

ONE WEEK MOLECULAR BIOLOGY WORKSHOP 2005

(December 2nd to December 6th, 2005)

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1 WEEK FLOWCHART:

p9	DAY 1:	am: LAB A: Purification of genomic DNA using phenol chloroform. pm: LAB A: Precipitation of DNA and dissolve in TE buffer. LAB B1: Set up digests.
p13	DAY 2:	am: LAB B1: Purification kits, CIP and ligation pm: LAB B2: Transformation procedures.
p18	DAY 3:	am: LAB B3: Look at plates and set up cultures LAB C1/C2: PAGE pouring and running protein samples. pm: LAB C2: Transfer of proteins. LAB C3: Western blot analysis
p24	DAY 4:	am: LAB B4: Mini Plasmid preparations am/pm: LAB D1: Total RNA prep LAB D2: Reverse Transcriptase + real time PCR.
p28	DAY 5:	am: LAB D2: look at real time data. am/pm: LAB E: DNA fingerprint assay (polymerase chain reaction).

CONTENTS BY STREAM:

- LAB A: PREPARATION OF GENOMIC DNA FROM CORN SHOOTS.
- LAB B: AN EXERCISE IN CLONING TECHNIQUES (INCLUDING RESTRICTION DIGESTS, PURIFICATION KITS, CIP ASSAYS, LIGATIONS, TRANSFORMATION, PLASMID PREPS)
- LAB C: DETECTION OF PROTEIN EXPRESSION FROM CLONED GENES BY WESTERN BLOTTING.
- LAB D: RNA WORK: ISOLATION/PURIFICATION AND REVERSE TRANSCRIPTASE ASSAY. INTRO TO REAL TIME PCR.
- LAB E: POLYMERASE CHAIN REACTION.

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 barefeet are allowed.
- 4 **EXTREME CAUTION:** must be taken when handling the following hazardous chemicals/materials in the lab:
 1. **Ethidium Bromide (EtBr).** Always wear gloves, and check gloves for leaks before handling. Handle only on covered countertops and EtBr staining of gels must use designated equipment only. Workbenches must be monitored with UV light (wear UV shield) after using EtBr; contaminated workbench covers must be changed promptly. Dilute gel staining solutions must be flushed in the sink by letting tap water run for sufficient amount of time. EtBr contaminated wastes must be disposed of in designated containers only, liquid and solid wastes are segregated.
 2. **Phenol and chloroform.** Leakproof gloves must be worn! Phenol-chloroform extractions should be done in the fumehood, and wastes must be disposed of in designated containers only.
 3. **Ultraviolet (UV) transilluminators.** Protective goggles or face shields must be worn before turning on any UV light source. Exposure must be kept a minimal.
 4. **Radioisotopes.** Leakproof gloves and protective goggles must be worn. Handling must be under the appropriate shielding. Disposal must be into designated containers only, with strict monitoring of possible spills.
 5. **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.
 6. **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.

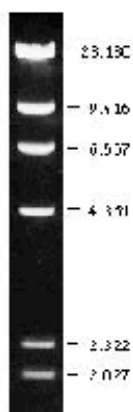
- 7 Take gloves off from hands before touching any fixtures in the lab (i.e. telephones, door knobs, common equipment unless designated otherwise, books, etc) whether or not you think the gloves are clean.
- 8 Wash hands thoroughly before leaving the laboratory.

Lambda DNA-Hind III Digest

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

Description: The Hind III digest of lambda DNA (cl857/ind 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 digested to completion with Hind III, phenol extracted and dialyzed against 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA.

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

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 dH₂O and subsequently heated.

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

Prestained Protein Marker, Broad Range (6–175 kDa)

Premixed Format

#P7708S 175 mini-blot lanes \$85 (USA)
 #P7708L 875 mini-blot lanes \$340 (USA)

Component Sold Separately:

Red Loading Buffer Pack

3X Red Loading Buffer (8 ml)
 30X Reducing Agent (1 ml)
 #B7709S \$15 (USA)

Description

Prestained Protein Marker, Broad Range is a mixture of purified proteins covalently coupled to a blue dye that resolves to 8 bands when electrophoresed. The protein concentrations are carefully balanced for even intensity. The covalent coupling of the dye to the proteins affects their electrophoretic behavior in SDS-PAGE gels relative to unstained proteins. The apparent molecular weight of the prestained proteins are given in the table below. For precise molecular weight determinations, use NEB's unstained Protein Marker, Broad Range (NEB #P7702) in addition to the prestained marker.

