

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

We will first use our purified PCR products to perform an In-Fusion PCR cloning reaction to fuse the two PCR products into a circular plasmid containing the functional genetic circuit. This In-Fusion reaction product will then be transformed into competent cells. We will perform colony PCR reactions to identify colonies that have the circuit of interest. Then we will grow overnight cultures from these colonies, perform minipreps to isolate the plasmids, and submit these plasmids for sequencing. Here's the overview for the week:

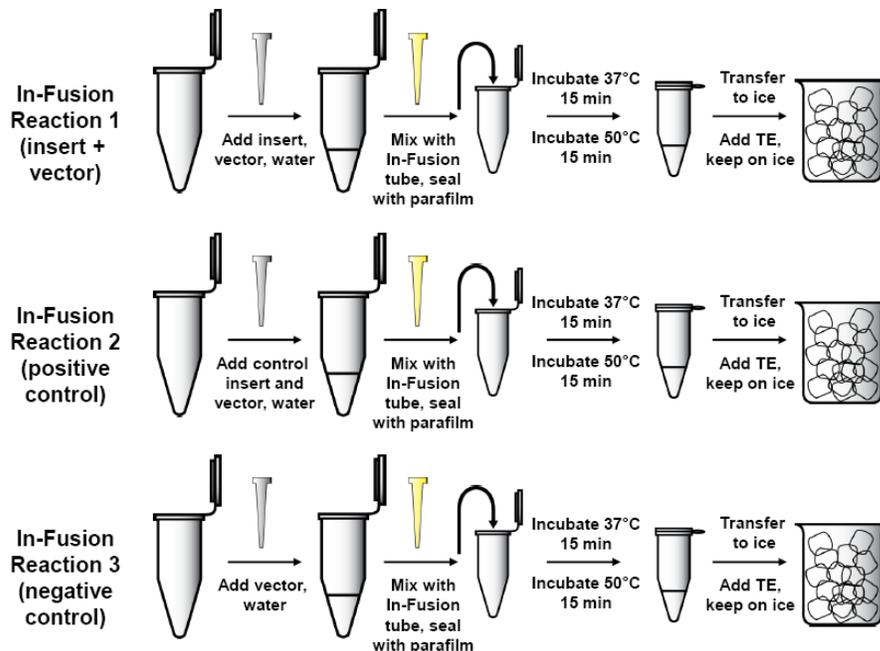
- Monday – In-Fusion PCR Cloning Reaction, Transformations
- Tuesday – Transformant Colony Imaging, Colony PCR and Making Overnight Cultures
- Wednesday – Colony PCR results, Making Glycerol Stocks, Minipreps, and DNA sequencing

### I. IN-FUSION PCR CLONING REACTION

#### Materials and Equipment

- In-Fusion PCR Cloning Kit
- Purified insert and vector PCR products (called "Insert" and "Vector" below)
- Molecular Grade Water
- Parafilm
- Non-shaking incubator (set to 37°C)
- Water bath (set to 50°C)
- Ice bucket with crushed ice
- TE Buffer (pH 8.0)

#### Protocol



- Write down the estimated sizes of the insert and vector PCR products by comparing the bands on the gel to the 1kb ladder bands. Do these match the predicted sizes of your insert and vector PCR products when looking at the primer sites on the template DNA? You do not need to answer this question now, but eventually you will want to see the Lab Introduction and APE files for more information on how to calculate the exact sizes of these PCR products. Then use these estimated PCR product sizes in the following formula to calculate the ng of insert DNA needed for the In-Fusion reaction. This formula calculates a 2:1 molar ratio of insert given 100 ng vector DNA.

$$\text{insert mass (ng)} = 2 \times \frac{\text{insert length (bp)}}{\text{vector length (bp)}} \times 100 \text{ ng vector}$$

- Next calculate the volumes of insert and vector DNA you will add to the In-Fusion reaction mix. These volumes are based on the concentrations you measured previously on the Nanodrop and the mass you calculated above.

$$i = \frac{\text{insert mass (ng)}}{\text{insert concentration } \left(\frac{\text{ng}}{\mu\text{l}}\right)} \quad v = \frac{100 \text{ ng vector}}{\text{vector concentration } \left(\frac{\text{ng}}{\mu\text{l}}\right)}$$

Write down how many  $\mu\text{l}$  of insert (i) and vector (v) you calculated in the table below. Finally, calculate the  $\mu\text{l}$  of water needed for a final reaction volume of 10  $\mu\text{l}$ .

