SC/BIO3140 (W2021)

Laboratory Manual

Laboratory Overview

Five laboratory projects will be conducted over the 10 weeks of this course. These projects are indicated below, along with their associated subtasks:

Lab 1 - Introduction to Laboratory Techniques (5%)

- Exercise your micropipetting skills
- Generate a maltose standard curve

Lab 2 - Protein analysis module (25%)

- Prepare samples for SDS-PAGE
- Cast 2 SDS-PAGE gels for Western blotting
- Western blotting transfer
- Western blotting detection
- SDS-PAGE with Coomassie Blue staining

Lab 3 - Restriction Mapping of α-Amylase Plasmid DNA (15%)

- Setup of pAMY8 DNA digest for restriction mapping
- Run of digested pAMY8 DNA on agarose gel
- Restriction map construction tutorial

Lab 4 - DNA Cloning and analysis module (25%)

- Isolation of genomic DNA from B. licheniformis
- Run of genomic DNA on agarose gel for quality check
- Determination of genomic DNA concentration and yield
- Setup of PCR for alpha-amylase cloning
- Run of PCR products on agarose gel and kit cleanup
- Double digest of PCR product and target pET15 vector
- Cleanup of restriction digests
- Gel confirmation of successful digestion
- Ligation and transformation
- Colony PCR
- **Enzyme activity of α-Amylase clones**

Lab 5 - Genetic mapping & Southern blotting (20%)

- PCR labeling of alpha-amylase probe with biotin
- Agarose gel for Southern blotting and transfer
- Southern hybridization
- Southern washing and detection

Parts of these projects will be conducted concurrently (read: at the same time) over the 9 sessions of the course, according to the schedule on the course website.

Laboratory Reports

Upon completion of a lab project, a full and detailed report must be submitted. Further details of how reports should be written will be given in class. TA's will announce exact deadlines for handing in lab reports. *Late reports will be deducted 25% for up to 24 hours and a further 10% per day each subsequent day.*

Session 1 - Laboratory 1 Activities

Brief overview

A colorimetric enzyme assay will be used to measure the units of α-amylase activity in samples of human saliva and in commercial preparations of α-amylase isolated from different species. The students will also learn how to use micropipettors properly by measuring the mass of different chemicals with a balance.

- Learn to use a micropipettor by measuring the mass of water, ethanol, and glycerol with a balance
- Generate a standard curve for maltose

Procedures: Micropipettor Use

1. Label three 1.5 eppendorf tubes “water”, “ethanol”, and “glycerol”
2. Measure the weight of tubes.
3. Place 1mL of water, ethanol, and glycerol into corresponding tubes.
4. Measure the weight of tubes.
5. Calculate the density of water, ethanol, and glycerol. Fill out the following table.

	Water	**Ethanol**	**Glycerol**
Weight difference (After – Before)			
Volume			
Density			

Procedures: Maltose Standard Curve:

1. Label six conical bottom 15-ml tubes from 1 to 6 as indicated in the table below:

Tube	**1 (Blank)**	**2**	**3**	**4**	**5**	**6**
Water	2 ml	1.8 ml	1.6 ml	1.4 ml	1.2 ml	1 ml
Maltose (2mg/ml)	0 ml	0.2 ml	0.4 ml	0.6 ml	0.8 ml	1 ml
Maltose Colour Reagent	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml

2. Add the appropriate volumes of water, maltose and maltose colour reagent into the tubes
3. Cap and vortex ALL tubes
4. **Incubate the tubes at 100 ^{0}C for exactly 15 minutes.**
5. Place the tubes on ice in order to achieve room temperature.
6. Add 9 ml of deionized water (dH_2O) to each tube, tightly cap the tubes, and mix the tubes by gently inverting several times. Aliquot 1 mL of mixture into standard cuvette.

7. Measure the absorbance at 540 nm wavelength using cuvettes in the spectrophotometer and fill in the following table (**first use tube 1 for the blank measurement**):

Tube	**1 (Blank)**	**2**	**3**	**4**	**5**	**6**
A_{540nm}						
mg of maltose	0	0.4	0.8	1.2	1.6	2

8. Calculate the mass (mg) of maltose in each sample and record the value in the table
9. Make the following plot:
    - X-axis: total mass (mg) of maltose
    - Y-Axis: A540 nm absorbance
    - Generate a best fit line to use it later to determine the amount of maltose present in the solutions on the basis of its absorbance

Session 1 – Laboratory 3 Activities

Procedure: Assembly of the restriction enzyme digestion reactions

The restriction digests are as follows:

Single cut:
*Hin*d III alone
*Eco*R I alone
Cla I alone
Bgl II alone

Double cut:
*Hin*d III and *Eco*R I together
*Hin*d III and *Cla* I together
*Hin*d III and *Bgl* II together
*Eco*R I and *Cla* I together
*Eco*R I and *Bgl* II together
Cla I and *Bgl* II together

1. Set up the above enzymatic reactions as follows, adjusting the amount of H_2O as necessary, and using the correct buffer for each reaction as indicated:

Component	One Enzyme	Two Enzymes
dH_2O	14.0 µl	12.0 µl
pAMY8 [0.5 µg/µL]	2.0 µl	2.0 µl
10X buffer	2.0 µl	2.0 µl
Restriction enzyme 1	2.0 µl	2.0 µl
Restriction enzyme 2	0	2.0 µl
Total	**20.0 µl**	**20.0 µl**

2. Place the 1.5 ml Eppendorf tubes in a 37°C incubator and leave them for 1.5 – 2 hrs.
3. Store the enzyme digests in the -20°C until next week.

Procedure: Casting 1% agarose gels

Please refer to Appendix A for details on how to prepare the agarose gel.
Once prepared, gels in running trays are to be wrapped with wet paper towels, placed in zip-lock bags and kept in the fridge for next week.

Please note, that this also can be done on Week 4 – Day 1. The decision when exactly to cast gels will be made by your TA and Lab Technician and announced in class.

Session 1 – Laboratory 4 Activities

Brief overview

The goal this week is to use a quantitative α-amylase assay to measure α-amylase activity of the recombinant strain of *E.coli* carrying the amyE plasmid, negative *E.coli* cells and *B.licheniformis*. In addition, biotin-labeled α-amylase probes will be generated to detect the α-amylase gene in restriction fragments of the *B.lichenformis* genome using the Southern Hybridization technique.

Procedure: Enzyme activity of α-amylase clones

1. You will be provided with three ml of cultures according to the chart that follows:

Tube #	Strain
1	AmyE *E.coli*
2	Negative *E.coli*
3	*B.licheniformis*

2. Spin down 1.5 ml of each of the cultures provided to you at 13,000 rpm for 2 minutes in a 1.5 ml eppendorf tube. Repeat it twice in the same tube.
3. Keep the supernatant on ice as it will serve as the culture medium in the assay.
4. Add 0.1 ml of 1x TBS to each tube of cells. Vortex for 30 seconds to fully resuspend the pellet of cells.
5. Transfer re-suspended cells into 2mL tubes containing 0.1 ml of glass beads and vortex the mixture for 1 minute.
6. Place the tube on ice for 1 minute.
7. Repeat the cycles of vortexing for 1 minute and placing the mixture on ice for four more times to ensure complete lysis of the bacteria.
8. Add 1.3 ml of 1X TBS to each tube and vortex to mix.
9. Spin the tubes at 13,000 rpm for 2 minutes. The glass beads should be firmly adhered to the bottom of the tube. Carefully transfer the bacterial lysate to another fresh 1.5 ml eppendorf tube and keep these tubes on ice while you set up the assay tubes.
10. Label eight 15 ml assay tubes , according to the table below:

Sample	Condition	LB broth	Culture medium	1X TBS	Lysate
1	Culture medium blank	1 ml	0 ml		
2	*amyE E.coli* medium	0.9 ml	0.1 ml		
3	Negative *E.coli* medium	0 ml	1 ml		
4	*B.licheniformis* medium	0 ml	1 ml		
5	Cell lysate blank			1 ml	0 ml
6	*amyE E.coli* lysate			0.9 ml	0.1 ml
7	Negative *E.coli* lysate			0 ml	1 ml
8	*B.licheniformis* lysate				1 ml

11. Add 1 ml of 1% starch solution to each of the assay tubes including the blank tubes
12. Vortex the tubes and place them in the boiling water bath (100°C) for exactly 12 minutes.
13. In the same order that the starch solution was added, add 1 ml of the maltose color reagent to each tube. Vortex the tubes and place them in the boiling water bath (100°C) for exactly 15 minutes.
14. Place the tubes on ice until they reach room temperature. Add 9 ml of deionized water and invert the tubes several times to mix their contents.
15. Use the appropriate blank to zero the spectrophotometer at 540 nm and measure the absorbance of the samples at 540 nm.
16. Record your absorption readings in the following table:

	amyE E.coli medium	Negative *E.coli* medium	*B.licheniformis* medium	*Amy E E.coli* lysate	Negative *E.coli* lysate	*B.licheniformis* lysate
A540 nm						
mg of maltose produced during assay						
Calculated mg of maltose produced by 1ml of test solution						

Session 2 – Laboratory 3 Activities

Brief overview

Genes are segments composed of DNA and are located on chromosomes. The α-amylase gene is part of the genomic DNA in many different strains of Bacillus. The size of the Bacillus genome is approximately 4000 kb. The α-amylase gene is 1.6 kb in size. The isolation of genomic DNA is the first step in the cloning of this gene and the subsequent laboratory exercises.