PROTEIN	SOURCE	APPARENT MW (Da)
MBP- β -galactosidase ¹	<i>E. coli</i>	175,000
MBP-paramyosin ¹	<i>E. coli</i>	83,000
Glutamic dehydrogenase	bovine liver	62,000
Aldolase	rabbit muscle	47,500
Triosephosphate isomerase	rabbit muscle	32,500
β -Lactoglobulin A	bovine milk	25,000
Lysozyme	chicken egg white	16,500
Aprotinin	bovine lung	6,500

¹ MBP - maltose-binding protein.

MBP- β -galactosidase - fusion of MBP and β -galactosidase.

MBP-paramyosin - fusion of MBP and paramyosin.

Contents

0.1–0.2 mg/ml of each protein in 70 mM Tris-HCl (pH 6.8 @ 25°C), 33 mM NaCl, 1 mM Na₂EDTA, 2% (w/v) SDS, 40 mM DTT, 0.01% (w/v) phenol red and 10% glycerol.

(see other side)



10–20%
SDS-PAGE

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 Ladder

#N3232S 100 µg \$55 (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

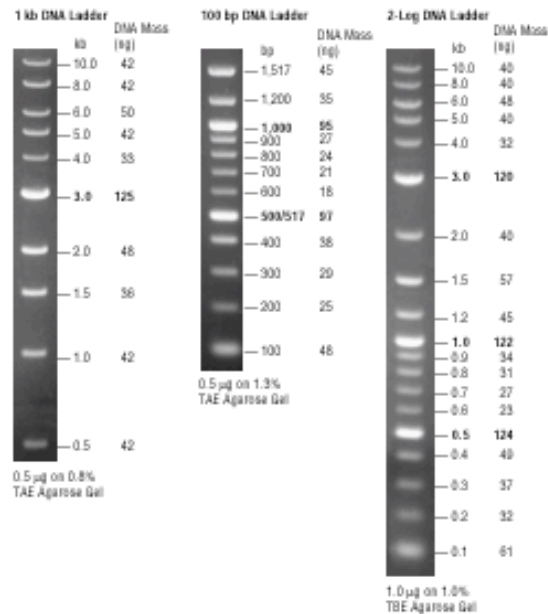
Description: The 2-Log DNA Ladder has a number of proprietary plasmids that are digested to completion with appropriate restriction enzymes to yield 19 bands suitable for use as molecular weight standards for agarose gel electrophoresis. This digested DNA includes fragments ranging from 100 bp to 10 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 proprietary 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 proprietary 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 Tris-HCl (pH 8.0), 1.0 mM EDTA. For long term storage store at –20°C. All DNA Ladders are stable for at least 3 months at 4°C.

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 Tris-HCl (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). Use α-³²P dATP or α-³²P dTTP for the fill-in reaction.

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

Reference:

1. 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.

Day 1:

LAB A - "some basics - getting genomic DNA"

PURIFICATION OF GENOMIC DNA FROM CORN SHOOTS.

The following procedure is modified from the Proteinase K digestion/phenol-chloroform purification process by Strauss (1987, pub. in Current Protocols in Molecular Biology. Ausubel et al. eds. pp2.2.1-2, which is still in routine use for the preparation of genomic material from many organisms. In this case, DNA will be isolated from corn shoots by lysis in Sarkosyl and proteinase K followed by phenol-chloroform extraction and ethanol precipitation. The DNA isolated by this procedure is sufficiently pure for restriction enzyme digestion. Both of these particular procedures whilst still in common use are often replaced by newer silica based technologies.

Stuff to know:

- *Phenol-Chloroform is bad for you. Note handling procedures discusses in class.*
- *We are most interested in genomic DNA which can shear very easily. Although, we are not using strict techniques to prevent shearing, we want to be relatively gentle with our samples today.*

Procedure:

1. Harvest 2 grams of shoot tissue from the seedlings provided. **Do this as quick as you can in order to keep things cold.** Add liquid nitrogen to cover the shoots, and grind the sample to a powder as rapidly as you can. Keep the sample frozen throughout this process.
2. Transfer the powder to a 50ml screwcap Nalgene Teflon tube. This is located in your ice bucket with 4.5ml of cold Digestion Buffer. **Make sure the powdered tissue is thoroughly dispersed in the buffer by gently swirling the tube each time the sample is added.**
3. Add 40ul of Proteinase K (25mg/ml stock solution) to give a final concentration of 0.2mg/ml. Mix gently. Add 0.5ml of 10% sarkosyl to give a final concentration of 1%. Again, mix the samples very gently.
4. Incubate the mixture at 55°C with gentle shaking for about 2 hours (generally people will do this step for much longer).
5. Take the sample to the fumehood. With gloved hands, add an equal volume of phenol mixture. **CAUTION: Phenol is toxic and extremely caustic. Avoid contact with skin. Open containers and dispense solutions of phenol-chloroform in the fumehood only!** *Phenol is also buffer saturated*

so take a close look at the container. You will notice that there may be two layers of liquid. The bottom layer is the organic phenol layer. Cap the tube securely and mix the contents thoroughly but gently by inversion until the mixture is homogeneous.

