Molecular Cloning Bootcamp 2

Today's mission: confirm whether we successfully built our dsRNA expression plasmids, then use these to make our dsRNA expressing bacterial strains. To do this we will (1) extract plasmids from the liquid cultures we started last time, (2) digest the plasmids with Age1, (3) run the digests on a gel to determine the sizes of our inserts, and finally (4) transform correct plasmids into dsRNA producing bacteria.

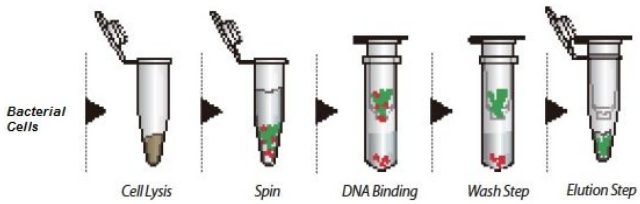
Part 1: Plasmid purification ("miniprep").

To do your miniprep, follow the step-by-step instructions provided at each lab station. I HIGHLY RECOMMEND reading the following overview before you begin.
Note: although this text was copied from the Qiagen Miniprep Handbook (http://www.qiagen.com/resources/download.aspx?id=22df6325-9579-4aa0-819c-788f73d81a09&lang=en), there are many brands of "miniprep" kits that work in the same way.

Principle

The miniprep procedure is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto silica in the presence of high salt. The procedure consists of three basic steps:

1. Preparation (cell lysis) and clearing (spin) of a bacterial lysate
2. Binding of DNA onto the column
3. Washing and elution of plasmid DNA



1. Preparation and clearing of bacterial lysate

The QIAprep miniprep procedure uses the modified alkaline lysis method of Birnboim and Doly (2). Bacteria are lysed under alkaline conditions, and the lysate is subsequently neutralized and adjusted to high-salt binding conditions in one step. After lysate clearing, the sample is ready for purification on the QIAprep silica membrane.

2. DNA adsorption to the QIAprep membrane

QIAprep 2.0 columns use a silica membrane for selective adsorption of plasmid DNA in high-salt buffer and elution in low-salt buffer. The optimized buffers in the lysis procedure, combined with the unique silica membrane, ensure that only DNA will be adsorbed, while RNA, cellular proteins, and metabolites are not retained on the membrane but are found in the flow-through.

3. Washing and elution of plasmid DNA

Salts are efficiently removed by a brief wash step with Buffer PE. High-quality plasmid DNA is then eluted from the QIAprep 2.0 column with 50–100 µl of Buffer EB or water. The purified DNA is ready for immediate use in a range of applications — no need to precipitate, concentrate, or desalt.

Part 2: Analytical digest

Once you have purified your plasmid DNA, you can digest it with Age1. This process should feel familiar. Hint: I to reduce variability, I recommend making a master mix of all of the reagents that will be in common between your different samples. Be sure to mix your water and buffer well before adding enzyme, then mix again

before aliquoting 10 ul into a new tube for each reaction. Add plasmid to each tube. Centrifuge briefly to make sure all contents are at the bottom of the tube. Incubate at 37C for 40 minutes.

Material	Volume for 1 sample	4.5X master mix
Sterile water	8 ul	
Cutsmart Buffer	1.5 ul	
AgeI	0.5ul	
Plasmid prep	5 ul	

Part 3: Gel electrophoresis

We will now run a gel to determine if we have the correct insert. To prepare your digests for gel electrophoresis, we add loading buffer. This serves three purposes: 1.) It adds a dye so we can see the sample to get it into the well, and estimate when to stop running the gel. 2.) It includes glycerol, which is dense. This keeps your sample in the well until the gel starts running and the DNA gets trapped in the agarose. 3.) It has a DNA dye that glows under blue light so we can see our digestion products.

1.) Add 4 ul of loading buffer to each digest and mix well.
2.) Load your samples in the well. Write down which wells contain your samples.
3.) While the gel runs, figure out what you expect to see if your plasmid is correct. What information do you need to know?
4.) We will image the gel once it's run. Did you get the right thing?

Part 4: Transform the RNAi production bacteria with your construct

We will now transform your vectors into RNAi production bacteria. This process should seem familiar!

Keep your cells cold! Any warming of competent cells is bad for the efficiency of the transformation. (Except for the heat shock step. Obviously.) Each group gets one tube of competent cells. I made these competent cells just for you guys! They contain an inducible T7 polymerase and are resistant to Tetracycline.

1. Add 1ul of your plasmid prep to your thawed tube of HT115 competent cells. Mix by flicking. DO NOT LET THEM WARM UP. Put your tube back on ice.
2. Heat shock cells by putting them into 42C heat block for 30 s.
3. Immediately return cells to ice. Incubate on ice for 2 min.
4. Add 200ul of LB media
5. Plate your transformations by pipetting onto selection media (LB+Amp+Tetracycline plates), adding a dozen or so sterile glass beads, and shaking to spread (instructors will demonstrate). Dump out used beads in dirty bead jar.
6. Put your plates at 4C. Lil will move them to the incubator to grow before next class.