose to solidify for 5 minutes before proceeding.

23. Mount the gel into the gel box per the instructions provided with the apparatus.

## WEEK 10 LAB E4 – 2D GEL STAIN

24. Fill the reservoirs with 1X Tris/glycine/SDS running buffer and begin the electrophoresis. The electrophoresis run conditions for this particular SDS-PAGE format is 200V constant voltage, which will take ~40 minutes. The migration of the Bromophenol Blue, present in the overlay agarose solution, is used to monitor the progress of the electrophoresis.

### **STAINING (AGAIN)**

25. Fill a staining tray with nanopure water for your gel run and set aside.

26. At the conclusion of the SDS-PAGE, open each gel cassette and place each gel into a tray with water.

27. Wash the gels 3 times for 5 minutes each. Add fresh water for each wash.

28. Add enough Bio-Safe stain to completely cover each gel. This is about 50ml of the stain.

29. Place each gel on a rocker or orbital shaker and shake for at least 60 minutes. The gels can be left in the stain overnight if desired.

30. We will then (on your behalf) discard the stain and wash the gels twice for 15-30 minutes with water. Longer water washes may be needed to remove remaining background. The gels can be stored in water for several days. We ask that you return the following week to look at your gels (10am).

## **APPENDIX**

## **MOL WT MARKERS**

#### Lambda DNA-Hind III Digest

#N3012S	150 µg	\$55 (USA)
#N3012L	750 µg	\$220 (USA)

Description: The Hind III digest of lambda DNA (cl857ind 1 Sam 7) yields 8 fragments suitable for use as molecular weight standards for agarose gel electrophoresis (1).

Lambda DNA-Hind III Digest visualized by ethidium bromide staining. 1.0% agarose gel.



Preparation:	The double-stranded DNA is dig	ested to completion	n with Hind III, pheno	l extracted and	dialyzed against '	10
mM Tris-HCI (	pH 8.0) and 1 mM EDTA.					

Fragment	Base Pairs	Daltons
1	23,130	15.00 x 10 <sup>6</sup>
2	9,416	6.12 x 10 <sup>6</sup>
3	6,557	4.26 x 10 <sup>6</sup>
4	4,361	2.83 x 10 <sup>6</sup>
5	2,322	1.51 x 10 <sup>6</sup>
6	2,027	1.32 x 10 <sup>6</sup>
7	564	0.37 x 10 <sup>6</sup>
8	125	0.08 x 10 <sup>6</sup>

Note:

Dilute in TE or other buffer of minimal ionic strength. The cohesive ends of fragments 1 and 4 may be separated by heating to 60°C for 3 minutes. DNA may denature if diluted in dH<sub>2</sub>O and subsequently heated.

Concentration and Shipping: 500 µg/ml. Supplied in 10 mM Tris-HCI (pH 8.0) and 1 mM EDTA. Store at -20°C.

# APPENDIX MOL WT MARKERS

Constituent proteins of natural prestained SDS-PAGE standards.							
			Presta	ined SD Standar	S-PAGE ds	Kaleid Stan	loscope dards
Protein	Source	Approximate MW* (kD)	High	Low	Broad	Prestained	Polypeptide
Myosin	Rabbit skeletal muscle	200.0	•		•	•	
-Galactosidase	E. coli	116.3	•		•	•	
Phosphorylase b	Rabbit muscle	97.4		•			
Serum albumin (BSA)	Bovine	66.2	•	•	•	•	
Ovalbumin	Hen egg white	45.0	•	•	•		
Carbonic anhydrase	Bovine	31.0		•	•	•	•
Trypsin inhibitor	Soybean	21.5		•	•	•	•
Lysozyme	Hen egg white	14.4		•	•	•	•
Aprotinin	Bovine pancreas	6.5			•	•	•
Insulin, B chain, oxidized	Bovine	3.5					•
* MW will vary from lot to lot	; see lot-specific calibrati	ion included with	n standa	rds.			
	- 113 kD	- 210	kD		- 216 k	D	

- 208 kD	- 92	- 210 KD	- 216 kD	
200 AD	- 52.9	- 125	- 132	- 36.4 kD - 26.6
- 126	02.0		- 78	
97 - 97	- 35.4	- 56.2		- 16
		- 35.8	- 45.7	- 8.4
	- 29	- 29	- 32.5	- 3.8
- 48		- 21	- 18.4	
	- 21.5	- 6.9	- 7.6	
Prestained SDS-PAGE	Prestained SDS-PAGE	Prestained SDS-PAGE	Kaleidoscope Prestained	Kaleidoscope Polypeotide
Standards, High Range	Standards, Low Range	Standards, Broad Range	Standards	Standards

Natural prestained standards. Molecular weights shown are of representative lots. Actual weights may vary.

## **MOL WT MARKERS**

### 100 bp, 1kb and 2-Log DNA Ladders

#### 2-Log DNA Ladder

#N3200S	100 μg	\$55	(USA)
#N3200L	500 μg	\$220	(USA)
1 kb DNA Ladde #N3232S	r 100 ωα	\$55	(USA)

#N3232S	100 µg	\$55	5 (USA)
#N3232L	500 µg	\$220	) (USA)

#### 100 bp DNA Ladder

#N3231S	50 µg	\$55	(USA
#N3231L	250 µg	\$220	(USA

#### Features

- Even Band Intensities
- No Extraneous High Molecular Weight Bands
- Value Load Only 0.5 µg/lane of 1 kb or 100 bp DNA Ladders
- Available in Bulk Quantities
- Easily Identifiable Reference Bands

Description: The 2-Log DNA Ladder has a number of proprietary plasmids that ae digested to completion with apprpriate restriction enzymes to yield 19 bands suitable for use as molecular weight standards for agarose gel electrophoresis. This digested DNA includes fragments ranging from 100 bp to 10 kb. The 0.5, 1.0 and 3.0 kb bands have increased intensity to serve as reference points.