6. Centrifuge at 10000rpm in a JA-14 rotor for 10 minutes at 4°C. Transfer the aqueous phase (top layer) into a fresh tube with a p1000 pipettor and blue tip taking care not to disturb the interphase. Repeat the extraction of the aqueous phase with phenol mixture once more (or until the interphase looks relatively clear). Transfer the last aqueous phase to a 15 ml screwcap conical FALCON tube.
7. Add an equal volume of a 24:1 mixture of chloroform:isoamyl alcohol. Mix thoroughly but gently. Centrifuge in the tabletop BECKMAN centrifuge for 5 minutes at 3000rpm.
8. Transfer the aqueous phase to a sterile siliconized 30ml Corex glass tube using a sterile siliconized Pasteur pipet. **NOTE: Take care not to disturb the interphase at this step. In your final aqueous sample, you do not want any organic material to carry over.**
9. Determine the volume of the aqueous phase using an empty glass pipette. Add 0.5 volume of 7.5M NH₄OAc. Mix briefly, then add 2 volumes of 100% ethanol. Cover the tube securely with parafilm and mix the contents gently by inversion. A white stringy precipitate should appear.
10. Prepare a sealed pasteur pipette (**do not use siliconized pasteur pipettes**) using a bunsen burner (*there will be a quick demo*), and allow the tip to cool. Once cool (wait a minute or two), you will use this pipette to carefully spool out your DNA.
11. Dip the spooled DNA into a solution of 70% ethanol. You can put 1.0ml of 70% ethanol in a microfuge tube. Carefully swirl the pipette tip in the solution taking care not to dislodge your DNA. **NOTE: the 70% wash will not dissolve the DNA pellet but will remove excess salts.**
12. Place the pasteur pipette with the tip facing up for approximately 10 minutes. This will allow our DNA pellet to dry so that all trace of ethanol has evaporated.
13. Dip the pipette tip in 1 ml TE (pH8) and resuspend until your DNA pellet has been dislodged from the pasteur pipette and is now sitting in your solution **NOTE you may need to use a sterile yellow tip to help dislodge the DNA.** Incubate at 55°C until pellet is dissolved. Will take a minimum of 1 hour, but we will incubate for a full overnight step.

LAB B1 - “some cloning, some kits”

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

DNA cloning is a powerful technique in molecular biology to generate specific recombinant DNA molecules intended for a variety of uses:

- a) To obtain large quantities of specific DNA sequences for use in studying gene structure and gene regulation, DNA/protein sequence determination, and for in vitro mutagenesis of nucleic acid sequence.
- 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 this section of the course, we will prepare fragments by digesting lambda DNA with HindIII. The fragments produced will represent our “insert.” We will also prepare a cut plasmid vector by digesting pUC18 with Hind III. With these two samples, we will attempt to ligate our “inserts” into our “cut vector.”

We will then detect the presence of successful ligations by transforming bacteria with our ligation mixture. Essentially only successful ligations (recirculized plasmid, or recircularize plasmid plus insert) will allow colonies to form upon transformation.

Stuff to know:

- *We will using the ChargeSwitch procedure, based primarily on pKa manipulation of a magnetic bead . It is important to note that there are other kit systems as well as the phenol/chloroform procedure that work well to clean plasmid / insert DNA.*
- *Tomorrow, you will load your samples onto the gel, but you may carry on to the next step as soon as you have loaded your sample, so we will prepare that gel ahead of time.*

LAB B1 – “and so it starts...”

Procedure:

1. You have two microcentrifuge tubes that contain the DNA to be digested. (one with a plasmid called pUC18, and one with lambda DNA to be cut up for our inserts).

2. Add the various restriction digestion components to each tube as follows:

Tube	H ₂ O(ul)	10x REACT#2	DNA	HindIII
pUC18	23	5ul	20ul (pUC18)	2ul
lambda	23	5ul	20ul (lambda)	2ul

note: both the volumes of pUC18 and lambda are at 20ul. You can even use these tubes to add the reagents to.

3. Incubate for overnight in 37°C waterbath. While the digestion is beginning, you will need to prepare one 0.8% agarose gel before you leave today.

