

**BioE 498 Lab: In-Fusion BioBrick Assembly and Re-engineering**

**I. PCR (POLYMERASE CHAIN REACTION)**

First we will use PCR to amplify our insert and vector DNA for use in the In-Fusion reaction later.

**Materials and Equipment**

Invitrogen High Fidelity Supermix

The following primers (sequences in red are non-homologous to the template DNA):

P1 GFP primer (5'-TACTAGAGTCACACAGGAAAGTACTAGA-3')

P2 GFP primer (5'-GAAGCCTGCAGCGGCCGCTACTAGTA-3')

P1 RFP primer (5'-TACTAGAGTCACACAGGAAAGTACTAGATGGCTTCTCCGAAGACGTTATCA-3')

P2 RFP primer (5'-GAAGCCTGCAGCGGCCGCTACTAGTA-3')

P3 primer (VF2: 5'-TGCCACCTGACGTCTAAGAA-3')

P4 primer (VR: 5'-ATTACCGCCTTTGAGTGAGC-3')

P5 primer (5'-TACTAGTAGCGGCCGCTGCAGGCTTC-3')

P6 primer (5'-TTTCCTGTGTGACTCTAGTATGTGCTCAGTATCTTGTTAT-3')

E0240 miniprep plasmid 1:1000 (GFP insert template DNA)

J04450 miniprep plasmid 1:1000 (RFP insert template DNA)

R0011 miniprep plasmid 1:1000 (vector template DNA)

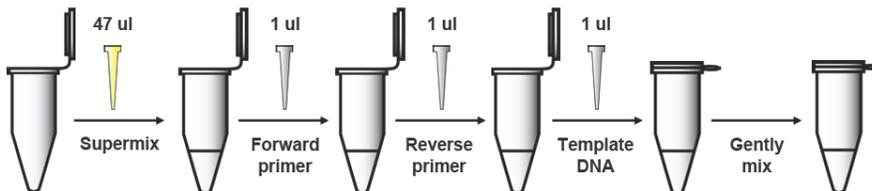
PCR Tubes

Tube Rack

Thermocycler

**Protocol**

1. Thaw Supermix, primers, and template DNA in your tube rack.
2. Put 6 PCR tubes in your PCR tube rack, label them with your Team Letter (A, B, C, D, E, or F) and PCR reaction number (e.g. for Team A, label tubes A1-A6).
3. Prepare each of the reaction mixtures below by micropipetting the indicated volumes of each reagent in the order shown. *Take a moment to think about the purpose of each control, discuss with your teammate, or ask an instructor to clarify.*



Reaction#	Name	Volumes (µl)								Template
		Supermix	P1	P2	P3	P4	P5	P6		
1	Insert	47	1	1						1 (insert)
2	Insert positive control	47			1	1				1 (insert)
3	Insert negative control	47	1	1						0

4	Vector	47								
5	Vector positive control	47			1	1	1	1	1 (vector)	
6	Vector negative control	47					1	1	1 (vector)	0

4. Mix each tube by gently flicking the bottom of the tube a few times.
5. Press down on each tube cap tightly so that your samples don't evaporate during the PCR reaction.
6. Put the tubes in the fridge until the instructor can put everyone's tubes in the thermocycler.
7. The thermocycler program that will be used is as follows:

- I. **Initialization:** 94°C for 3 min
- II. **Cycle 30X**
  - Denaturation:** 94°C for 30 sec
  - Annealing:** 55°C for 30 sec
  - Elongation:** 68°C for 3 min
- III. **Termination:** 68°C for 10 min
- IV. **Final Hold:** 4°C

## II. AGAROSE GEL ELECTROPHORESIS

---

Next we will run the PCR products out on a gel to see what size they are and how much DNA we have of each sample.

### Materials and Equipment

Gel Box with gel tray and 10 tooth comb  
 250 mL glass bottle  
 Scale  
 Weigh boat  
 Agarose  
 Spatula  
 1X Buffer with Sybr Safe (50 mL 10X TAE Buffer, 450 mL DI water, 5 ul Sybr Safe)  
 Microwave  
 Kimwipe  
 Parafilm  
 1kb ladder (NEB)  
 2X Loading Buffer (6X Loading buffer diluted 1:3 in DI water)  
 Transilluminator  
 Photography hood  
 Camera

### Protocol

#### i. Prepare a 1% agarose gel with Sybr Safe

1. Wedge gel-casting tray into gel box so that the sides of the tray with the gaskets form a seal with the sides of the gel box. Make sure the gaskets are completely inside the groove first or the gel will leak.

2. Get a 250 mL glass bottle and bring it over to the scale with a weigh boat and agarose. Put the weigh boat on the scale and zero the scale. Scoop out 1 gram of agarose into the weigh boat and pour it into the bottle.
3. Pour 100 mL of 1X Buffer with Sybr Safe into the bottle and swirl a few times to mix the agarose solution.