**Example:** Say you have 100 ng/ $\mu\text{l}$  insert that is 1kb and 100 ng/ $\mu\text{l}$  vector that is 2kb. We calculate insert mass (ng) = 2 X (1000/2000) X 100 = 100 ng insert mass. For the insert and vector  $\mu\text{l}$ , divide the ng you want by the ng/ $\mu\text{l}$  you have. In this example, since we want 100 ng of the insert and vector, have 100 ng/ $\mu\text{l}$  of insert and 100 ng/ $\mu\text{l}$  of the vector, we would need 1  $\mu\text{l}$  of each to get 100 ng of each. Then to make 10  $\mu\text{l}$  total volume with water, we need 1  $\mu\text{l}$  insert, 1  $\mu\text{l}$  vector, and 8  $\mu\text{l}$  water.

- Label three 1.5 mL tubes with your team letter and #1, 2, and 3.
- Add the following reaction volumes from the table to each of the three 1.5 mL tubes. The positive control is a control insert and control vector that comes with the In-Fusion Kit that will tell you whether you did the reaction correctly after transformation (see below). The negative control has only the vector PCR product without the insert. This will tell you how many transformants you get due to "background" plasmids that you used as template DNA in the PCR reaction. The insert+vector reaction should have many more transformants since both fragments need to fuse together to form a circular plasmid necessary for transformation.

In-Fusion Reaction#	Name	Volumes ( $\mu\text{l}$ )					
		Insert	Vector	Control Insert	Control Vector	Water	Total Volume
1	Insert+Vector	$\frac{\quad}{(i)}$	$\frac{\quad}{(v)}$			$\frac{\quad}{(10-i-v)}$	10
2	Positive control			2	1	7	10
3	Negative control		$\frac{\quad}{(v)}$			$\frac{\quad}{(10-v)}$	10

5. In your tube rack you will find 3 In-Fusion reaction tubes with aluminum seals. Label them 1-3 on the side of the tube.
6. Add your 10  $\mu$ l total volume from the #1 1.5 mL tube to the #1 In-Fusion reaction tube and mix well by pipetting up and down 5 times. Do the same with the #2 and #3 tubes.
7. Peel off the parafilm paper next to your reaction tubes and seal the top of each reaction tube by placing the parafilm on top of the tubes, slowly pulling down in each direction, and pressing the parafilm against the sides of the tube. Then press the top of each tube with your finger so that there is a good seal.
8. Record the time (there's a clock on the wall). Incubate the reaction tubes for 15 min at 37°C in the incubator.
9. Transfer your reaction tubes to the 50°C water bath in a tube boat for 15 min.
10. Transfer the tubes to ice.
11. Add 40  $\mu$ l TE buffer (pH 8.0) to each reaction tube and mix well by pipetting up and down 5 times.
12. Put the tubes back on ice.

## II. TRANSFORMATION

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Next we will transform the In-Fusion reaction into both electrocompetent and chemically competent cells. The electrocompetent cells produce *lacI* from the chromosome so that you can control the circuit's function with the addition of IPTG to express either GFP or RFP. However, the transformation efficiency of this strain is not optimal and it's possible you won't get any successful transformants that have your In-Fusion plasmid. So, we will also use chemically competent cells that come with the In-Fusion Kit to maximize the number of successful transformants since they are optimized for In-Fusion reaction transformations. This will also allow you to see the difference between electro and chemical transformations.

### Materials and Equipment

In-Fusion reactions (3)

GFP Circuit Teams: Transformation positive control plasmid 1:10 (R0011+E0240 GFP circuit)

RFP Circuit Teams: Transformation positive control plasmid 1:10 (J04450 RFP circuit)

*E. coli* DH5 $\alpha$  *lacI*<sup>q</sup> electrocompetent cells (5 X 40  $\mu$ l)

Fusion-Blue chemically competent cells (5 X 50  $\mu$ l)

SOC liquid media

Electrocuvettes (5)

LB + 100  $\mu$ g/mL ampicillin plates (10)