**While the DNA samples are incubating with restriction enzymes, 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. Weigh out 0.4g of agarose and transfer to a 100ml flask. Add 50mls of 1x TBE buffer and swirl gently to disperse the agarose. Microwave the mixture on high power until it boils and the agarose is completely dissolved. Look for the occurrence of “chunkies” in your mixture. The dissolving step is a fine line between boiling your sample enough to dissolve your material, but not boiling it too much so that liquid starts to evaporate.*

*Allow the solution to cool to 60°C by incubating in a 60°C waterbath for about 10 minutes. Then add 1ul of 10mg/ml Ethidium Bromide stock **WARNING: EtBr is a carcinogen! Do not handle without wearing gloves and avoid spills.***

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

**Pour the cooled agarose into the plate – don't forget the comb! The gel will take approximately 20 minutes to set. We will leave it overnight at 4C in the dark.*

Day 2:

LAB B1 - “purification, gels, and more enzymes”

- 1, Take out your digests, and you will transfer 2ul of each reaction mix into two new microfuge tubes that contain 8ul of distilled water. Add 2ul of DNA loading buffer (blue stuff) to each of the new tubes, and load these samples onto the gel you poured. **NOTE: this gel is a visual check to compare and contrast your DNA samples (after digestion) with some control uncut DNA samples (in your ice bucket, 10ul of labeled control pUC18, 10ul control lambda, both blue in colour, to be loaded with your digests).**
2. With the rest of your digest samples (i.e. the majority of your digest sample, ~48ul), you will perform nucleic acid purifications as according to the chargeswitch procedure.

CHARGESWITCH:

3. To your pUC18 and lambda samples, add 50ul Purification Buffer (N5).
4. Add 10ul ChargeSwitch Magnetic Beads to the tube and pipet up and down gently to mix without forming bubbles.
5. Incubate at room temperature for 1 minute.
6. Place the sample on MagnaRack for 1 minute or until beads have formed a tight pellet.
7. Without removing the tube from the MagnaRack, carefully remove and discard the supernatant without disturbing pellet by angling the pipette such that the tip is away from pellet.
8. Remove the tube containing the pelleted magnetic beads from the MagnaRack.
9. Add 150ul Wash Buffer (W12) to the tube and pipet up and down gently to mix the sample without forming bubbles.
10. Place the sample on the MagnaRack for 1 minute or until beads have formed a tight pellet.
11. Without removing the tube from the MagnaRack carefully remove and discard the supernatant without disturbing the pellet of beads.

12. Repeat steps 9 to 11 once.
 13. Remove the tube containing the pelleted magnetic beads from the MagnaRack.
 14. Add 20ul Elution Buffer (E5: 10mM Tris-HCl, pH8.5) to the tube and pipet up and down gently to mix the samples without forming bubbles.
 15. Incubate at room temperature for 1 minute.
 16. Place Rack on MagnaRack for 1 minute or until beads have formed a tight pellet.
 17. Without removing the tube from the MagnaRack, carefully transfer the supernatant containing the purified DNA product to a sterile microcentrifuge tube without disturbing the pellet of beads. These are your purified products.
- END OF CHARGESWITCH**
18. Set aside the purified lambda digests in your ice bucket until the ligation step. With your pUC18 plasmid sample, transfer 4ul into another microcentrifuge tube (THIS IS IMPORTANT: you will use this stuff in the ligations later. This will be your digested non-CIP pUC18 sample).
 19. With the remaining 16ul, begin a dephosphorylation assay with your purified plasmid sample: To the pUC18 tube (you should have 16ul of it now), add 5ul of 10x CIP buffer, 28ul sterile dH₂O, and 1ul of 0.1U/ul CIP (calf intestinal phosphatase)
 20. Incubate at 37°C for 1 hour.
 21. Purify the CIP'd or dephosphorylated plasmid by ChargeSwitch (step 3) procedure as before.
 22. O.K. a quick check of what you have for your ligations... You should have purified dephosphorylated pUC18 (from step 21). You should have purified lambda DNA fragments (from step 18), and you should have purified pUC18 that has not been dephosphorylated (from step 18).
 23. Label five tubes A to E (or give them descriptive names to help you keep track of what they are).

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 before adding the ligase.**

	A	B	C	D	E
nonCIP plasmid	1	1	0	0	0
CIP pUC18	0	0	2	2	0
lambda DNA cut	0	5	5	5	0
control pUC18 + CIP	0	0	0	0	3
control lambda DNA cut	0	0	0	0	4
5x ligase buffer	2	2	2	2	2
dH2O	6	0	0	0	0
T4 DNA ligase	1	1	1	1	1

27. Incubate 15 to 30 minutes at room temperature.

LAB B2 - “getting DNA in bugs”

TRANSFORMATION OF COMPETENT CELLS

This week 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.

