Molecular Biology Lab Course Schedule Overview

August 10 -15th, 2009

Wesleyan University GLSP

Professor Michael A. McAlear

Monday

Check in, introductions

Lecture: Basics of molecular biology

Lecture: Restriction enzymes

Exp 1: Run a digest of plasmid DNA, Agarose gel

Tf *E. coli* with ryPCNA plasmid

Tuesday

Lecture: The Polymerase Chain Reaction

Exp 3: Do PCR reaction,

Exp 1: Get *E. coli* tf off plates, into LB Amp

Lecture: DNA sequencing

Exp 2: Dilute, plate, irradiate yeast

Lecture: Yeast life cycle, genetics

Wednesday (1/2 day)

Exp 1: transfer tf to LB AMP

run PCR on gel

Thursday

Induce *E. coli* with IPTG (10:00 AM)

Exp 4: Inoculate yeast onto plates

Lecture: Protein purification

Exp 1: Lyse *E. coli* cells (1:00 PM)

Put extracts on Ni beads and wash

Run Protein gel, stain overnight

Lecture: Protein synthesis

Test strains on SC-Ura etc.

Friday

Exp 1: Destain protein gel

Do colony PCR on E. coli, yeast with PCNA primers

Exp 2: Count viable yeast colonies, plot viabilities

Exp 4: Score yeast plates for growth

Lecture: DNA microchips

Saturday

Wrap up

Experiment 1

Making recombinant proteins in *E. coli*

The point of this experiment is to demonstrate how the tools and techniques of modern molecular biology allow us to engineer bacteria to produce large quantities of foreign proteins of interest. In this case, the foreign protein of interest will be yeast PCNA, a DNA replication factor, but it could just as well be a human growth hormone, a blood clotting factor, etc. The yeast PCNA gene will be introduced into *E. coli* on a recombinant plasmid, and the coding sequence of the PCNA gene will include a specialized N-terminal tag (encoding a series of histidines) that will facilitate the purification of the PCNA protein. The engineered bacterial strain will be grown up, lysed and the PCNA protein will be purified away from the thousands of native *E. coli* proteins. We will use agarose gels to analyze the recombinant plasmids, and we will use polyacrylamide gels to visualize the protein bands. The chromatographic purification strategy will be based on the attraction between histidines, and immobilized Ni^{++}.

Day 1, (Monday)

Part 1

Take 2 ul of a sample of the plasmid pMM10 DNA, and perform a restriction digest with the enzymes EcoRI, BsmaI

2 ul	DNA pMM10
15 ul	H2O
2 ul	10 x Buffer
1 ul	Enzyme
20 ul	

Incubate the reaction at 37 C for 2 hrs, and then load onto an agarose gel. Include a molecular weight marker on the gel, as well as a sample of the plasmid DNA that was not digested with the enzyme. After electrophoresis, visualize the DNA bands by staining with ethidium bromide, and compare them with the known restriction map.

Part 2

Take 1 ul of the plasmid DNA and use it to transform a sample of competent *E. coli* cells. Include a negative transformation control that does not have any plasmid. After transformation, spread the *E. coli* mixture onto LB plates containing the antibiotic ampicilin. Incubate overnight at 37 C.

Day 2, (Tuesday)

Observe and record the number of colonies growing on the transformation plates. Take a single transformant from the plasmid DNA sample plate, and use it to inoculate a 5 ml culture of LB Amp liquid media. Grow the 5 ml culture overnight at 37 C.

Day 4, (Thursday)
(Get culture of pMM 13 ready for this (BL21 DE3))

Early (8:00 AM) take 2 ml from culture, and add it to 30 mls of fresh LB Amp liquid media. Let the cells grow for 2 - 3 hrs at 37 C, and then add 30 ul of 1 M IPTG. Let the culture grow for another 3 hrs, and then spin down the cells in a centrifuge. Remove the excess LB media. Resuspend in 2.5 ml NBB (Native Binding Buffer) and 2.5 ul of 100 mg/ml stock of egg white lysozyme, transfer to a 15 ml tube and incubate on **ice** for 15 min with agitation.