Procedures: Agarose gel electrophoresis of the DNA digest for restriction mapping

1. Prepare 1X LAB Running Buffer from 25 X LAB stock provided. Place it on ice or into the fridge to keep it cold.
2. If it is not done on Week 3 Day 2, prepare 2 x 1% agarose gels (please refer to Appendix A for details on how to prepare the agarose gel). **Important: Use 1 X TAE to cast gels.**
3. Prepare the samples by mixing 17 µl enzyme digest with 3.4 µl 6X DNA loading dye.
4. Place each tray with the gel in the electrophoresis tank, fill the tank with pre-chilled Running Buffer until the buffer is ~1-2 mm deep over your gel (= 225 mL/electrophoresis tank).
5. Load your samples in the following order:

Gel 1:	**Gel 2:**
50 bp DNA Ladder, QL, 10 µL/well	50 bp DNA Ladder, QL, 10 µL/well
*Hin*d III alone	*Cla* I alone
*Eco*R I alone	*Bgl* II alone
1 Kb DNA ladder, QL, 10 µL/well	1 Kb DNA ladder, QL, 10 µL/well
*Hin*d III and *Eco*R I together	*Eco*R I and *Cla* I together
*Hin*d III and *Cla* I together	*Eco*R I and *Bgl* II together
*Hin*d III and *Bgl* II together	*Cla* I and *Bgl* II together
100 bp DNA Ladder, QL, 10 µL/well	100 bp DNA Ladder, QL, 10 µL/well

6. Run the gels at 200 Volts for exactly 30 minutes or less. Start and stop running your two gels at the same time! BE AWARE of high voltage.
7. Photograph your gel using the imager in the laboratory and save this image for your laboratory report.

a) Assembly of the restriction map:

1. Using the 1 Kb and 50 bp (and/or 100 bp) DNA Ladders, calculate the lengths of the restriction fragments
2. List the single and double digest fragment lengths in two separate tables
3. Follow TA instructions for assembly of restriction map. This will be submitted in your laboratory report.

Session 2 – Laboratory 4 Activities

Lyse cells

1) Pellet cells:

1.1 Transfer 25 mL of B. licheniformis overnight culture into an Oak Ridge centrifuge tube [Discard used 50 mL test tube into Biohazard Solid Waste container];
1.2 Balance tubes and centrifuge at room temperature for 5 min at ~3 500 g (use Sorvall RC 6 Plus centrifuge with SS34 rotor set at 5 500 rpm);
1.3 Decant the supernatant into Biohazard Liquid Waste container;
1.4 Keep the pellet on ice.

2) Wash cells:

2.1 Add 2 mL of TE buffer, vortex thoroughly for 20 sec to completely disperse bacterial cells, so there are no visible cell clumps;
2.2 Add 20 mL of TE buffer, vortex for 10 sec;
2.3 Balance tubes and centrifuge at room temperature for 5 min at ~3 500 g (use Sorvall RC 6 Plus centrifuge with SS34 rotor set at 5 500 rpm);
2.4 Decant the supernatant into Biohazard Liquid Waste container;
2.5 Add 2mL of TE buffer to the pellet, vortex to resuspend cells, keep them on ice.

3) Lyse cells in P:C:I (25:24:1) presence:

3.1 Transfer cell suspension into 15 mL round bottom tube (#1) containing 1 mL of glass beads [Place used Oak Ridge centrifuge tube into 10% Bleach solution for o/n disinfection];
3.2 In fume hood, add 1 mL of P:C:I, close the tube securely with plug cap to prevent leakage and vortex vigorously for 1 min exactly, then place the tube on ice for 1 min exactly for a rest. Be careful to avoid Phenol burns;
3.3 Repeat cycle of vortexing (1 min) and resting on ice (1 min) three more times;
3.4 Centrifuge the contents of tube (2 mL bacterial cell lysate + 1 mL glass beads + 1 mL P:C:I) at room temperature for 5 min at 6 000 x g to separate phases (use Sorvall RC 6 Plus centrifuge with SS34 rotor and rubber adapters for 15 mL round bottom tubes set at 6 000 x g);
3.5 Very carefully, without jostling remove the tube from centrifuge rotor. There will be three layers in the tube - the bottom layer with glass beads and organic phase, the middle opaque layer with denatured proteins and other cellular debris and the upper clear aqueous layer with DNA. Carefully, without disturbing layers transfer the top aqueous layer (~2 mL) into fresh 15 mL round bottom tube (#2), keep it on ice. Be sure not to carry over any P:C:I during pipetting. [Discard the 15 mL tube #1 with phenol, glass beads and cell debris (Place it in Solid P:C:I Waste rack)].

Extract DNA to purify it

4) P:C:I extraction #1:

4.1 Add 2 mL (1 volume) of P:C:I.
Cap and gently invert the tube #2 to mix aqueous and organic phases;
4.2 Centrifuge the tube (~4 mL) at room temperature for 5 min at 6 000 x g to separate phases (use Sorvall RC 6 Plus centrifuge with SS34 rotor and rubber adapters for 15 mL round bottom tubes set at 6 000 x g);
4.3 Save the top aqueous layer (~2 mL) in fresh 15 mL round bottom tube (#3), keep it on ice.
[Discard the 15 mL tube #2 (Place it in Solid P:C:I Waste rack)].

5) P:C:I extraction #2:

5.1 Add 2 mL of P:C:I. Cap and gently invert the tube #3 to mix aqueous and organic phases;
5.2 Centrifuge the tube (~4 mL) at room temperature for 5 min at 6 000 x g to separate phases (use Sorvall RC 6 Plus centrifuge with SS34 rotor and rubber adapters for 15 mL round bottom tubes set at 6 000 x g);
5.3 Save the top aqueous layer (~2 mL) in fresh 15 mL round bottom tube (#4), keep it on ice.
[Discard the 15 mL tube #3 (Place it in Solid P:C:I Waste rack)].

6) C:I extraction #1:

6.1 Add 2 mL of C:I. Cap and gently invert the tube #4 to mix aqueous and organic phases;
6.2 Centrifuge the tube (~4 mL) at room temperature for 5 min at 6 000 x g to separate phases (use Sorvall RC 6 Plus centrifuge with SS34 rotor and rubber adapters for 15 mL round bottom tubes set at 6 000 x g);
6.3 Save the top aqueous layer (~ < 2 mL) in fresh 15 mL conical test tube, keep it on ice.
[Discard the 15 mL tube #4 (Place it in Solid P:C:I Waste rack)].

Precipitate DNA

7) Precipitate DNA:

7.1 Transfer 1.5 mL of the aqueous phase with genomic DNA into three 2 mL microtubes (3 x 0.5 mL/each tube);
7.2 Add 150 μL (0.1 volume) of 3M Sodium Acetate, pH 5.2 (3 x 50 μL/each tube), cap and gently invert tubes to mix;
7.3 Add 4.125 mL (2.5 volumes) of ice-cold 95% Ethanol (3 x 1.375 mL/each tube), cap and slowly
invert tubes to mix;
(7.- o/n incubation at -20^0 C step will be omitted. Question for students – Why?)
7.4 Centrifuge tubes (3 x 1.925 mL) at +4^0 C for 20 min at 16 000 x g, but first mark the spot for expected location of DNA pellet on outside of microtubes
(use Refrigerated benchtop microcentrifuge set at 13 000 rpm);

7.5 Decant (or carefully pipet off) the supernatant without disturbing DNA pellets (to avoid dislodging/losing them);

8) Wash DNA pellets:

8.1 Add ice-cold 70% Ethanol (3 x 0.4 mL/each tube) to wash DNA pellets by gentle flicking on tubes;

8.2 Centrifuge tubes at room temperature for 10 min at 16 000 x g, but first place tubes in rotor so that marked spots for pellets are in proper position
(use Benchtop microcentrifuge *Spectrofuge 24D* set at 13 000 rpm);
PS Even though it is preferable to centrifuge at $+4^0$ C (Refrigerated benchtop microcentrifuge), this step can be done at room temperature (Regular benchtop microcentrifuge).