Stuff to know:

- *Competent cells are very delicate. Be gentle when handling them.*
- *The two transformation procedures have separate 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:

- 1 Prepare 9 fresh microcentrifuge tubes and label them from A(t) to I(t) – these will be the tubes where the heat shock transformation reactions take place. Keep on ice.
- 2 Competent cells (labeled “M”) will be brought out and placed in ice bucket near the start of the class. Carefully transfer 20ul of competent cells to each of your 9 transformation tubes. Be very gentle – heat shock competent cells are notoriously delicate.
- 3 - To tubes **A(t) to E(t)**, add 1ul of the diluted ligation mixes (from step 2);
 - to tube **F(t)**, add 5ul (0.05ng) of BRL control DNA (pUC19);
 - to tube **G(t)**, add 5ul (0.5ng) of undigested pUC18 control;
 - to tube **H(t)**, add 1ul unligated cut pUC18 (the purified nonCIP pUC18 that was saved from yesterday);
 - to tube **I(t)**, add 1ul sterile distilled H2O.

- 4 Leave the cell/DNA mixtures on ice for 30 minutes. Place SOC media in the 37°C waterbath to prewarm. During this time we will perform the electroporation procedure.

The following procedure is as recommended for use with the BRL CellPorator. Electroporation will be done using only the same BRL control DNA as in tube F(t). This way, a direct comparison between the two procedures can be done.

- Label two microcentrifuge tubes (one will be for your plasmid, and one will be a water control). To each tube add 20ul of electrocompetent cells (labeled "E")

- To one tube, add 1ul (0.01ng) of BRL control DNA. To the other tube, add 1ul of dH₂O. Mix gently, by tapping the bottom of the tubes.

- At this point, there will be demonstration on how to use the electroporator and how to set up the cuvettes.

- Pipet the cell mixtures into the corresponding electroporation cuvettes – try not to introduce air bubbles.

- Place the cuvettes in the electroporator and deliver voltage through your sample under the following settings. (400V, 330uF capacitance, low W impedance, fast charge rate, 4000 W resistance on voltage booster)

- After electroporation, carefully pipette out your samples back into your labeled tubes. Add 980ul of SOC media to each of your two tubes and incubate tubes for about 1 hour at 37°C with shaking. (if you have trouble pipetting out your zapped competent cells, you can also add SOC to the cuvette and take out the solution in that manner).

- 5 NOW,... back to your original 9 tubes. .. After the 30minutes on ice, 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!**
- 6 Immediately transfer to ice. Then add 480ul of the prewarmed SOC media to each tube. Mix gently.
- 7 Incubate at 37°C for about 1hr with shaking (this will roughly coincide with your electroporated samples).
- 8 You now have a total of 11 tubes to take care of (nine of which were heat shocked, and two of which were electroporated).
- 9 For your heat-shocked samples, place 50ul 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 the turntable and "hockey sticks" (This will be quickly demonstrated).

- 10 For your electroporated samples, first take 50ul of your sample and add it to 950ul of SOC (i.e. you are diluting it further by 20 fold). Plate 50ul of the dilution onto appropriately labeled LB plates containing ampicillin and X-gal.
- 11 Incubate all 11 plates overnight at 37°C in an inverted position (agar side up).

Day 3:

LAB B3 - “set up culture for minipreps”

Procedure:

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 2mls of LB broth + 50ug/ml ampicillin with a single colony of transformed bacteria. **NOTE: don't forget you will be inoculating a total of 7 cultures (see above).** 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.

LAB C - “Looking at proteins”

DETECTION OF PROTEIN EXPRESSION FROM CLONED GENES BY WESTERN BLOTTING

Western blotting of proteins is analogous to Southern Blotting of DNA. 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.

Stuff to Know:

- Essentially, you will be running two polyacrylamide gels with the intent of performing a western blot on one of them. The samples that you will be running are three different bacterial cultures all containing 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 alone. This will express the GST domain protein by itself. (~26kDa)*
 - ii. *pGEX-2T LCK. This will express a GST / wildtype LCK fusion protein (~85kDa)*
 - iii. *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...)*
- You will be using that nasty neurotoxin, acrylamide. Please be careful with this stuff!

Procedure:

LAB C1 - “pouring a protein gel”

Gel Part:

1. You will first pour two polyacrylamide gels 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):

5mls 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-acrylamide
25ul 10% ammonium persulfate
5ul 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.

LAB C2 - "run and transfer our proteins"

Procedure:

- Take your protein gels out from the fridge. We will be running bacterial samples in them today. Use the below instructions for sample preparation.
 - You will have three microfuge tubes labeled "GST", "N32" and "LCK." In each tube is 50ul of the aforementioned bacterial culture.
 - To each tube, add 25ul of a 3x sample buffer (blue stuff) to each tube. Now, take your three samples and your prestain standards ("STD" tube with pink liquid), and boil them for a minimum of 8 minutes.
- 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.
- You are now ready to load your samples. You can load using your p20 pipette 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 one will be stained for total protein, and the other will be used for western analysis**).

