Biology 322 Fall 2012 Spontaneous and Induced Mutation Part II

Rifamycin (rifampin) is a major drug used in the treatment of tuberculosis infections, and increasing rifamycin resistance represents a worldwide clinical problem. Resistance to this antibiotic is caused by mutations in the *rpoB* gene, encoding the beta-subunit of RNA polymerase. We will follow up on our *rifR* mutations by determining the location and nature of mutatgenic changes that can confer resistance to the antibiotic without severely compromising the ability of RNA polymerase to perform its cellular function

The *lac*- phenotype (inability to metabolize lactose as an energy/carbon source) in *E. coli* results from mutations in either the *lacZ* or the *lacY* gene. We will measure the frequency of reverse mutation from *lac*- → *lac*+.

MONDAY OCT 29 MEET IN LAB at 10am

- Set up overnight cultures of selected *rifR* strains (for genomic DNA preps on Tuesday)**. Each student sets up three cultures—one spontaneous and one induced *rifR* mutant strain and a culture of** a *rifS* colony – further instructions will be given in class.
- Streak *lac*- candidates on MacConkey plates to confirm mutant phenotype and isolate single colonies (pure clones). ***Each student should streak at least 2 lac- candidates.***
- Discuss growth of *E. coli* on minimal medium. *Look at this web site comparing minimal media for E. coli versus a human cell*

http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/M/Media.html

---

BEFORE TUESDAY OCT 30

- You must have a detailed DNA prep protocol **recorded in your lab notebook before you arrive for lab** on Tues 10/30 (see page 2-3 for details).
- **No google.doc submission is required for this lab period**

TUESDAY OCT 30

- Prep DNA from *rifR* and *rifS* lines using protocol recorded in your lab notebook. Each student performs three DNA preps.
- Determine DNA concentration using Nanodrop setup (more info on page 3)
- Setup PCR to amplify a portion of the rpoB gene
- Set up overnight cultures of *lac*- lines – ***one culture per student***

BEFORE THURS NOV 1

- **In your lab notebook and submitted as a Google doc:** Work out a protocol for measuring the frequency of spontaneous reversion mutations in a culture of *lac*- cells. Be sure to specify the type of media that you will be using to grow up cultures of *lac*- cells and to **select** for revertants and how you will carry out the plating portion of the assay. You must decide on dilutions etc.

THURS NOV 1

- Agarose gel analysis of genomic DNA preps and PCR products generated by amplification of RNA pol *(rpoB)* gene (see additional info below)
- Set up *lac*- → *lac*+ reversion experiment

- Start or continue Part III: *Using public databases to gather information required for our sequence analysis of the rifR mutant lines*

Using a Qiagen DNeasy Kit to prepare genomic DNA from rifR mutant lines.

LINK to DNeasy Kit Handbook:
http://fire.biol.wwu.edu/trent/trent/DNeasykit.pdf

1. ***Browse through the DNeasy kit instructions:*** note that this kit can be used to prepare genomic DNA from a variety of tissue/cell sources
2. ***Read important info below.***
3. ***In lab notebook write out protocol for prepping genomic DNA from E. coli cells.*** You must have a detailed protocol recorded in your lab notebook before you arrive for lab on Tues 10/30
4. This protocol should not be in paragraph form: list individual steps and leave room between each step (or on the side of the page) for notes. Do not submit this protocol to google.docs

Important info:

- At the top of your protocol put this NOTE: ***Check the board for late-breaking info on the protocol***
- ***B***e sure to read the ***Introduction*** (pgs 9-11). We will be doing the DNeasy Mini Procedure (which uses spin columns)
- Is E. coli gram negative or gram-positive? [Think about: why would these two classes of bacterial cells require different lysis procedures?]
- All reagents will be prepared for you including the proteinase K solution.
- You will be starting with about **~1 ml** of an overnight culture, which we have previously determined is NOT too many cells. With spin columns, overloading the column with too much cell lysate can substantially reduce yield (see pg. 15-16)
- After pelleting cells***, pipet off supernatant.***
- After adding buffer ATL (lysis buffer), ***pipet up and down*** to disrupt cell pellet and generate a homogenous cell suspension.
- After adding proteinase K (what's this for?), we will let the lysis proceed for about an hour *(good thing we're not doing rodent tails!)* in a 56ºC waterbath. Vortex every 20 minutes.
- We will not be adding RNase to our DNA preps as contaminating RNA is typically not a problem for subsequent PCR runs
- Write out column-binding and elution steps very carefully. Make sure you don't omit any steps.
- Plan to do a single 200ul elution.

Qiagen Hype: *Qiagen* miniprep columns contain a unique silica-gel membrane that binds up to 20 µg DNA in the presence of a high concentration of chaotropic *(what does this mean?)* salt, and allows elution in a small volume of low-salt buffer. RNA, proteins and metabolites are not retained on the membrane. QIAprep membrane technology eliminates time-consuming phenol–chloroform extraction and alcohol precipitation, as well as the problems and inconvenience associated with loose resins and slurries. High-purity plasmid DNA eluted from QIAprep modules is immediately ready to use — there is no need to precipitate, concentrate, or desalt.