8.3 Carefully remove all Ethanol without dislodging/losing pellets and let them air dry for 5-15 min.

9) RNase A treatment:

9.1 Resuspend DNA in 120 μL of H_2O, nuclease-free with 0.1 μg/mL RNase A (3 x 40 μL/each tube) by gentle pipetting up and down 5-10 times or so.

9.2 Proceed to RNAase treatment of gDNA to remove RNA (Incubate tubes at $+37^0$ C for 1 hour);

9.3 Collect all 120 μL of your gDNA stock in one fresh 1.5 mL microtube and store at -20^0 C.

(9.- o/n incubation at $+4^0$ C step to enhance complete DNA redissolving will be omitted. Question for students – Why?)

Procedures: Agarose gel electrophoresis of genomic DNA from *B.licheniformis*:

1. Prepare a 0.7 % agarose gel (see Appendix A!)
2. To assess the quality of your genomic DNA you must make a few dilutions to run on the agarose gel. Add 45 µl of TE buffer to each of two 1.5 ml eppendorf tubes. Label the first as "10^{-1}"dilution and the second as "10^{-2}"dilution. To the first tube add 5 µl of your DNA and mix it well by pipetting up and down 5 times. Then transfer 5 µl of DNA in TE buffer from the first dilution over to the second tube and mix well by pipetting carefully another 5 times.
3. Transfer 5 µl of each dilution to a new 1.5 ml eppendorf tube and add 5 µl of 2X DNA Gel loading dye to each tube and mix well by pipetting up and down a few times. Centrifuge the tube to collect all the sample at the bottom of the tube.
4. Load the entire sample into a separate well. Load 10 µl of the 1Kb DNA molecular weight marker and run the gel at 100 volts for 1 hr.
5. Photograph your gel and keep this photograph for your laboratory report. Your DNA preparation should look like the DNA in the figure below:

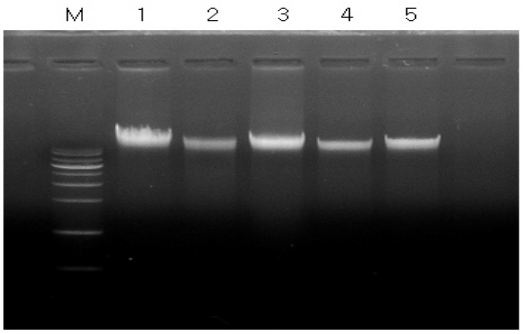


Figure: Agarose gel electrophoresis of genomic DNA isolated from different orgamisms:
M: 1 kb ladder marker, line 1: Chicken whole blood, line 2: Human whole blood, line 3: *E. coli*, line 4: *L. brevis*, line 5: *Streptomyces hygroscopicus subsp*

Session 3 – Laboratory 4 Activities

Procedure: Quantitation of genomic DNA from *B.licheniformis*:

1. Firstly, use 0.5 ml of TE buffer as the BLANK measurement in the Quartz cuvette provided.
2. After you are finished with the BLANK measurement proceed to the next step
3. Mix 2 µl of your genomic DNA preparation plus 498 µl of TE buffer in a 1.5 ml eppendorf tube. Mix well by pipetting up and down at least 5 times.
4. Transfer all the solution (approximately 0.5 ml) to the Quartz cuvette and measure the absorbance at 260 and 280 nm of wavelength
5. Now you are ready to quantify your DNA concentration

a) The absorbance at 260nm is used to calculate the concentration of nucleic acids. At a concentration of 50 µg/ml and a 1 cm path length* dsDNA has A260 = 1. The absorbance value is also dependent on the amount of secondary structure in the DNA due to hypochromicity.

b) To ensure the concentration reading is accurate, the absorbance reading should be within the linear range of the spectrophotometer. The Lambert-Beer law relates the absorption of light to the properties of the material through which the light is travelling. The law states that there is a logarithmic dependence between the transmission of light through a substance and the product of the absorption coefficient of the substance and the distance the light travels through the material (i.e. the path length). The law tends to break down at very high concentrations, especially if the material is highly scattering. So for concentrated solutions the absorbance value and therefore the concentration can be inaccurate. It is often useful to prepare and measure a series of dilutions to check not only the concentration but the accuracy of the dilutions as well. Inaccurate dilutions can occur if the DNA is not homogeneously re-suspended. For reliable spectrophotometric DNA quantification A260 readings should lie between 0.1 and 1.0.

a) To improve the accuracy of DNA concentration determination allowance should be made for any impurities in the solution. This can be estimated by adjusting the A260 measurement for turbidity which is measured at an absorbance of A320. The equation below can be used:

b) Concentration (µg/ml) = (A260 reading – A320 reading) × dilution factor × 50µg/ml

c) Total yield is obtained by multiplying the DNA concentration by the final total purified sample volume.

d) DNA Yield (µg) = DNA Concentration × Total Sample Volume (ml)

Procedure: Cloning of the α-amylase gene from *Bacillus* by PCR

1. Prepare a diluted genomic DNA sample with a final concentration of 0.25 µg/µl
2. Set up the PCR reactions as indicate in the table below:
   * First, prepare 3.5X rxns master mix for the first three components and use 29 µl per each reaction. Your TA will provide details.

Component	Negative control	pAMY8 positive control	*B.licheniformis*
2X PCR master Mix	25 µl	25 µl	25 µl
Forward Primer, 10 µM	2 µl	2 µl	2 µl
Reverse Primer, 10 µM	2 µl	2 µl	2 µl
Template DNA	N/A	0.5 µg in 5 µl	0.5 µg in 2 µl
dH_20	21 µl	16 µl	19 µl
Total Volume	50 µl	50 µl	50 µl

3. Mix the samples well and briefly spin the tubes down (1000 rpm for 1 minutes)
   Now you are ready to proceed with the PCR reaction using the thermal cycler (program 3140 PCR1)

Procedure: Agarose gel electrophoresis of the unpurified α-amylase PCR product

1. Prepare a 1% agarose gel
2. Prepare three samples by mixing 5 µl of the *unpurified* PCR product with **5** µl 2X DNA loading dye.
3. Load the three PCR samples on the gel and load one lane with the 1 Kb DNA molecular weight marker.
4. Run the gel at 100 volts for 20 minutes.
5. Photograph your gel using the imager in the laboratory and save this image for your laboratory report.

Procedure: PCR reaction cleanup

1. Add 5 volumes of Buffer PB to 1 volume of the PCR reaction and mix. For example, add 250 µl of Buffer PB to 50 µl PCR reaction (or 225 to 45).
2. To bind DNA, load the samples into the MinElute columns and centrifuge at 10,000 rpm for 1 min (The maximum loading volume of the column is 800 µl. For sample volumes greaterthan 800 µl simply load again).
3. Discard flow-through. Place the MinElute column back into the same tube.
4. To wash, add 750 µl Buffer PE to the MinElute column and centrifuge for 1 min.
5. Discard flow-through and place the MinElute column back in the same tube.
6. Centrifuge the column for an additional 1 min at maximum speed.
7. Place the MinElute column in a clean 1.5 ml microcentrifuge tube.

To elute DNA, add 44 µl Buffer EB (10 mM Tris·Cl, pH 8.5) to the center of the membrane, let the column stand for 1 min, and then centrifuge for 1 min at maximum speed. Label the side of the tube with your name.

Procedure: Restriction enzyme digest setup (NdeI/BamHI-HF double digest) for the PCR product and pET15b vector

1. Set up the restriction enzyme digestion in a 1.5 ml eppendorf tube as indicated in the table below:

Component	**Volume**
Purified PCR product (DNA)	40 µl
10 X NEB CutSmart buffer	5 µl
BamHI-**HF** enzyme	1
NdeI enzyme	1 µl
dH_20	3 µl
Total	50 µl

Component	**Volume**
pET15b vector (DNA) 3 µg	3 µl
10 X NEB CutSmart buffer	5 µl
BamHI-**HF** enzyme	1
NdeI enzyme	1 µl
dH_20	39 µl
Calf intestinal phosphatase	1ul
Total	50 µl

2. Place the 1.5 ml eppendorf tubes in a 37 °C incubator and leave them overnight. Next morning the Laboratory Technician will place tubes at -20 ^{0} C.