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

**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.*

- 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 ~ 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.

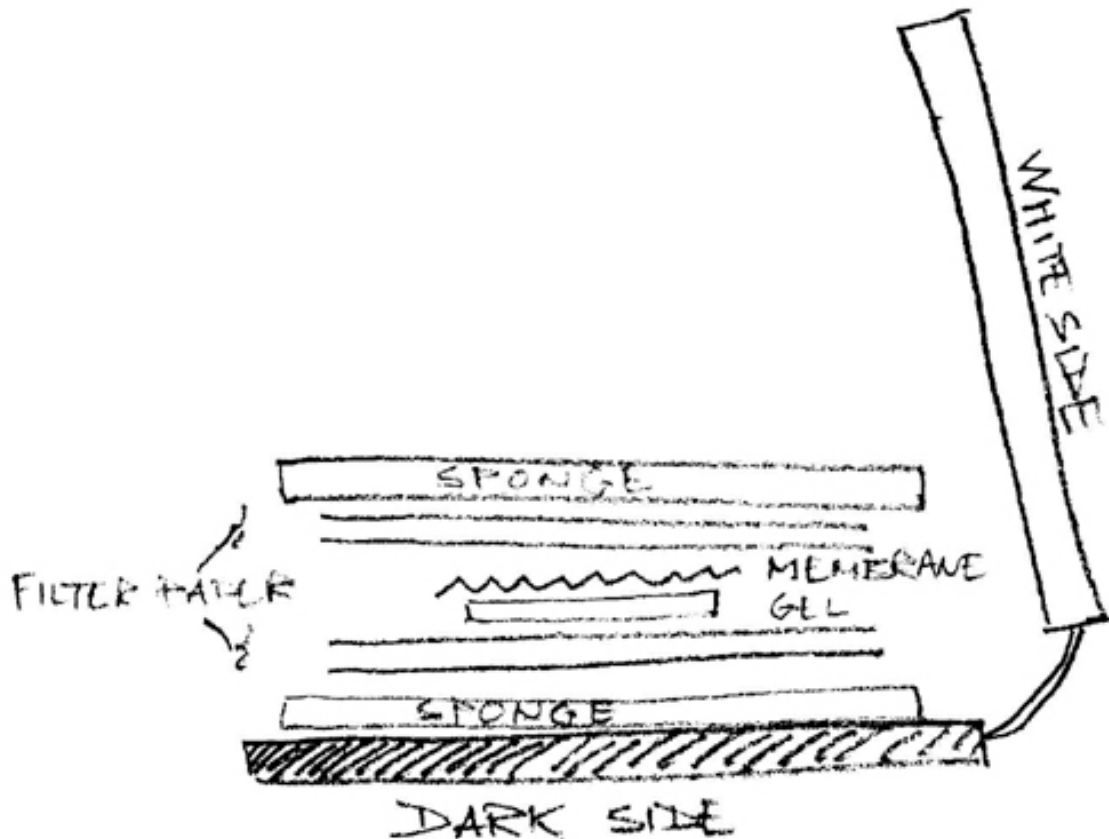
at this point, one of your gels will be used to begin the transfer

procedure, while the other will be stained in the following manner.

- carefully place the acrylamide gel into a plastic container filled with coomassie solution (you only need enough stain to cover the gel). Let the gel incubate, rocking, for a minimum of 20 minutes. After 20 minutes, pour the stain back into a special container marked "used stain." Add destain solution to your gel and incubate for 20minutes to overnight. Repeat until the stain has gone from the gel.

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 accidentally 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. BIORAD has been clever enough to make sure all their transfer systems are colour coordinated. The plastic transfer cassette ALWAYS has a black side. This does not represent evil. This represents negative charge: your proteins will migrate away from the black side.



So when you set up the transfer, do so in the following manner (this will be demonstrated). Put gloves on...

- a) In the large plastic container, place your plastic transfer cassette with the black side flat. Add transfer/methanol buffer to submerge the cassette. Take one of the prewetted sponges (rinse in tap and then in distilled water), place on top of black side.
In subsequent steps, make sure everything is submerged in transfer buffer.
- b) Add two pieces of Whatman filter paper on top of the sponge. Place your gel next. AND THEN place your membrane on top of the gel. Place two more pieces of Whatman filter paper on top and make sure you get rid of bubbles in between this sandwiched set-up. Place other prewetted sponge on top of all of this, and close the cassette.
- c) Place cassette in transfer holder (remember to make sure everything is colour coordinated). Add ice holder. Fill chamber up with transfer/methanol buffer (you may have to use the stuff in your big plastic container). Plonk the whole thing in your ice bucket. Put the lid on and set voltage at 100V. The transfer will take approximately 1 hour.
During this time, it is appreciated if you wash the plates and gel apparatus with tap water rinsed with distilled water in the sink.