Put the flask in the microwave with the cap **loosely** tightened to minimize boil over. Microwave on high power for 1 minute. For optimal results, the solution should boil for about 5-10 seconds. Stop the microwave early if the solution starts to boil over. **WARNING: if the cap is screwed on too tightly, pressure buildup can occur which allows the solution to become superheated beyond boiling temperature. When the cap is unscrewed and exposed to atmospheric pressure the solution will boil over potentially resulting in serious burns.**

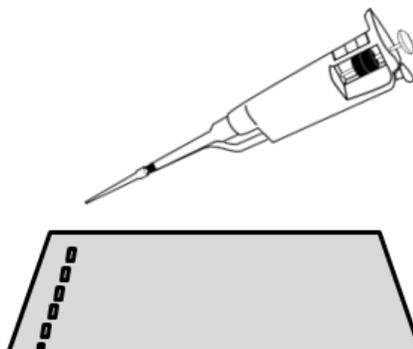
4. The bottle will be hot, so pick up the bottle with a paper towel. If the solution is completely clear, swirl it a few times and let it sit for 1 minute on the bench. Otherwise, put it back in the microwave and microwave for an additional 20 seconds, or until the solution starts to boil.
5. Carefully pour the entire solution into the casting tray.
6. Remove any bubbles by taking a Kimwipe and spreading it gently across the gel solution surface.
7. Position the gel comb with 10 teeth (with "1.5" rightside up) in the end slot of the gel box (it should be about halfway up the length of the teeth).
8. Do not move casting tray while the agarose solution is solidifying.
9. After about 20-30 minutes the gel should have solidified, but test this by gently touching the gel with a gloved hand and make sure it's firm but not squishy.
10. Remove comb from gel by slowly pulling the ends up so that you get clean wells. Pull out the casting tray from the gel box and rotate it 90 degrees with the wells at the top of the gel box.
11. Pour 1X Buffer with Sybr Safe buffer into the gel box so that the gel is completely submerged.

## ii. Running the gel

1. Bring your PCR products over to the gel and cut out a small piece of parafilm. Peel off the top layer and put the parafilm face-up near the gel box.
2. Pipet 5 ul of the 1kb ladder (already containing loading buffer) into the second and ninth wells in the gel (keeping the first and tenth wells at each end free).
3. Pipet 5 ul of 2X Loading Buffer in 6 different spots on the parafilm. Pipet 5 ul of each PCR product into a different loading buffer spot. Pipet each 10 ul PCR product:buffer mixture into wells 3-8 (in between the two 1kb ladder wells).
4. Attach the cover of the gel box and firmly connect the electrodes to the leads, making sure the positive pole (red) is at the opposite end of the gel from the wells. Then connect the other leads to the power supply with the cable colors matching the colors on the power supply.
5. Run the gel at 160 V until the dye has run about halfway down the gel, about 30 minutes.
6. Disconnect the leads from the power supply and remove the gel box cover. Remove the gel tray holding the gel from the gel box. Drain the buffer off into the gel box while holding the gel in place.

### Gel Sample Layout

Lane 1: Blank



- iii. Imaging
- |         |                 |  |
|---------|-----------------|--|
|         | <b>Lane 2:</b>  | 1 kb ladder (5 $\mu$ l)  |
|         | <b>Lane 3:</b>  | Insert (5 $\mu$ l DNA + 5 $\mu$ l loading buffer)                  |
|         | <b>Lane 4:</b>  | Insert positive control (5 $\mu$ l DNA + 5 $\mu$ l loading buffer) |
|         | <b>Lane 5:</b>  | Insert negative control (5 $\mu$ l DNA + 5 $\mu$ l loading buffer) |
| tray    | <b>Lane 6:</b>  | Vector (5 $\mu$ l DNA + 5 $\mu$ l loading buffer)                  |
|         | <b>Lane 7:</b>  | Vector positive control (5 $\mu$ l DNA + 5 $\mu$ l loading buffer) |
|         | <b>Lane 8:</b>  | Vector negative control (5 $\mu$ l DNA + 5 $\mu$ l loading buffer) |
| of      | <b>Lane 9:</b>  | 1 kb ladder (5 $\mu$ l)  |
| 3. Turn | <b>Lane 10:</b> | Blank  |

down on top of the hood.

- Turn on the transilluminator.
- Adjust the zoom and focus on the camera until a clear picture is in the display.
- Take the photo and turn off the transilluminator.
- Transfer the image to a computer with the USB cable.
- Take a look at your gel image and see if the bands are the predicted size.
- Estimate the concentration of each band by comparing it to the 1kb ladder fragments (see 1kb ladder spec sheet above the gel imaging station).