- The E. coli cells are lysed by the action of the proteinase K in the enzymatic lysis Buffer ATL.
- Buffer AL contains the guanidinium salts that help the DNA to bind to the silica matrix in the DNeasy Mini Spin Columns, along with the added ethanol.
- Column Wash buffer AW1 helps to wash any carry-over proteins from the column,
- Column wash Buffer AW2 helps to wash any residual salts from the column.
- Buffer AE is 10 mM Tris·Cl, 0.5 mM EDTA, pH 9.0.

Questions about what tubes to use at each step?

Check with CT

Assessing quality and yield of your genomic DNA preps

The quality and yield of your genomic DNA prep will be assessed in two different ways

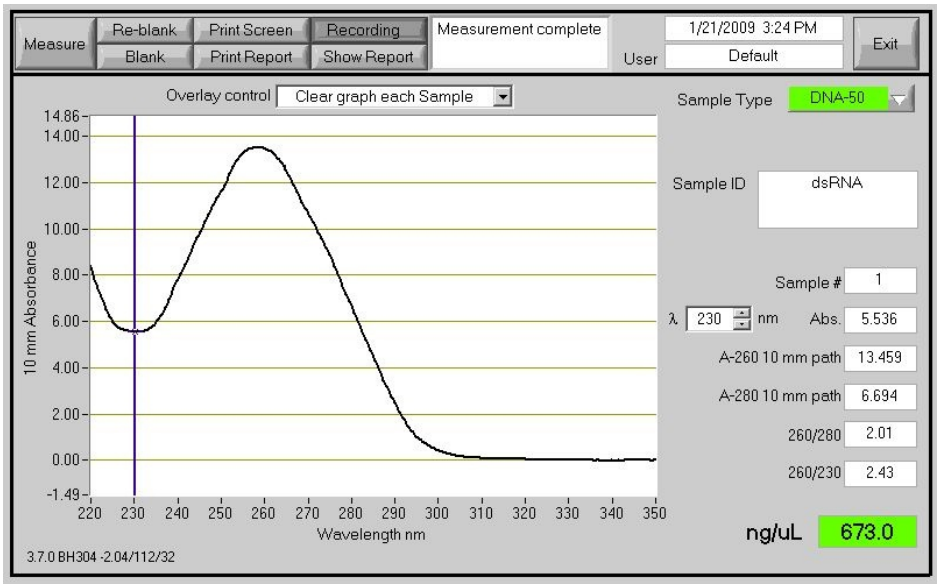
1. ***Nanodrop spectrophotometric analysis***: UV absorbance spectrum
2. ***Agarose gel electrophoresis*** of genomic DNA (and PCR products)

Assessing DNA quality and quantity using absorbance in the ultraviolet range

- The most common technique to determine DNA yield and purity is also the easiest method—absorbance. All that is needed for measurement is a spectrophotometer equipped with a UV lamp
- DNA concentration can be estimated by measuring the absorbance at 260nm (A260)
- DNA purity can be estimated from the A260/A280 ratio. An A260/A280 ratio between 1.7 and 2.0 generally represents a high-quality DNA sample.

Carefully follow instructions posted next to the Nanodrop instrument

- ***Ask for assistance if you need it***
- Print out window showing info below.
- Printer not working? Write down ng/ul A260/280 ratio and inspect curve and compare to figure below



PCR amplification of a portion of the rpoB gene in rifR mutant lines
See also Part 3 handout

PRIMERS: rpoB R&L PCR product from: 1351 to 2235 in rpoB gene. **Length:** 885 bp.
CGTCGTATCCGTTCCGTTGG rpoB-L,
TTCACCCGGATACATCTCGTC rpoB-R

PCR rxn Master Mix

	25ul rxn	x ?? rxns	Final Concentration
10x buffer	2.5ul		
25 mM MgCl2	1.5 ul		
10 mM dNTP 2.5mM each	0.5 ul	l	
10 uM Primer 1	1.0 ul		
10mM Primer 2	1.0 ul		
Taq 6Units/ul	0.25 ul		
PCR H_2O	16.25 ul		
total	23ul		
DNA per tube	2ul		

Thermocycler info

1	94oC	2 min
2	94oC	30 sec
3	55oC	30 sec
4	72oC	1 min
		25 cycles 2-4
5	72oC	5 min
6	4oC	hold

Agarose Gel Analysis of genomic DNA preps and PCR products

Agarose Gel conditions: same as with aha3 experiment – check your records for details

Each student will run 6 samples on an agarose gel plus Hi Lo size standards:

- Three genomic preps: run 10 ul + loading dye (stock is 6X)
- PCR run of each prep: run 5ul plus loading dye
- ***DO NOT add gel loading dye directly to your tubes of genomic DNA or PCR products.***
- Instead, retrieve the appropriate amount from the tube and mix it with loading dye on a piece of parafilm as described by your instructor