8. When an hour 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.

LAB C3 - “develop our westerns”

Procedure:

Western Part: (Quick Method devised by Millipore for use with Immobulon P membrane).

1. After drying your membrane, add your primary antibody solution (10mls of anti-p56lck rabbit antisera “54-3B” at 1/2500 dilution in TBS+Tween20 and 5% BSA) 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 approximately 20mls (you can measure it the first time, and eyeball it from that point on) TBS+Tween. Shake by hand for about 10 seconds and dump the solution into the sink.
3. Add your secondary antibody solution to your membrane (10ml Goat anti-rabbit 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 6 to 8 washes. Wash once in buffer that does not have Tween20 detergent (we will use 0.1M Tris pH9.5). Your membrane is now ready for substrate detection protocol which will be outlined in class (**we will be using the ASBI-Fast Red**).

Day 4:

LAB B4 - “quick miniplasmid preps”

MINI PLASMID PREPARATIONS USING LYSOZYME/HIGH HEAT PROCEDURE.

Today is a relatively easy day, where 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.

Stuff to know:

- Another very 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, we are only doing a relatively impure prep in that we are not spending additional time purifying our sample (we are only doing a precipitation as an enrichment step). This is good enough for our purpose of performing a restriction digest. If the sample is to be used for more fussy procedures (i.e. sequencing, ligations, etc), it is recommended that you perform a purification step (i.e. kit, phenol/chloroform, etc).

Procedure:

1. Vortex each culture thoroughly (the ones you prepared yesterday from picked colonies), and transfer 1ml of each culture into a clean microfuge tube. Store the rest of the culture in the refrigerator as backup.
2. Spin the cells down for 30 seconds at maximum speed in the microcentrifuge. Remove all of the supernatant by pipeting out the last bit of media left.
3. Add 200ul of STET buffer to your cells and resuspend by vortexing.
4. To your cell mixtures, add 20ul of 20mg/ml lysozyme solution in 25mM Tris-HCl, pH8. Vortex briefly, and place tubes in a boiling waterbath for 60 seconds. **Do not overboil and don't forget to use a lid lock.**
5. Immediately spin for 10 minutes at maximum rpms in the microcentrifuge.
6. Remove the pellets (predominantly chromosomal DNA) with the flat end of a sterile toothpick. You will find that the pellet will stick nicely to the toothpick without mixing in with the supernatant. Discard the pellet.
7. Add an equal volume of ice-cold isopropanol to the supernatants. Mix by inversion several times. Chill for 10 minutes at -20°C.

8. Spin down DNA precipitates for 5 minutes at maximum speed in the microcentrifuge. Wash pellet with 250ul of 70% ethanol. Spin down for 1 minute. Remove the supernatant.
9. Air-dry the pellets or dry in SpeedVac for 10 minutes.
10. Dissolve the pellet in distilled water (for small pellets use 20ul)
11. You will now set up 7 restriction digests with the following recipe for each digest:

12ul	dH ₂ O
2ul	10X React2 Buffer
5ul	plasmid prep
1ul	HindIII (10U/ul)

Incubate for 1 hour 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 containing 8 times (since we have 7 samples) the amount of everything except the DNA. (i.e. 96ul of water, 16ul of the React Buffer, etc, etc.). This way, to your DNA you can just add 15ul 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.
12. Prepare a 0.8% Agarose gel as previously outlined in Day 1 (don't forget the ethidium bromide).
13. After the digest incubation, add 4ul of DNA loading buffer to each tube and load samples - along with a IHindIII marker - to your agarose gel. Run gel at 100V for approximately 1 - 2 hours. Visualize and photograph gel.

LAB D - "Reverse Transcriptase/Real Time-PCR procedure"

In this procedure you will perform a reverse transcriptase real time polymerase chain reaction experiment using a 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.

Stuff to know:

- As mentioned before, RNA is extremely sensitive to degradation (We'll get into this in class). Make a special effort to be as aseptic as possible – i.e. wear gloves all the time and change them regularly.