Session 4 – Laboratory 4 Activities

Procedure: Reaction cleanup for the digestion products

*Two reactions are to be cleaned-up.

1. Add 300 µl of Buffer ERC to the enzymatic reaction and mix. The maximum volume of enzymatic reaction that can be processed per MinElute column is 100 µl. If the enzymatic reaction is in a volume of <20 µl, adjust the volume to 20 µl. If the enzymatic reaction exceeds 100 µl, split your reaction, add 300 µl of BufferERC to each aliquot of the split reaction and use the appropriate number of MinElute columns.

2. Check that the color of the mixture is yellow (similar to Buffer ERC without the enzymatic reaction).If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.

3. Place a MinElute column in a 2 ml collection tube in a suitable rack.

4. To bind DNA, apply the sample to the MinElute column and centrifuge for 1 min at 13,000rpm. To obtain maximal recovery, transfer all traces of sample to the spin column.

5. Discard the flow-through and place the MinElute column back into the same tube.

6. To wash, add 750 µl Buffer PE to the MinElute column and centrifuge for 1 min.

7. Discard the flow-through and place the MinElute column back in the same tube. Centrifuge the column for an additional 1 min at maximum speed.

8. Place the MinElute column in a clean 1.5 ml microcentrifuge tube.

9. To elute DNA, add 20 µl Buffer EB (10 mM Tris·Cl, pH 8.5) to the center of the membrane, let the column stand for 1 min, and then centrifuge for 1 min.

1. Keep your clean digestion products at -20^{0}C.

Procedure: Agarose gel electrophoresis of the DNA digest:

1. Prepare a 1% agarose gel
2. Prepare two samples by mixing 3 µl of the clean double cleaved PCR product (and clean single cleaved gDNA) with 3 µl of 2X DNA loading dye per each sample.
3. Prepare two samples by mixing 5 µl of the clean double cleaved pET15b vector (and clean single cleaved pRL498 vector) with 5 µl of 2X DNA loading dye per each sample.
4. Load the PCR samples on the gel and load one lane with 10 µl of 1 Kb DNA molecular weight marker.
5. Run the gel at 100 volts for 20 minutes.
6. Photograph your gel using the imager in the laboratory and save this image for your laboratory report.

Procedure: Ligation reaction set up for the PCR product and genomic DNA:

1. Keep all the reaction components on ice!!!!
2. Set up the 4 ligation reactions as indicated in the tables below:
   * First, prepare 5X rxns master mix for T4 DNA Ligase, 10X Buffer and H2O and use 9 µl per each reaction. Your TA will provide details.

	Sample A	**Sample B (Vector only control)**
Component	**Volume**	**Volume**
Cleaved PCR product	10 µl	-
Vector (pET15b)	1 µl	1 µl
10X T4 ligase buffer	2 µl	2 µ
T4 DNA ligase	1 µl	1 µl
dH_20	6 µl	16 µl
Total	**20 µl**	**20 µl**

	Sample C	**Sample D (Vector only control)**
Component	**Volume**	**Volume**
Genomic DNA library	10 µl	-
Vector (pRL498)	1 µl	1 µl
10X T4 ligase buffer	2 µl	2 µ
T4 DNA ligase	1 µl	1 µl
dH_20	6 µl	16 µl
Total	20 µl	**20 µl**

3. Place the sample in the thermal cycler and incubate overnight at 16 °C. The program is called 16 and is programmed into the thermal cycler. The laboratory technician will keep your ligation products in the -20 °C for you to use next day.

Session 4 – Laboratory 5 Activities

a) **Assembly of restriction enzyme digestion reactions**:

1. Set up the following two enzymatic digests using genomic DNA from *B.licheniformis*

Component	**Volume**
Genomic DNA (10 µg)	~ 8-10 µl
10 X NEB CutSmart buffer	5 µl
HindIII-**HF** enzyme	4 µl
dH_20	31 µl
Total	50 µl

Component	**Volume**
Genomic DNA (10 µg)	~ 8-10 µl
10 X NEB CutSmart buffer	5 µl
EcoRV-**HF** enzyme	4 µl
dH_20	31 µl
Total	50 µl

2. Place the 1.5 ml eppendorf tubes in a 37 °C incubator and leave them overnight.
3. Next morning your Laboratory Technician will place tubes at -20 ^{0} C.

b) **PCR labeling of probe DNA with Biotin**:

1. Set up your PCR reactions as follows:
   * First, prepare 3.5X rxns master mix for the Primers, 2X PCR MM and H_2O and use 48 µl per each reaction. Your TA will provide details.

Component	**Negative control**	**pAMY8 positive control**	***B.licheniformis***
2X PCR master Mix	25 µl	25 µl	25 µl
Forward BIO-Primer, 10 µM	5 µl	5 µl	5 µl
Reverse BIO-Primer, 10 µM	5 µl	5 µl	5 µl
Template DNA		2 µl (0.1 µg)	2 µl (1 µg)
dH_20	15 µl	13 µl	13 µl
Total Volume	**50 µl**	**50 µl**	**50 µl**

2. Run your PCR reaction using the thermocycler (BIO program).
3. **When competed (or next morning) your Laboratory Technician will place tubes at -20 ^{0} C.**

Session 5 - Laboratory 4 Activities

Procedure: Bacterial transformation protocol

1. You will find SOC broth in the 42°C water bath for use as outgrowth medium (step 8).
2. Thaw two aliquots of SoloPack Gold cells on ice (half of tube for each of 4 Ligation reactions).
3. When the cells have thawed, swirl the tubes to gently mix the cells.
4. Add 5 (or 10 µl – TA will let you know which volume to use) of the experimental DNA to each of the tubes of cells.
5. Swirl the tubes gently, then incubate the tubes on ice for 30 minutes.
6. Heat-pulse the tubes in a 42°C water bath for 30 seconds. The temperature and duration of the heat pulse are critical for maximum efficiency. For consistent results, remove any ice trapped around the outside bottom of the tube.
7. Incubate the tubes on ice for 2 minutes.
8. Add 250 µl of preheated (42°C) SOC broth and incubate the tubes at 37°C for 1 hour with shaking at 225-250 rpm.
9. Plate ≤200 µl of the transformation mixture on LB agar plates containing the appropriate antibiotic (**the PCR product and pET15b transformation reactions will be plated on LB-AMP$^+$ plates whereas the genomic DNA library ligation will be plated on LB-KAN$^+$-Starch plates**).
10. Incubate the plates at 37°C overnight.

***** Your TA will transform a separate of tube of bacteria: one half of it with the pUC18 control plasmid to test for competent cells transformation efficiency and the second half - with the pET15b positive control plasmid *****

Week 5 - Laboratory 5 Activities

Brief overview

A blot is a technique in molecular biology used to detect biomolecules, and for detecting, analyzing, and identifying proteins. In a standard blot the biomolecules to be detected are first separated by electrophoresis and transferred onto the membrane or paper substrate. This is then followed by detection by either nucleotide probes (for a northern blot and southern blot) or antibodies (for a western blot).

Procedure: Agarose Gel Electrophoresis

1. Prepare a 0.8% agarose gel
2. Prepare samples by adding the appropriate amount of 6X DNA loading dye to have in Total Volume of samples final concentration of DNA loading dye equal to 1X.
3. Boil all samples at 100^0C for 2 min (or at 90^0C for 5 min) before loading.
   *the biotinylated marker is ready-to-use product, do not boil it. Use entire volume of aliquot provided to you to load on gel.
4. Load your samples in the following order:

	volume	6X DNA loading Dye	Total Volume
Biotinylated marker	Min 5 µl or more	N/A	N/A
HindIII digested DNA	20 µl		
EcoRV digested DNA	20 µl		
pAMY8 PCR product	5 µl		
B.licheniformis PCR product	5 µl		
Water PCR product	5 ul		
B.licheninformis genomic DNA	10ul (5ug)		
pAMY8 plasmid	10ul (1ug)		

5. Important! Remember to keep the rest of your original samples at -20^{0}C. You will need them to use in future experiments on Week 10 Day 2 and Week 11 Day 1.
6. Run the gel @ 100 Volts until the bromophenol blue band is ¾ down on the gel.
7. Photograph your gel using the imager in the laboratory and save this image for your laboratory report to compare against your final southern blot exposure.

