SB.001 Cloning Lab
BioBrick Creation: Cloning into a BioBrick Vector

October 9, 2006

One of the great features of parts is that they are able to self-replicate. No more expensive fabs for our genetic circuits. All we need to do is get one bacteria that has the correct plasmid in it and then it will multiply and generate more plasmids for us. In this section of the lab, we will clone the PCR product that contains the part you harvested, but with BioBrick ends, into a standard BioBrick Cloning Vector. In this lab you will use the skills you used before: restriction digestion, gel extraction and ligation.

A few short steps are required each of the two days after the lab. You can select a couple members from your group to do them, or all go together.

1 Prelab

Problem 1. We will be doing a serial quick digestion. You will cut both the part (as linear PCR product), and the plasmid. You will need 1 μl of enzyme per μg of DNA, to keep the level of glycerol (from the enzyme storage buffer) low, we are going to use only 2 μl of enzyme. Write the amount of DNA you got from the PCR reaction, and the concentration of DNA in your BioBrick plasmid prep. We want a final volume of 50 μl for our digestion reactions. Write the "recipe" for your reaction mixtures, with the following constraints: Total Volume is 50, no more than 5 μl enzyme, and each contains 5 μl of 10x buffer.

Problem 2. Ligation Equation. In order to optimally ligate your DNA together, you will have to add appropriate amounts of the insert and the plasmid. The equation is described here:

$$L = 6\frac{m_i l_v}{m_v l_i} \tag{1}$$

Where m_l and m_v are the masses of the insert and vector, and l_i and l_v are the lengths of the insert and vector.

2 Lab

Please read the entire lab before starting as time is limited, and several steps have lapses of time that you do not have to do anything for that step, but you should be preparing for other steps (such as making gels and such).

Part 1: Restriction Digestion. We will be doing a quick digestion. You will cut both the parts (as linear PCR product), and the plasmid. Follow the recipie you wrote in Problem 1. Take a 1.5 ml tube, add water if you are using any to dilute your DNA, followed by the DNA, and 5 μl each of the NEBuffer, and if the enzyme requires it, 10x BSA. Finally, add the required enzyme. Incubate the reaction at 37° for 1 hour. Then follow the Qiagen PCR Purification protocol to clean up your sample, and elute into 40 or 45μl depending on whether or not your enzyme requires BSA. Add the second buffer and enzyme to those tubes and incubate for 30 minutes. Then add 2 μl Shrimp Alkaline Phosphotase (SAP) to the Vector tube, and incubate 30 more minutes. Do not add SAP to the insert!

Part 2: Gel Purification While your first digestion proceeds, make a 0.7% agarose gel(about 185 mg in 25 ml TAE, 25 μl SYBR Safe dye), with a least three sets of two wells that are taped together to make three larger wells. Once your digestions are done, add 6 μl of 10x loading dye to each tube, flick the tubes and spin down gently. Pipette the mixture 10 μl at a time into the wells, placing the tip just above the well and gently expelling the DNA into the well, it is important that the DNA not splash around, so you do not lose any. Add the 1 kb DNA ladder in another well. Run the gel for 30-45 minutes at 100V. Excise the gel fragments (your insert should be a single clear, for the plasmid you may see two bands, the one that appears to be longer is probably correct because linear DNA runs slower than the supercoiled plasmid DNA which is un-cut) and proceed with the Qiagen Gel Extraction protocol to purify your DNA. When cutting the gel fragments, try to get as close to the exact band as possible, as the less agarose is in the sample the better. Make a quick slice both above and below the band and then on the inner gel side and near the edge. Then do the other band. You should be quick in youIf you have a questions as to which band to cut, ask the TAs to take a look at your gel. Do not keep the UV lamp on longer than absolutely necessary

Part 3: Quantification Measure, using the nanodrop device, the amount of DNA for both the insert and vector. Based on the ratio of Insert to Vector DNA you found in the pre-lab, write the amount of each you will use in your ligation reaction.

Part 4: Ligation Using the formula from Part 3, prepare a series of ligation mixtures, with various ratios of vector to insert according to the table below. Some reactions lack various components and are used as negative controls.

Ligation Mixtures

Mixture	1	2	3	4	5	6	7
10x Buffer	1	1	1	1	1	1	1
30% PEG 8000	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Vector	0	1	1	1	2	1	1
Insert	1	$a/2$	a	$2a$	a	0	0
Ligase	1	1	1	1	1	1	0
Water	To 10μl	...	...	...	...	...	...

Part 5: Transformation Thaw 7 aliquots of chemically competent cells on ice. Label each tube, and add the corresponding ligation mixture. Keep the aliquots on ice for 10 minutes. Heat shock each aliquot for 40 seconds at 42°C. Add 750 μl of LB, mix gently and incubate at 37°. Centrifuge cells at 9,000 RPM for 5 minutes, and resuspend in 20 μl of LB.

Part 6: Plating Take out seven LB+Agar+Antibiotic plates from the refrigerator when you are done with the transformation. Take the cell mixtures and pipette them onto the plates. Gently spread each mixture using a cell spreader, sterilizing it with alcohol and a flame before each plate.

Part 7: Analysis and Growth Send a representative or two to the lab the next day to see what is growing on your plates. Scan your plates and include the image with your lab report. You should have a significantly larger number of colonies on the experimental plates vs. the controls (at least an order of magnitude). Assuming you were successful, take 5 test tubes, add 5 ml of Amp+LB media to each. Pick 5 colonies with which to innoculate the tubes. Let them grow overnight.

Part 8: Plasmid Isolation and Analysis Take the overnight cultures, use 1 ml of each to create freezer stocks (1 ml culture + 333 μl of 60% glycerol). Use the rest in a Qiaquick plasmid purification kit. Elute in 80 μl of EB and measure the concentration of DNA using the nanodrop. Do a 5 minute analytical digestion using the two restriction enzymes you used earlier. If the part is there, you should see the plasmid and the insert on an analytical gel. Take a picture of the gel. For each of the wells that looked correct on the gel, make up a tube with 10 μl H_2O, 1 μ sequencing primer, and 2 μl of plasmid DNA. For any digests that were unsuccessful, discard the freezer stock you made. Fill out the sequencing form from the course website, use short labels for your tubes and enter the same names on the sequencing form. Place the sequencing form in the pocket on the refrigerator door and the tubes in the tube rack in the refrigerator.

Part 9: The DNA Sequence Within two days you will get an email containing two files for each sample you submitted. One has the chromatograph (the amount of fluorecence the laser in the sequencer picked up at each point in time), and the other has the sequence as determined by the sequencing software. Find the sequence of your component by the

restriction sites you used. Verify that the sequence between the restriction sites matches the components you wanted to import. If there are 'N"s in your sequence, look at the chromatograph itself and see if you are able to discern which base is present. If you have at least one sequence that is entirely correct for each of the parts that you created, congratulations, you've succeeded! If not, you'll have a chance later to debug your procedure with the TA's.

Part 10: The Registry You need to keep the community up to date with regard to the status of your parts, so you should update the status of your parts online to show that they have been constructed in DNA and what plasmid you put them in, and that you have sequence verified them. If your cloning wasn't entirely successful, write up to which stage it worked.