LAB D1 – “Total RNA preparation direct from cheek cells”

Procedure:

- 1 You will perform a cheek cell rinse using the saline solution, cup, and 1500rpm centrifugation spin (flowchart shown in class).
- 2 Carefully decant the supernatant without disturbing the pellet. **You want the pellet!** After the supernatant has been removed, flick the bottom of the tube gently to loosen up the pellet – this will help in the resuspension of the pellet later. Add 1mls ice-cold PBS to the loosened pellet and resuspend cells gently by swirling the FALCON tube.
- 3 Transfer the suspension to a microfuge tube. Spin the cell mixture using the microcentrifuge at a setting of 3 (**this is important!**) for 5min. The PBS step is used to essentially give your cells a bit of a rinse. Discard the supernatant.
- 4 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. **NOTE: Trizol contains phenol so take care!** Incubate the microfuge at room temperature for 5 minutes. Add 200ul of chloroform, shake vigorously for 15seconds and incubate at room temperature further for about 3 minutes.
- 5 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.
- 6 Transfer the upper phase to a fresh microcentrifuge tube. Take care not to disturb the interphase when retrieving the aqueous phase.
- 7 Precipitate the RNA by adding 500ul of isopropanol (isopropyl alcohol), and letting it incubate at room temperature for 3 minutes.
- 8 Centrifuge at 12000rpm for 10 minutes at 4°C. Wash the pellet with 1ml of 70% ethanol and spin again at 12000rpm for 5 minutes.
- 9 Keep the pellet and air dry for approximately 10 minutes (Do NOT overdry). Dissolve the pellet in 100ul of DEPC treated distilled water.

LAB D2 – “The RT-PCR part.”

1. Using BioRad's iScript cDNA synthesis kit, each group will set up 3 reverse transcriptase reactions using the following table.

TABLE:

iScript Reaction Mix	4ul
iScript Reverse Transcriptase	1ul
Nuclease-free water	5ul
RNA template (100fg to 1ug total RNA)	10ul*

- There will be a demo at this point with the MyiQ system, but the assay will be performed under the following parameters:

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

- Once the reverse transcriptase portion is finished, we can then set up our real time PCR reactions. Roughly, we will use the following reaction set up, but specific details will be announced in class. **Note:** There will be a demo on setting up the software on the MyiQ system, as well as an opportunity to analyse our data tomorrow.

Component	Volume per reaction	Final concentration
iQ SYBR Green Supermix	12.5 µl	1X
Primer Mix	0.5 µl	100 nM–500 nM
Sterile water	9.5 µl	
DNA template	1.0 µl	
Total Volume	25 µl	

Primer Mix contains 6.25 mM of each primer (Forward and Reverse). Adding to mixture results in a Dilution of 25 and an ultimate concentration for each primer of 250 nM. The recommended amount of primer based on the MyiQ Supermix is 100 - 500 nM of each primer.

- Load samples into MyiQ cycler programed 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 steps 2 – 4 (x 50)		
STEP 6	72C	1 minutes	Final Elongation
STEP 7	50C	30 seconds	
MELT FUNCTION +1°C/30 seconds for 45°C			

Day 5:

LAB E - “who has the most crap”

A SIMPLE FINGERPRINT ASSAY USING POLYMERASE CHAIN REACTION

Last day! To ease off in our final day (apart from checking out yesterday's real time data), today's procedure will follow a simple handout that will be given in class. In short, we will be attempting to do a simplified fingerprint assay of our own genomic DNA. Have fun...

Buffers and solutions:

Phosphate-buffer saline (PBS) pH7.3

137mM NaCl
2.7mM KCl
4.3mM Na₂HPO₄·7H₂O
1.4mM KH₂PO₄

Digestion Buffer

100mM NaCl
20mM Tris-HCl, pH8
25mM EDTA, pH8
Added after resuspending cells:
1% SDS and 0.2mg/ml Proteinase K

TE (pH8)

10mM Tris-HCl, pH8
1mM Na₂EDTA

Phenol-Chloroform Mixture:

25 parts buffer equilibrated phenol
24 parts chloroform
1 part isoamyl alcohol

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

10x MOPS Running Buffer

0.4M Mops
0.1M NaOAc
10mM EDTA
up to 1L with DEPC treated ddH₂O

Resolving Buffer:

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

Stacking Buffer:

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

3x Sample Loading Buffer:

150mM Tris pH6.8
6mM Na₂EDTA
3% SDS
3% b-mercaptoethanol
24% glycerol
speck of bromophenol blue.

10x Electrophoresis Buffer

30g Tris Base
144g Glycine
10g SDS
Add dH₂O to 1L.

Coomassie Blue Stain

3.75g Coomassie Brilliant Blue R-250
750ml Methanol
600ml dH₂O
Stir 2 to 3 hours. Filter through Whatmas #1 filter paper.

Destain Solution

5% Methanol
10% acetic acid
85% dH₂O

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 MgCl₂

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 H₂O

10ml of 250mM KCl

Adjust pH to 7.0 with 5M NaOH

Adjust volume to 1L

Autoclave. (Just before use, add 5ml sterile 2M MgCl₂, and 20ml of filter-sterilized 1M glucose.

STET

8% sucrose

5% Triton X-100

50mM Na₂EDTA

50mM Tris-HCL (pH8)