Procedure: Southern Blot transfer protocol

1. Transfer gel to a tray containing ~100 mL of Southern denaturing solution. Gel needs to be covered by the solution. Shake gently for 25-30 minutes.
2. Decant denaturing solution carefully and add 100 ml of Southern neutralizing buffer. Shake gently for 15-30 minutes.
3. Soak the following while gel is shaking in Southern neutralizing buffer:

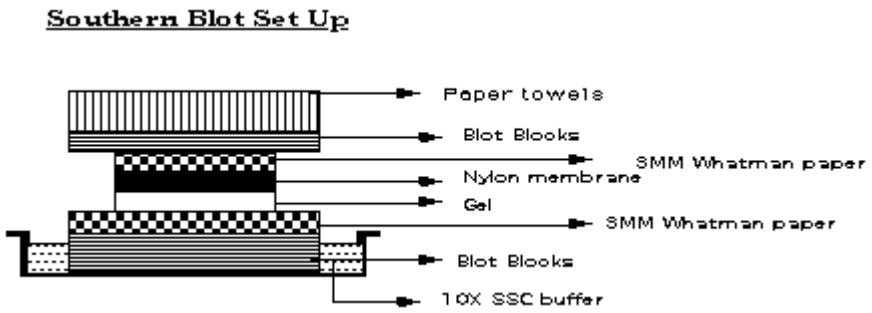
   a) transfer membrane in dH_2O
   b) thick blotting paper in Southern transfer buffer (20X SSC)
   c) thin blotting papers (6X long for "Wick" and 9 for blot) in Southern transfer buffer.

4. In a big Lasagna dish with Southern transfer buffer on bottom set up your Southern Blot (one dish will be used for minimum two Work Stations):
5. First, place the DNA running tray (serves as a support for your blot) upside down and cover it with a soaked wick. In order for your wick to work and draw solution up for transfer, the level of Southern transfer buffer in a dish should not be as high as your blot.
6. Place on top of wick the pre-soaked thick blotting paper. Make sure that paper is cut exactly the size of the gel.
7. Place gel face down onto thick blotting paper. Remove the bubbles between the gel and thick blotting paper. (Use glass Pasteur pipette to carefully roll out all bubbles).
8. Cover the inverted gel with the nylon transfer membrane that was soaked in dH_2O. Make sure that the membrane is cut exactly the size of the gel. Remove **ALL** of the bubbles between the gel and membrane.
9. Place 9 sheets of pre-soaked thin blotting paper on top of the membrane. Make sure that paper is cut exactly the size of the gel.
10. Place a stack of dry paper towels on top of blotting paper. Make sure that paper is cut exactly the size of the gel.

11. Place large plate and weight on top of the entire set-up to ensure constant contact of gel to nylon transfer membrane and even movement of buffer through the paper towel. Let it stand overnight.

Look at the following diagram for assistance with setting up your southern blot:

*Please note, this diagram slightly differs in minor details from our set up.



***Important! Steps 12-17 must be done next day in the morning!*

12. Disassemble the blot after the transfer is complete and discard everything except for the nylon membrane that carries your DNA.
13. Place the membrane in a dish containing 40 ml of 6X SSC buffer for 5 minutes.
14. Air dry the membrane.
15. Place the membrane between 2 filter papers and bake @ 80°C for 30 minutes.
16. Keep your membrane in the freezer until next week.
17. Mark the edge in contact with the gel with a pencil. This side contains the bound DNA.

Session 6 – Laboratory 2 Activities

Procedure: Preparation of the samples:

1. Each group is given a 1.5 ml tube of an overnight culture of *Bacillus amyloliquefaciens* and a 1.5 ml tube of an overnight culture of *Bacillus licheniformis*. Centrifuge the cultures at 13,000 rpm for 1-2 minutes to pellet the bacterial cells.
2. Discard the supernatant and add 100µl of TE buffer (10mM Tris-HCl, pH 8, 1mM EDTA). Vortex vigorously to resuspend the bacterial cells. Transfer resuspended cells into tube with glass beads.
3. Pulverize the *Bacillus* cells with the glass beads by vortexing for 1 minute. Subsequently place the tube on ice for 1 minute. Repeat this process 5 more times. Now that the bacterial cells have been lysed proceed to the next step.
4. Spin down the lysate (13,000 rpm for 3 minutes) to ensure that the beads are in the bottom of the tube and transfer the bacterial lysate into a new 1.5 ml eppendorf tube (only the soluble component. Avoid the pellet at the bottom).
5. Label the new tubes as follows: *B.amyloliquefaciens* and *B.licheniformis*.
6. Transfer 20 µl of bacterial lysate to a new tube and pipette another 20 µl of 2X SDS-PAGE loading buffer. Mix well by pipetting and briefly vortex the sample for 15 seconds.
7. Collect some saliva (**one from each partner labelled A and B**) in the weighing cups provided. Transfer your own saliva to a 1.5 ml eppendorf tube by using a 1 ml tip that has a broad end (use the scissors provided to cut off the fine end of the tip-ask your TA for help if you do not understand this step !!!)
8. Mix 20 µl of the saliva with 20 µl of 2X SDS-PAGE loading buffer and vortex the sample for 15 seconds.
9. Add 20 µl of the following samples to new, appropriately labeled 1.5 ml eppendorf tubes:
   a) Commercial α-amylase from *Bacillus licheniformis*
   b) Commercial α-amylase from *Aspergillus oryzae*
   c) Commercial α-amylase from porcine pancreas
   d) Unknown sample #1
   e) Unknown sample #2
10. Add 20 µl of 2X SDS-PAGE loading buffer and vortex the samples for 15 seconds.
11. Heat all the samples for 5 minutes at 95-100°C. Protein molecular weight marker is ready-to-use NEB product, do not heat it.
12. Spin down all the tubes @13,000 rpm for 1 minute to ensure that all the samples settle to the bottom of the tubes (some of the sample evaporates due to the boiling!!!)
13. Keep your sample stocks (2 from Step 5, 2 from Step 7 and 3 commercial α-amylases with 2 unknown samples provided for you) at -20^{0}C.You will use them at a later date.

Session 6 – Laboratory 4 Activities

Procedure: Colony PCR protocol:

1. Pick 4 colonies from the LB- AMP^+ transformed with your PCR product and re-suspend them individually in 20 µl of dH_20 each. You will use these mixtures as Colony Samples for the PCR outlined below. For pET15b control use a colony from TA's pET15b plate.
2. Set up the following six PCR reactions as depicted below:
   * First, prepare 7X rxns master mix for the Primers, 2X PCR MM and H_2O and use 18 µl per each reaction. Your TA will provide details.

Component	**Negative control**	**pET15b positive control**	**Colony Samples (X4)**
2X PCR master Mix	10 µl	10 µl	10 µl
T7 Forward Primer, 10 µM	2 µl	2 µl	2 µl
T7 Reverse Primer, 10 µM	2 µl	2 µl	2 µl
Template DNA	N/A	2 µl	2 µl
dH_20	6 µl	4 µl	4 µl
Total Volume	**20 µl**	**20 µl**	**20 µl**

3. Mix well all the ingredients in the PCR tube and place them in the thermal cycler. The program for this PCR is called 3140PCR2.
4. While waiting the reaction to go to completion prepare a 1% agarose gel.

Procedure: Agarose gel electrophoresis of the colony PCR products:

1. Prepare a 1% agarose gel (please refer to earlier text for details on how to make the agarose gel)
2. Prepare six samples by mixing 10 µl of the PCR product with **10** µl 2X DNA loading dye.
3. Load all six PCR samples on the gel and load one lane with the 1 Kb DNA molecular weight marker and one more lane with 100 bp DNA Ladder.
4. Run the gel at 100 volts for 20 minutes.
5. Photograph your gel using the imager in the laboratory and save this image for your laboratory report.

Session 7 – Laboratory 2 Activities

TA Demo: SDS-PAGE gel preparation, followed by SDS-PAGE setup

SDS-PAGE set up:

You will learn and cast Polyacrylamide gel in order to use it later. The type of Polyacrylamide Gel Electrophoresis you will perform employs the most popular discontinuous buffer system (SDS-PAGE buffer system by Laemmli), therefore you need to cast a gel which consists of two portions: 10% Separating (or Resolving, or Running) SDS-Polyacrylamide Gel and 4% Stacking SDS-Polyacrylamide Gel.

Your TA will provide a demonstration of how to make SDS-PA gel. Pay close attention to TA demo, and all hints and notes in the following protocol to ensure a trouble-free gel casting. The temperature of ingredient solutions, the order of mixing and the timing and way of dealing with the monomer solution while pouring are all important factors for success.