Sonicate at setting 5 for 4 times with 10 second bursts. Allow 20 seconds between bursts and keep on ice throughout. The sonicator should not touch any part of the tube. Freeze tube in a methanol/dry ice bath and then thaw in a 40°C water bath. Repeat 2 more times. To break up DNA and make the solution less viscous, aspirate through a 20 gauge needle with a syringe. Repeat aspiration 2 more times.

Pellet cell debris in a clinical centrifuge. Transfer 1 ml of cleared lysate into a new 1.5 ml plastic tube. Save another 50 ul aliquot of crude extract in another tube for your gel.

We will use the batch method for isolation of the pure protein. Remove and discard supernatant on top of your 0.3 ml aliquot of Invitrogen ProBond Nickel column resin.

Add 0.5 ml sterile water, invert tube to mix, spin quickly in table top and discard water. Repeat this wash 2 times.

Wash the column material with 1.0 ml of NBB, as above.

Add 1.0 ml of your extract to resin, make sure the top is on securely and put on ice for 15 min (mix a few times). Spin in table top, take off 1 ml of liquid and save (unbound lysate).

Add 1.0 ml NBB and incubate on ice with shaking for 5 min. Spin and remove liquid (NBB wash 1). Repeat with another 1 ml of NBB (label NBB wash 2).

Remove unbound proteins from resin with a 0.5 ml wash with Native Wash Buffer (NWB). Shake for 5 min in the cold room.. Collect and label " NWB eluate 1". Keep on ice.

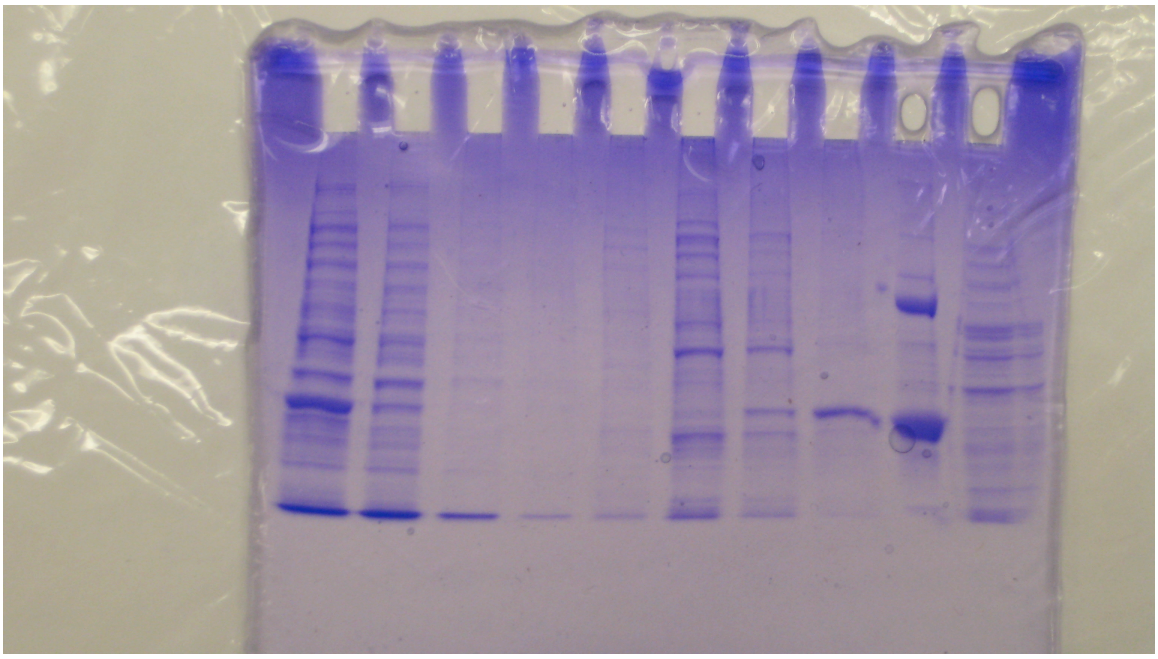
Elute bound protein with NWB containing imidazole. First, use 0.5 ml of 200 mM imidazole (5 min. shake). Collect eluate on ice and label (NWB 200).