LB + 100  $\mu$ g/mL ampicillin + 1 mM IPTG plate

Bunsen burner

Beaker with ethanol and cell spreader

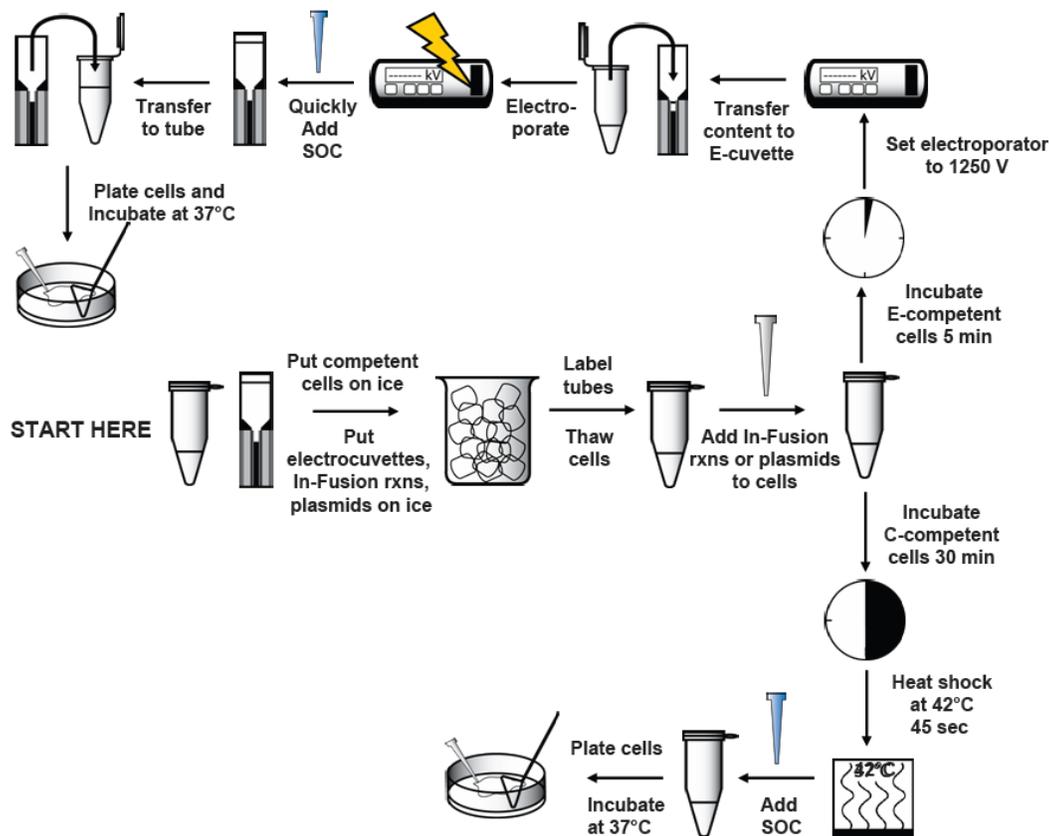
Electroporator

Non-shaking incubator (set to 37°C)

Heat block (set to 42°C)

Ice bucket with crushed ice

Protocol



1. Remove 5 tubes of electrocompetent cells (marked with two blue dots) and 5 tubes of chemically competent cells (yellow tubes) from the  $-80^{\circ}\text{C}$  freezer and put **immediately** on ice. Make sure the bottom of the tubes are completely submerged in ice and not just lying on top of the ice.
2. While the cells are thawing on ice (about 15-20 minutes), remove 5 electrocuvettes from the  $-80^{\circ}\text{C}$  freezer, unwrap them from packaging, and put on ice.
3. Put your transformation positive control (labeled #4) tube on ice next to your 3 In-Fusion reaction tubes.
4. Label the tops of the electrocompetent cells tubes 1A, 2A, 3A, 4A, and 5A and label the tops of the chemically competent cells tubes 1B, 2B, 3B, 4B, and 5B (see table below).
5. Prewarm 10 LB+Amp plates and 1 LB+Amp+IPTG plate (in the fridge) by putting them in the  $37^{\circ}\text{C}$  non-shaking incubator (if these are not in there already).
6. Test to see if your cells are thawed by gently flicking the bottom of one electrocompetent cells tube and one chemically competent cells tube. If the liquid moves up the tube by flicking, then the cells are thawed.
7. If thawed, transfer  $2.5\ \mu\text{l}$  of the #1 In-Fusion reaction tube into the #1A and #1B competent cells tubes, mix by flicking the tube gently a few times, and incubate the tube on ice.
8. Do the same for the #2-4 tubes into the #2A-4A and 2B-4B competent cells. The positive control plasmid is #4. Do not add any DNA to tubes #5A and 5B – these are your negative controls.