Warning!
Acrylamide is a NEUROTOXIN. It can be absorbed through unbroken skin. Thus, exercise caution when handling unpolymerized acrylamide**: wear gloves during the entire process of casting your gel**.
Also, **do NOT pour unpolimerized acrylamide down the sink! Any liquid leftovers must be collected in the Acrylamide Waste container located in the Fume Hood.**

Procedure:

1. First, assemble your gel cassette using glass plates (Short plate and 1.5 mm Spacer plate), Casting Frame and Casting Stand according to your TA's instructions. Also, you may find useful a copy of BIO-RAD Assembly guide provided to you.
   Note: we use two (gray and green) gaskets instead of just one gray.
   Note: make sure both glass plates (Short plate and 1.5 mm Spacer plate) are absolutely clean. If not, use Windex glass cleaning solution provided in Spray bottles and KimtechWipes.

   Next, check your gel cassette for leaks (optional?) and mark the level to which Separating gel is poured.

   Can be done by several ways:
   a) Fill the assembled gel cassette with 6.5 mL of dH_2O to test whether it is sealed properly and also to mark the level of water with a marker. Then drain the water by carefully tilting the entire assembly on its side or even inverting it up-side down over your liquid waste container at your workstation. Use KimtechWipes to pad dry any remaining water. Let your gel cassette dry.
      Note: 6.5 mL is the volume of Separating gel to be poured into gel cassette with 1.5 mm Spacer Plate. It was determined for you by the Laboratory Technician.

Note: With a leak occurring, you have to disassemble the gel cassette, pad dry plates and start again.

b) Insert a comb into the gel cassette. Mark the Short plate 1 cm below the comb teeth. Remove the comb. Now check for leaks with water (no need for measuring exact volume) or take a risk and proceed without leak proofing (not recommended though).

It is time to get ingredients needed to make a gel (stored at 4^0C) and place them on ice. Each group is provided with 5 aliquots of solutions.

Note: Volumes of reagents aliquoted for you are designed to be enough to make one gel. If you need to re-do gel casting, use spare aliquots.

Note: TEMED is located in the Fume Hood. Do not take it out, simply add TEMED into your solution over there.

2. Gel Casting.

A)-Prepare the Separating gel monomer solution by combining all reagents except 10%APS and TEMED.

-Add them into a 125 mL "Separating gel" flask in an order corresponding to the order of ingredients listed in the Table below.

Table SDS-PAGE Formulations for casting one gel (1.5 mm thickness).

Ingredients	10 % Separating gel	4% Stacking gel
dH_2O *(adjust accordingly)	2.95 mL	2.7 mL
1.5M Tris-HCl, pH 8.8	1.9 mL	N/A
1.0M Tris-HCl, pH 6.8	N/A	0.47 mL
30% Acrylamide/Bis	2.5 mL	0.5 mL
10% SDS	75 µL	37.5 µL
10% APS	75 µL (or 37.5 µL?)	37.5 µL (or 18.75 µL?)
TEMED**	3.75 µL	3.75 µL
Total Volume	~7.5 mL	~3.75 mL

*Volume of dH_2O is 2.9875mL = ~2.988mL, if your TA recommends to use 37.5 µL of 10% A*PS.

**In Total Volume calculation the volume of TEMED is ignored.

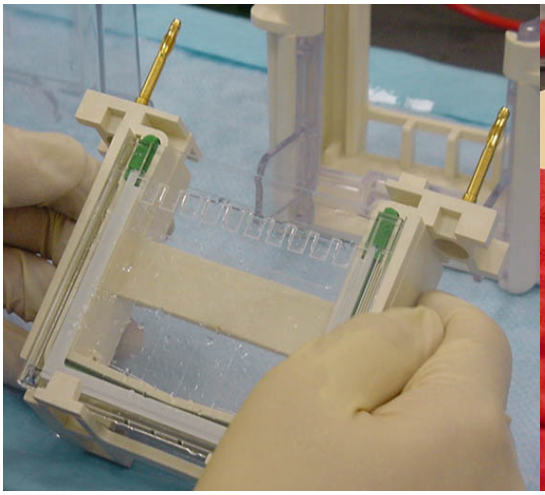
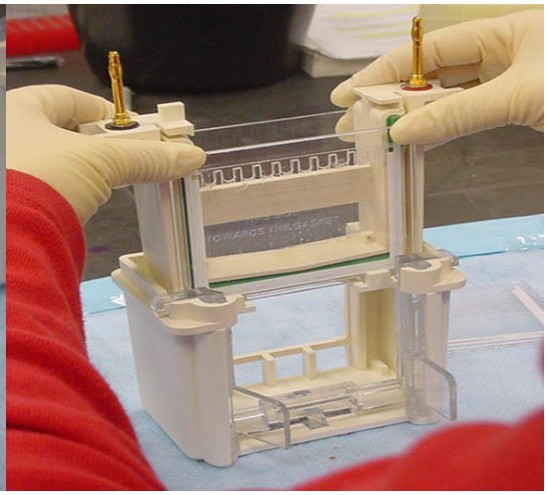
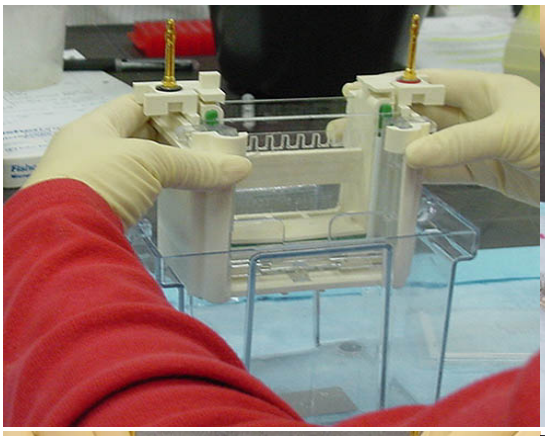
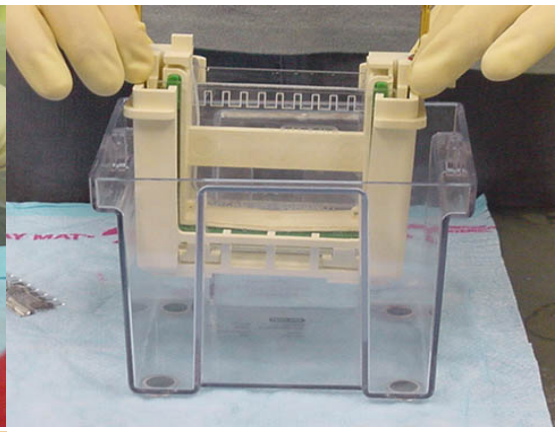
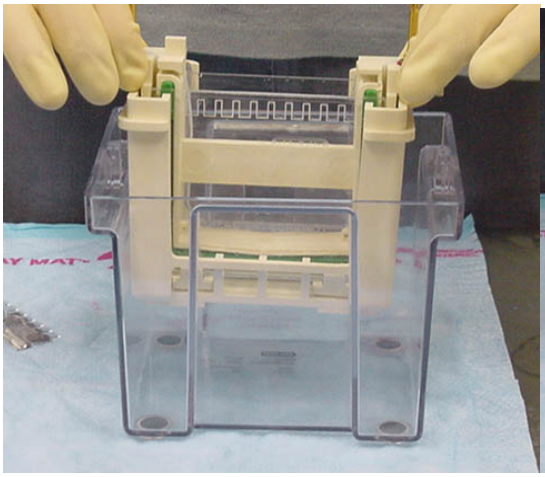
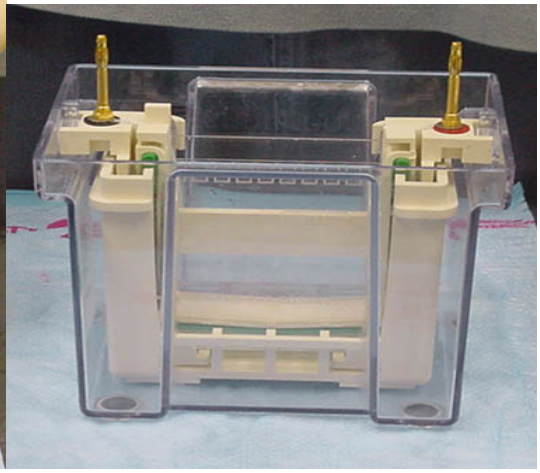
-Mix the contents of the flask by careful swirling few times.