The 1 kb DNA Ladder has a number of perpretary plasmids that are digested to completion with appropriate restriction enzymes to yield 10 bands suitable for use as molecular weight standards for agarose gel electrophoresis. The digested DNA includes fragments ranging from 0.5–10.0 kilobases (kb). The 3.0 kb fragment has increased intensity to serve as a reference band.

The 100 bp DNA Ladder has a number of poprietary plasmids that are digested to completion with appropriate restriction enzymes to yield 12 bands suitable for use as molecular weight standards for agarose gel electrophoresis. The digested DNA includes fragments ranging from 100–1517 base pairs. The 500 and 1,000 base pair fragments have increased intensity to serve as reference points.

Storage Conditions: Markers are supplied in 10 mM rfs-HCI (pH 8.0), 1.0 mM EDP. For long term storage store at -20°C. All DNA Ladders are stable for at least 3 months at 4C.

Concentration: Selling concentration: 500µg/ml for 100 bp and 1 kb ladders. 1,000µg/ml for 2-Log DNA Ladder



Sample Preparation: The double-stranded DNA is digested to completion with appropriate restriction enzymes, phenol extracted and equilibrated to 10 mM Tis-HCI (pH 8.0) and 1 mM EDTA.

Notes on Use: All fragments have 4-base, 5' overhangs that can be end labeled using T4 Polynucleotide Kinase (NEB #M0201) or filled-in using DNA Polymerase I, Klenow Fragment (NEB #M0210) (1). Usex=[<sup>22</sup>P] dATP or  $\alpha$ =[<sup>22</sup>P] dTTP for the fill-in reaction.

We recommend loading 1 g of the 2-Log DNA Ladder diluted in sample bufer. This ladder was not designed for pecise quantitation of DNA mass but can be used for approximating the mass of DNA in comparably intense samples of similar size.

#### Reference:

 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., (pp. 10.51–10.67). Cold Spring Harbor: Cold Spring Harbor Laboratory.

## **APPENDIX**

## **BUFFER RECIPES**

#### TE (pH8)

10mM Tris-HCl, pH8 1mM Na<sub>2</sub>EDTA

#### Loading / Stop Buffer

50% Glycerol 0.1M EDTA 1% SDS (optional) 0.1% bromophenol blue

#### **10x TBE Electrophoresis Buffer**

108g Tris base 55g Boric acid 40ml of 0.5M EDTA (pH8) Water to 1L

#### **Resolving Buffer:**

0.75M Tris-base 0.21% SDS Adjust to pH8.8 with HCI

#### Stacking Buffer:

0.13M Tris-base 0.12% SDS Adjust to pH6.8 with HCI

#### 3x Sample Loading Buffer:

150mM Tris pH6.8 6mM Na<sub>2</sub>EDTA 3% SDS 3% b-mercaptoethanol 24% glycerol speck of bromophenol blue.

#### **10x Electrophoresis Buffer**

30g Tris Base 144g Glycine 10g SDS Add dH<sub>2</sub>O to 1L.

#### **TBS (Tris Buffered Saline)**

50mM Tris pH7.5 150mM NaCl (+0.05% Tween 20 for TBS+Tween20)

Alkaline Phosphatase Buffer (for Western Substrate development) 0.1M Tris pH9.5 100mM NaCl 5mM MgCl<sub>2</sub>

#### LB Agar

Make 1L of LB broth, and add 15g of Bacto-agar before autoclaving. Cool to 55-60°C before adding ampicillin, X-gal or IPTG (if necessary)

#### SOC medium

20g Bacto-tryptone 5g Bacto-yeast extract 0.5g NaCl 950ml distilled H<sub>2</sub>O 10ml of 250mM KCl Adjust pH to 7.0 with 5M NaOH Adjust volume to 1L Autoclave. (Just before use, add 5ml sterile 2M MgCl<sub>2</sub>, and 20ml of filtersterilized 1M glucose.

## **APPENDIX 1<sup>ST</sup> PAPER FORMATTING**

## Title of Paper [in Arial font size 22, bold]

Authors, real and/or fake [Arial font size 10, regular] Institution, real and/or fake [Arial font size 10, italics]

Fake date of submission. Received | Accepted [Arial font size 10, regular]

Abstract paragraph goes here. Generally no longer than half a page, but shorter is also o.k. [Arial, font size 10, bold]. Abstract paragraph goes here. Generally no longer than half a page, but shorter is also o.k. [Arial, font size 10, bold].

Keyword: Fictional theme, subject, subject, etc [Arial, font size 10, bold].

### Introduction [main subheadings in Arial, font size 14, bold]

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### Methods [main subheadings in Arial, font size 14, bold]

Must include one example of a high throughput technique. i.e things with an "-omics" label.

#### Methods subheading [main subheadings in Arial, font size 10, bold]

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### Results [main subheadings in Arial, font size 14, bold]

Figure/Table legends [Arial, font size 8, regular]. *Figures can be created from scratch or hacked/modified from existing figures. If the latter, please include attribution in the reference section.* 

### Discussion [main subheadings in Arial, font size 14, bold]

Conclusions [main subheadings in Arial, font size 14, bold]

Acknowledgements [main subheadings in Arial, font size 14, bold]

# Literature Cited/References [main subheadings in Arial, font size 14, bold]

References [Arial, font size 8, regular] Akey JM, Zhang G, Zhang K, Jin L, and Shriver MD. 2002. Interrogating a high density SNP map for signatures of natural selection. Genome Res. 12:1805–1814.