Transformation#	Name	Volumes ( $\mu$ l)				+ control plasmid #4	Incubation time
		In-F #1	In-F #2	In-F #3			
<b>Electro</b>							
<b>1A</b>	In-Fusion Rxn #1	2.5				5	
<b>2A</b>	In-Fusion Rxn #2		2.5			5	
<b>3A</b>	In-Fusion Rxn #3			2.5		5	
<b>4A</b>	Positive control				2.5	5	
<b>5A</b>	Negative control					5	
<b>Chemical</b>							
<b>1B</b>	In-Fusion Rxn #1	2.5				30	
<b>2B</b>	In-Fusion Rxn #2		2.5			30	
<b>3B</b>	In-Fusion Rxn #3			2.5		30	
<b>4B</b>	Positive control				2.5	30	
<b>5B</b>	Negative control					30	

9. Record the time. After 5 minutes (or longer if other groups are using the electroporator, this will not decrease your transformation efficiency), bring your ice bucket over to the electroporator with your P1000 pipette, SOC media in a rack, 1.5 mL tube rack, and P1000 pipette tips.
10. Pipette 450  $\mu$ l of SOC media and have one team member hold the pipette with SOC for later while the other team member does steps 11-16. If you don't have another team member, put your pipette with the SOC media down on the bench **slowly and carefully** so the media doesn't go into the pipette.
11. Set the electroporator to 1250 V and pull out the electrocuvette holder on the right side.
12. Transfer the contents of the #1A electrocompetent cells tube into the **bottom gap** of one electrocuvette.
13. Place the electrocuvette into the electrocuvette holder in the correct orientation (with the "bump" of the electrocuvette facing towards the back).
14. Slide the electrocuvette holder into the electroporator all the way until it stops.
15. Press the "Pulse – Press 2X" button **two** times to deliver a pulse to (electroporate) the cells.
16. After the long beep, **quickly** remove electrocuvette from the electroporator by removing the holder.
17. **Immediately** have the other team member add the 450  $\mu$ l of SOC buffer to the electrocuvette, pipetting up and down 3 times to mix the culture.
18. Pipette the entire cells and SOC media mixture back into the original tube (this will require two pipetting steps) and close the lid.
19. Repeat steps 10-18 for tubes #2A, 3A, 4A, and 5A.
20. Remove your plates from the non-shaking incubator. Label the bottom of your 10 LB+Amp plates with your team letter, transformation number, and date.
21. Label the bottom of your one LB+Amp+IPTG plate with your team letter, #1A, +IPTG, and date. We're going to look at the difference between your insert+vector In-Fusion #1A transformation with and without IPTG.
22. Remove the #1A LB+Amp plate lid and pipet 200  $\mu$ l of the #1A transformation tube on to the center of the plate.

23. Remove the cell spreader from the beaker of ethanol, tap it gently on the side of the beaker to remove excess ethanol, then dip it into the Bunsen burner just long enough for the ethanol to catch fire. Hold the spreader away from the burner and let the ethanol burn off.
24. Touch the spreader to the plate to allow any heat to dissipate, then spread the liquid culture evenly over the surface of the agar plate. The best way to do this is to simultaneously turn the plate clockwise with one hand and spread the culture from the top of the plate to the bottom continuously.
25. Put the top of the plate back on and set plate aside.
26. Repeat steps 22-25 for the rest of the tubes, starting with the additional 200  $\mu$ l of the #1A transformation tube on to the labeled LB+Amp+IPTG plate.
27. Incubate the plates in the non-shaking 37°C incubator **upside down** overnight.
28. After 30 minutes (or longer, it won't decrease transformation efficiency), you can start the chemical transformations. Do this by heat shocking tubes #1B, 2B, 3B, 4B, and 5B in the 42°C heat block for **exactly** 45 seconds and place the tubes **directly on ice** for one minute.
29. Add 450  $\mu$ l SOC media to each tube one at a time and mix by pipetting up and down 3 times.
30. Plate these 5 chemical transformants tubes on your remaining 5 LB+Amp plates the same way as before (using 200  $\mu$ l again).
31. Incubate the plates in the non-shaking 37°C incubator **upside down** overnight.
32. Clean up! Make sure everything is put or thrown away in the Biohazard waste bin, your bench has been wiped down with ethanol, and put your team box with the In-Fusion reactions in the -20°C freezer.

### III. TRANSFORMANT COLONY IMAGING

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The next step is to analyze your transformants from yesterday's transformations. The transformants on these plates will have plasmids with the amp resistance gene since they were all plated with ampicillin. There should be colonies on your positive control plates (#4A and 4B) and none on your negative control plates (#5A and 5B). Hopefully there will be colonies on your #1A and #1B plates because these cells hopefully contain your plasmid of interest with the functional genetic circuit. The #2A and 2B plates are the positive control of the In-Fusion reactions, so there will hopefully be colonies on these plates since the vector fragment contains an amp resistance gene. The #3A and 3B plates will show you the background plasmids used as template DNA in your vector PCR reaction and hopefully will be much less than plates #1A and #1B. Note that the colonies of the A plates from your electrotransformations are much larger than the B plates from your chemical transformations. This is because the Fusion-Blue chemically competent cells grow into smaller colonies. Unfortunately these colonies (on the B plates) can not be visualized as glowing or not because the colonies are too small.