-Immediately prior to pouring the gel, add 10% APS (the initiator of polymerization) and TEMED (the catalyst).
-Swirl GENTLY (no more than three short times!) to mix and initiate polymerization.
Note: Remember, vigorous/energetic mixing will introduce not wanted air bubbles with oxygen (inhibitor of polymerization) into your solution.
-Immediately pour the solution into gel cassette to fill it up to the marked level using disposable transfer pipette. Do it carefully and smoothly to prevent it from mixing with air.
-Overlay this layer with Isopropanol. Add it VERY SLOWLY and EVENLY to prevent mixing. Isopropanol will exclude oxygen and ensure a flat interface between the Separating and Stacking gels.
-Allow the gel to polymerize for 35 to 45 minutes. A line will become visible at the top of the gel as it polymerizes.
-Pour off the alcohol overlay into your liquid waste container at your workstation. Rinse the gel surface with dH_2O and drain the water by inverting the gel.
Note: At this point, you have a choice (well, your TA will let you know) to postpone making the Stacking gel until the next week. If you need to stop, just add 2 ml of diluted 1:4 1.5M Tris-HCl, pH 8.8 on top of the Separating gel to keep it hydrated, cover it with comb, put in a few damp brown paper towels, place in a Zipper bag and store at 4^0 C (upright in a pegged rack).

B)-Prepare the Stacking gel monomer solution.
-Add required ingredients (except 10% APS and TEMED) into 25 mL “Stacking gel” flask in a-proper order.
-Swirl gently to mix.
-Insert a piece of filter paper to dry the area in between the glass plates above the Stacking gel completely. Pay attention not touch the surface of the gel. (Optional)
-Add 10% APS and TEMED, swirl GENTLY to mix and immediately pour the solution between glass plates (on top of the Separating gel) until the rim of the Short plate is reached.
-Insert the comb into the gel cassette. Take care not to splash out the Stacking gel monomer solution. It is easiest to insert a comb between spacers starting at an angle and insert well 1, then well 2 and so on until it is completely inserted. Do it slowly, carefully and try to avoid trapping air bubbles under the comb. Prepare Kimtech Wipes pads to collect spills and pad dry at the bottom of your gel cassette.
-Let the Stacking gel to polymerize for 25 to 35 min.
-Cover your gel in a few damp brown paper towels to keep it hydrated and place in a Zipper bag. Store at 4^0 C (upright in a pegged rack) to use it next week (Week 2, Day 1)
Note: Obviously, the last step is omitted, if you are making the Stacking portion of your gel on the day of its use.
Note: On the day of running SDS-PAGE, gently remove the comb and rinse wells thoroughly with running buffer.

Procedure: Loading and running of the protein samples:

1. Wear gloves when handling polyacrylamide gels
2. Assemble the gel cassettes in the electrophoresis devices provided as depicted below:

Procedure: SDS-PAGE apparatus assembly and loading*:*

1. Fit gel into the inner frame. The long gel plate is facing towards you, and the shorter one is facing away from you. It is important to get a tight seal between the green gasket and the white frame on the gel. The gel and the buffer dam on the other side will form the inner buffer chamber.
2. **Step two**: The buffer dam and the gel form the inner buffer chamber. The frame is then slid into the support. Note that the gel is held in place to get a tight seal. Once the frame is inserted, the levers are closed to LOCK the frame in place. The levers must be closed at the same time. You should get some resistance.
3. **Step three**: 1X SDS-PAGE running buffer is added to the inner chamber and students should check for a tight seal. If there is no buffer leakage in the inner chamber proceed and add more buffer to the outer chamber. (Students will be provided with 10X buffer stock for Lab Section) You are now ready to proceed.
4. Remove any leftover gel and air bubbles from the wells by gently flushing the wells with running buffer using your pipette tip or a Pasteur pipette.
5. Load 15 µl of each sample using your micropipette in the following order:

Lane number (from left to right)	Contents
1	*B.amyloliquefaciens* lysate
2	*B.licheniformis* lysate
3	Commercial α-amylase from *Bacillus licheniformis*
4	Commercial α-amylase from *Aspergillus oryzae*
5	Commercial α-amylase from porcine pancreas
6	Saliva sample A
7	Saliva sample B
8	Unknown sample #1
9	Unknown sample #2
10	Protein molecular weight marker

6. Run the gel at 125 Volts for 1 hour until the bromophenol blue dye is at the bottom of the gel
7. While the gel is running prepare and activate your PVDF membrane. Typically the PVDF membrane is soaked in methanol for 30 seconds. Then the PVDF membrane is soaked in deionized water for 2 minutes. Lastly the membrane is place in cold transfer buffer for at least 5 minutes prior to the transfer (see next procedure c), first four steps)

Procedure: Transferring of the proteins from the polyacrylamide gel to the PVDF membrane:

1. Soak the PVDF membrane in 50 ml of methanol (this will activate the membrane) for 30 seconds.
2. Collect the methanol and place the PVDF membrane in 100 ml of deionized water to rinse off the methanol. Let the membrane sit in the water for exactly 2 minutes.
3. Place the PVDF membrane in 30 ml of ice-cold transfer buffer for 5 minutes.
4. Now you are ready to proceed to the next step.
5. Assemble transfer sandwich by orientating cathode, fiber pad, filter paper, gel, membrane, filter paper, fiber pad and anode so protein transfer goes in the direction of cathode to anode (see illustrated diagram). Pay special attention to prevent or remove air bubbles during assembly, as bubbles will cause interruptions in protein transfer (Anode is typically color-coded in red, while cathode is typically color-coded in black. Place the transfer sandwich into the transfer unit and place into the gel box. The transfer process will generate heat, so to avoid band smearing, use a chilling unit, pre-chilled transfer buffer, or carry out the transfer in a cold room.
6. Fill the unit with sufficient transfer buffer (see recipe) to cover the entire membrane(s).
7. Transfer for 1.5 hours at 110 V (the transfer can proceed at 30 V overnight (usually do this in the cold room)).

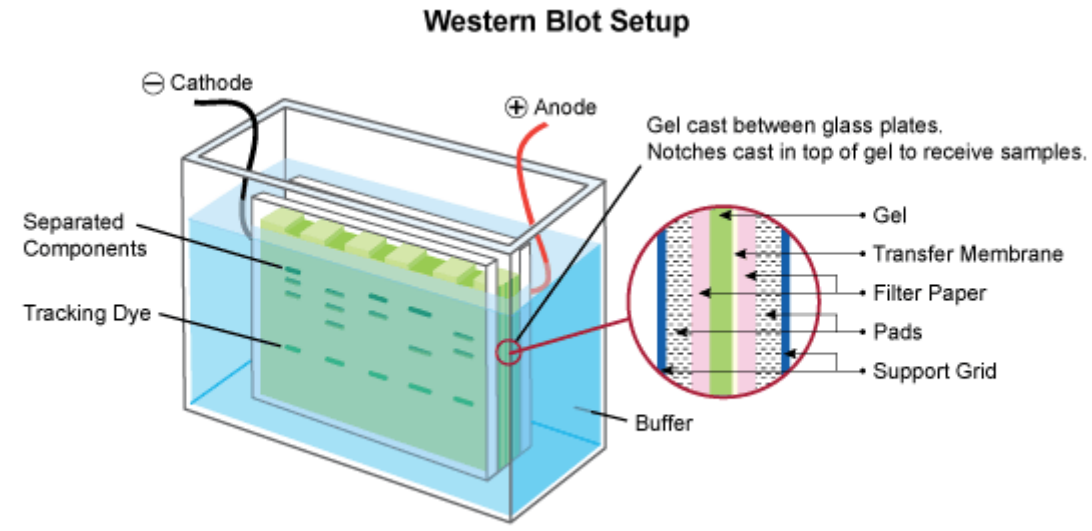


Diagram 1: *Illustration of Western Blot Setup.*

Session 7 – Laboratory 5 Activities

Procedures: Hybridization of the probe:

1. Following advice of your TA or lab tech, place your blot (with others) into one glass hybridization tube with the DNA side facing the inside of the tube.

2. Add ~20-40 ml 2x SSC and mix at room temperature for 5 minutes. Make sure that the buffer covers the top of the membranes and adjust the volume to make up for this.

3. Denature the provided aliquot (0.8-1 mL) of 10 mg/ml sheared herring sperm DNA by heating the DNA in a dry bath at 100°C for 10 minutes. Chill on ice. Add 500 µl of the denatured herring sperm DNA to 9.5ml of Southern Prehybridization Solution (50% formamide, 5 x SSC, 5 x Denhardt's reagent, 20 mM sodium phosphate, pH 6.5).