Proceed with 0.5 ml of 400 mM imidazole, 0.5 ml of 600 mM imidazole and finally use 0.5 ml of 800 mM imidazole. Save and label eluates

Run the protein samples on an SDS PAGE acrylamide gel. Include a molecular weight marker (PCNA) and a sample of an *E.coli* strain (whole cells ok) that does not have the recombinant PCNA plasmid. After electrophoresis, stain the gel overnight with Coomasie Blue protein stain.

Day 5, (Friday)

Destain the protein gel, and analyze the protein bands. Determine whether the chromatography procedure yielded the recombinant PCNA protein in a more purified form.



crude X, unbound lysate, NBB1, NBB2, NWB, NWB200, NWB400, NWB600, PCNA, E. coli

Experiment 2

Radiation sensitive yeast strains

One way that we can determine the role that specific genes play in cells is to investigate the properties of cells that carry mutations in such genes. For example, if gene *X* plays a role in DNA replication or DNA repair, one might expect that cells carrying a mutation in gene *X* may not be able replicate or repair its DNA very well. Such a defect could be tested by challenging the mutant cells with a DNA damaging agent, and then determining how well the cells survive. In this experiment, we will investigate the UV sensitivity of strains carrying mutations in two known DNA replication proteins (*RAD9* and *CDC44*). A series of different yeast strains will be diluted, and plated onto YPD plates. The plates will be irradiated with increasing doses of UV light, and the colonies will be allowed to grow up for 3 days. The number of surviving colonies will be determined. While this experiment uses yeast as a model organism, the exact same principles apply to human diseases that predispose individuals to UV induced skin cancer (i.e Xeroderma Pigmentosa).

YMM10 Mat **a** *ade2 leu2-3,112 ura3-52*

YMM16 Mat **a** *his4 lys2-801 ura3-52 cdc44-1.URA3*

YMM59 Mat **a** *leu2-3,112 ura3-52 rad9::LEU2*

Day 1, (Tuesday)

We will have prepared cultures of three yeast strains that are growing in liquid YPD media. The samples will be sonicated to disperse the clumps of cells, and the cell density/ml of culture will be determined. To do this, take a sample of early log cultures for each strain, dilute the culture 10 x in sterile water (i.e. add 100 ul of cells to 900 ul of water) and then sonicate. The sonication is necessary to get an accurate reading of the cell number, because it breaks up clumps of cells that can be hard to count.

Take the 1 ml of the diluted cell culture in an eppendorf tube. Put the eppi tube into the holder and position the microtip 2/3 rds down into the tube, and close door. Sonicate sample with the microtip for 10 seconds at setting "4" (set dial at "4", hit start, count, then hit stop). Clean off the tip with a kimwipe that has been wetted with ethanol. This level of sonication will break the weak attachments between cells that have undergone cell division, but that have not yet separated from each other. It will not kill the cells, nor will it separate buds from mother cells. If after sonication large clumps of cells are still seen, samples can be resonicated again at the same or a higher setting ("5.0").

Count the cell number with a hemacytometer and then dilute the cells (serial 10 x dilutions) as necessary to a density of about 4,000 cells/ml in sterile water. You will need a total of 1.5 mls from each strain at this cell density.

Plate 100 ul of the diluted cells from each strain onto each of 14 YPD plates (total of about 300 cells per plate). Label and irradiate duplicate plates with UV light at 0, 50, 100, 150, 200 J/m^2. Can also test saran wrap, sunscreen. Incubate plates at 30°C

Day 5, (Friday)

Count colonies, plot viability. Determine whether certain mutations affect the survival rate.

Experiment 3

The Polymerase Chain Reaction

Tuesday

Take colonies from different strains, mix in NTPs, primers etc. Do PCR reaction, run on gel, analyze products.

Experiment 4

Genotype and phenotype of mutant yeast strains

Wednesday

Inoculate yeast strains onto plates

Friday

Score plates, correlate phenotype with genotype