### the gel

- Slide the gel from the on to the middle of the transilluminator.
- Position the hood on top the transilluminator. on the camera and position the lens looking

## III. PCR PRODUCT PURIFICATION

Purifying the PCR products will remove polymerases, dNTPs, and the buffer that can interfere with downstream reactions.

### Materials and Equipment

Qiagen PCR Purification Kit

( **IMPORTANT!!!** Before starting, add ethanol (96–100%) to Buffer PE according to bottle label. )

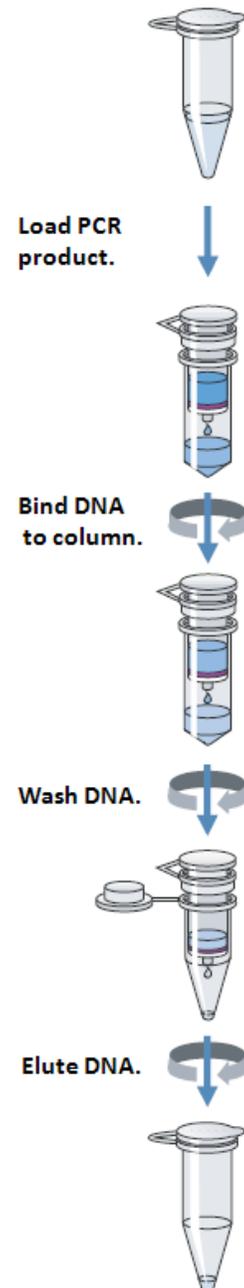
Microcentrifuge

( Set to 13,000 rpm )

### Protocol

( Adapted from the Qiagen PCR Purification Kit protocol using a microcentrifuge. )

- We will purify only the insert and vector PCR products (not the PCR controls). Label two 1.5 mL tubes with your PCR product name.
- Transfer your 45  $\mu$ l PCR product from the PCR tube to the new 1.5 mL tube.
- Add 225  $\mu$ l of Buffer PB to your 45  $\mu$ l PCR product and mix by pipetting up and down a few times.



4. Place a QIAquick spin column in a provided 2 ml collection tube.
5. Set the microcentrifuge to 13,000 rpm and leave it there for all subsequent spins.
6. To bind DNA, apply the Buffer PB:PCR product mixture to the QIAquick column and centrifuge for 1 min.
7. Discard flow-through into a 50 mL Falcon tube. Place the QIAquick column back into the same tube. Collection tubes are re-used to reduce plastic waste.
8. To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 1 min.
9. Discard flow-through and place the QIAquick column back in the same tube.
10. Centrifuge the column for an additional 1 min. **IMPORTANT:** Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
11. Place QIAquick column in a clean 1.5 ml microcentrifuge tube that is labeled with the sample name.
12. To elute DNA, add 30  $\mu$ l H<sub>2</sub>O to the center of the QIAquick membrane and centrifuge the column for 1 min. **IMPORTANT:** Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA.
13. Measure concentration of your purified PCR products (see below) and afterwards store your DNA at  $-20^{\circ}\text{C}$ .

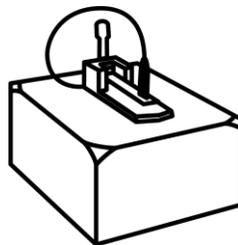
## IV. SPECTROPHOTOMETRIC QUANTITATION OF DNA

---

The nanodrop uses water surface tension to pull apart a droplet of the solution and then examine the sample via spectrophotometer. This allows us to quantitate the amount of DNA in our purified PCR products using only 1  $\mu$ l of sample.

### Materials

Nanodrop and workstation  
Molecular grade water  
Purified PCR products  
Kimwipes



### Protocol

1. Open the Nanodrop software (*ND-1000*).
2. Select "Nucleic Acids".
3. Initialize the Nanodrop by pipetting 1  $\mu$ l of molecular grade water on to the sensor and putting the arm down, then select OK.
4. Lift up the arm and wipe the water off the sensor and the sensor located on the arm with a Kimwipe.
5. Blank the Nanodrop by pipetting another 1  $\mu$ l of water on to the sensor and placing the arm down, then hit "Blank".

- Lift up the arm and wipe the water off the sensor and the sensor located on the arm with a Kimwipe.
- Pipet 1 ul of your purified insert PCR product on the sensor and put the arm down.
- Type in the name of the sample and your Team Name in the box on the right.
- Make sure the "Recording" button on the top center is selected.
- Click "Measure".
- Lift up the arm and wipe the water off the sensor and the sensor located on the arm with a Kimwipe.
- Repeat steps 7-11 for the purified vector PCR product.
- Select "Report" to see your Nanodrop measured samples.
- Write down the concentrations of your samples.
- Store your purified PCR products in the  $-20^{\circ}\text{C}$  freezer.