4. Discard the 2 x SSC solution from the hybridization tube**(**s**)** and add the Southern Prehybridization Solution containing the herring sperm DNA to your membranes.

5. Incubate both membranes at 42°C for at least 1 hour.

6. Near the end of the one hour, add 200 µl of your heat denatured sperm DNA (10 mg/ml) to 9.8 ml of the Southern Hybridization Solution (45% formamide, 5 x SSC, 1 x Denhardt's reagent, 20 mM sodium phosphate, pH 6.5).

7. Denature the rest (~40 µl) of your BIO-PCR probe (i.e. the PCR reaction that produced a band at ~433bp like your *B. licheniformis* DNA BIO-PCR product) by heating at 100°C for 10 minutes. Quick cool your probe on ice.

8. Add all of your denatured BIO-PCR probe to the Southern Hybridization Solution containing the denatured herring sperm DNA which was made in step 6.

9. Discard the Southern Prehybridization Solution from the hybridization tube into Prehyb/Hyb Waste container (located in Fume Hood) and replace it with the Southern Hybridization Solution/herring sperm DNA mixture containing your DNA probe.

10. Incubate the membranes at 42°C until your next lab (Week 11 Day 2).

Session 8 – Laboratory 2 Activities

Procedure: Immunological detection of α-amylase proteins

1. Using blotted membrane generated prior to this lab:
2. Place the PVDF membrane in 50 ml of TBS buffer and let it equilibrate for 5 minutes.
3. You will be provided with 100 ml of Western Blocking Buffer (WBB - 5% dry milk in TBST). First, aliquot it into two provided tubes (1^0Ab tube and 2^0Ab tube) – 20 mL/each tube for later use. Then use the rest of WBB for Step 4.
4. Discard the TBS and add 50 ml of western blocking buffer (we do this in order to minimize background) for 30 minutes-hour at room temperature
5. Discard the blocking buffer and incubate the membrane with 18-20 ml of primary antibody (1° Ab-primary antibody) solution using new square Western Blot dish. Add the primary antibody to membrane and incubate 1 hour at room temperature with shaking using the shakers provided in the laboratory.
6. Remove the primary antibody solution and wash membrane three times for 5 minutes with TBST.
7. Discard the TBST and incubate the membrane with the secondary antibody (2° Ab-secondary antibody) solution for 1 hour at room temperature with shaking.
8. Remove the 2° antibody solution and wash membrane three times for 5 minutes each in TBST buffer.
9. Add AP reaction buffer to the PDVF membrane and GENTLY rock the membrane for 2 minutes at room temperature.
10. Discard the AP reaction buffer and add 18 ml of AP substrate solution (BCIP/NBT) and allow the reaction to proceed for 15-30 minutes in the dark (place your dish in your drawer so that there is little light exposure) until you can visualize the bands on the membrane.
11. To stop the reaction, discard the AP substrate solution and add AP stop buffer. Rinse membrane one more time with AP stop buffer.
12. Discard the AP stop solution and allow the membrane to air dry. Take an image of your membrane for your laboratory report results section.

Session 8 – Laboratory 5 Activities

Procedure: Washing of the nylon membrane:

1. Discard the Hybridization Solution into Prehyb/Hyb Waste container (located in Fume Hood).
2. Add 50 ml Southern Wash Solution 1(2 x SSC, 0.1% SDS), rotate gently for 2 minutes at room temperature.
3. Discard wash and repeat step 2 with Wash Solution 1.
4. Discard Wash Solution1and add 50 ml of Southern Wash Solution 2 (0.2 x SSC, 0.1% SDS), rotate gently at room temperature for 2minutes.
5. Discard wash and repeat step 4 with Wash Solution 2.
6. Discard Wash Solution 2 and add 50 ml of Southern Wash Solution 3 (0.16x SSC, 0.1% SDS) which has been pre-warmed to 65°C. Incubate in an oven for 10 minutes at 65°C.
7. Discard wash and repeat step 6 with Wash Solution 3.

Procedure: Detection of the probe using enhanced chemiluminescence:

1. Rinse the membrane in the Hyb tube in 20ml of Tris-NaCl (100 mM Tris-HCl, pH 7.5, 150 mM NaCl) for 30 seconds.
2. You will be provided with ~65 mL of Biotin Blocking Solution (BBS - 3% BSA in Tris-NaCl). First, aliquot exact 30 mL of BBS into pre-labeled tube for HRP-Streptavidin for use in Step 4. Now, the rest of BBS stock can be used for Step 3.
3. Discard the rinse solution and add Biotin Blocking Buffer. Incubate in an oven at 65°C for 1 hour.
4. Prepare 1:5,000 dilution of HRP-Streptavidin in 30 ml of BBS.
5. Discard the Biotin Blocking Solution, transfer membranes into two separate containers and add 15ml of Biotin Blocking Solution containing 1:5,000 dilution of HRP-Streptavidin to each blot.
6. Gently rock at room temperature for 10 minutes.
7. Wash membranes with 15- 20 ml of Tris-NaCl.
8. Gently rock at room temperature for 5 minutes.
9. Repeat the Tris-NaCl wash three more times.
10. Discard the Tris-NaCl solution, place your membranes on sheet protector and incubate them with Immobilon Forte Western HRP Substrate (~2 mL/Southern Blot and ~1 mL/Dot Blot) for ~2 minutes. DO not rinse the blot after this step.
10. Use UVP ChemStudio imager in the laboratory to observe chemiluminescent signal and record results (bring a memory stick as a printer is not available). (Alternative option: Place the blots in an imaging cassette and expose signals on imaging film in the dark room.) Either way, TA is to do this.

Session 9 – Laboratory 2 Activities

Brief overview

This week covers the analysis of α-amylase proteins by use of Coomassie Blue staining which allows for the visualization of all of the polypeptide bands in an SDS-polyacrylamide gel.

Procedure: Preparation of SDS-PAGE Gel #2

Cast, load, and run SDS-PAGE gel #2 in the same way as for gel #1 (see Week 7).

Procedure: Coomassie Blue staining of the SDS-PAGE gel

1. Assemble and run your SDS-PAGE gels exactly as outlined above. The **only difference** is that after completing the electrophoresis part, the gel is **not transferred** to a PVDF membrane but taken out of the plates and used immediately for the staining with Coomassie Blue stain.
2. Add 50 ml of Coomassie Blue stain to the shallow plastic container provided.
3. Open the gel cassette by gently prying the plates apart with the small plastic spatula provided. Please ask your TA for help if you feel uncomfortable doing this part (**the plates are extremely fragile so in order to avoid injuries ask for help!!!)**
4. Submerge the plate. Gel-side down, into the stain solution and gently pry the gel off the plate by inserting the spatula under one corner of the gel.
5. **DO NOT MICROVAWE THE GEL!!!!!**
6. Place the container on the gel rocker and let it stain 20 minutes.
7. Discard the staining solution in the disposal vessel provided (**in the fume hood**) and briefly wash the gel with 200 ml of deionized water.
8. Add 50 ml of Coomassie destain solution and place a few Kim wipes into the container to absorb the dye.
9. Rock the gel on the gel rocker for 15 minutes. Protein bands should become visible within this time frame. The gel can be stored in destain solution indefinitely.
10. Photograph your gel after you place it on plastic wrap. Use the image for your laboratory report.
11. Discard of the destain solution in the disposal container provided (**in the fume hood**).

Appendix A
Preparation of Agarose Gel

Casting an agarose gel:

- First, prepare 1X LAB buffer from 25X stock provided. Typically, you need to make 300 mL of 1X LAB (40 mL to make an agarose gel and 225 mL to use as a running buffer in electrophoresis tank)

- Use the 125 ml flask that is provided to you.

- Measure 40 ml of 1X LAB buffer using a graduated 50 ml cylinder and pour into the flask

- Weigh the proper amount of agarose in order to prepare the desired gel by weight per volume (w/v).

Use the table below as a guide:

Gel Percentage	**0.7 %**	**0.8%**	**1%**	**1.2 %**
Agarose (g)	0.28	0.32	0.4	0.48
1 X LAB (ml)	40	40	40	40

- Swirl the agarose in the flask to disperse evenly

- Microwave the flask on high for 30-60 seconds until the agarose is melted. Remove the flask using gloves (**Be careful!!! Hot liquid!!!!**) and allow the agarose solution to cool off.

- When the solution is relatively cool then add 2 µl of 10 mg/ml ethidium bromide solution and make sure that you swirl carefully so that the ethidium bromide is evenly distributed. (CAUTION**: ETHIDIUM BROMIDE is a potent MUTAGEN!!!!)**