#### Materials and Equipment

Blue light or UV transilluminator  
Imaging system with computer

#### GFP Circuit Protocol

1. Look at your plates from yesterday and write down your best estimate of the number of colonies on each plate. Think about your results – do they make sense?

2. Take your plates over to the imaging system.
3. Put the #1A plate with IPTG on the transilluminator and take a photo. Adjust the exposure time so that you can see individual colonies clearly and distinguish glowing from non-glowing colonies.
4. If there are glowing colonies, then these have the plasmid of interest! If there are not any glowing colonies, then hopefully the #1B plate has the plasmid of interest and you will need to perform colony PCR to find out (see protocol below).
5. Take a photo of your #1A plate without IPTG. There should not be any glowing colonies. This is a control to see if there is actually IPTG on your plate.
6. Take photos of the rest of your plates to document your results.

### **RFP Circuit Protocol**

1. Look at your plates from yesterday and write down your best estimate of the number of colonies on each plate. Think about your results – do they make sense?
2. Take your plates over to the imaging system.
3. Look at your #1A plate – there will hopefully red glowing colonies that you can see visually. However, to document your results, put the #1A plate with IPTG on the transilluminator and take a photo. Adjust the exposure time so that you can see individual colonies clearly and distinguish glowing from non-glowing colonies.
4. If there are glowing colonies, these may be from the original RFP-expressing J04450 plasmid and may not have the circuit of interest with the new promoter and RBS. You will need to perform a colony PCR reaction to find out (see protocol below). If there are not any glowing colonies, then hopefully the #1B plate has the plasmid of interest and you will need to perform colony PCR to find out.
5. Take a photo of your #1A plate without IPTG and note the differences – this plate should not contain any glowing colonies. This is a control to see if there is actually IPTG on your plate.
6. Take photos of the rest of your plates to document your results.

## **IV. COLONY PCR AND MAKING OVERNIGHT CULTURES**

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Colony PCR is a technique that will allow you to determine the insert size within a plasmid and allows you to distinguish between colonies that have your plasmid of interest vs. those that do not. Colony PCR is just like a regular PCR reaction except that we are using the template DNA from the plasmids inside cells instead of from a diluted miniprep plasmid, as before. The first step of the PCR reaction programmed in the thermocycler is 94°C for 3 minutes. This heats up the cells in the PCR reaction and they lyse, causing the template DNA (in this case plasmid DNA) to be used in the PCR reaction.

For Colony PCR we are using the Fermentas Master Mix which does not contain a high fidelity polymerase, so it is cheaper to use than the Invitrogen High Fidelity mix that we used before. The Fermentas mix is still suitable for colony PCR since we are not using the DNA for constructing genetic circuits, only to screen colonies for the correct insert, and thus do not need to have high fidelity PCR products. We will concurrently make overnight cultures of the colonies that you are testing so that you can extract the plasmids from the cultures that hopefully contain the correct plasmid, then send these off to be sequenced.

### **Materials and Equipment**

Plates with transformant colonies

Fermentas 2X PCR Master Mix

PCR tubes (8)

GFP circuit teams: P3 and P4 (VF2 forward and VR reverse) primers, R0011+E0240 control plasmid

RFP circuit teams: P1 and P6 (RFP-insert forward and R-vector reverse) primers, J04450 control plasmid

LB+Amp in 125 mL bottle with 50 mL

15 mL Falcon Tubes (7)

Thermocycler

Shaking incubator (set at 37°C)

## Protocol

### GFP Circuit Teams

If you had glowing colonies on your #1A plate with IPTG, then you do not need to do colony PCR because your circuit has been assembled correctly and it is already in the host strain of interest (those expressing *lacI*). Instead of colony PCR, just make an overnight culture in 6 mL LB+Amp media of at least one glowing colony (see protocol below). If you had no glowing colonies on your #1A plate with IPTG, then you will need to perform colony PCR on 6 colonies from your #1B plate and your R0011+E0240 control plasmid as a positive control. The negative control will have no template DNA. Use the P3 (VF2) and P4 (VR) primers for your forward and reverse primers, respectively, in your colony PCR reaction below. If the PCR product size from a colony matches the size from the positive control, then there is a good chance you have the plasmid of interest.

### RFP Circuit Teams

If you had glowing colonies on your #1A plate with IPTG, then perform colony PCR on 5 glowing colonies you have, one non-glowing colony (as a control), and your E0240 plasmid as a positive control. If you had less than 5 glowing colonies on your #1A plate with IPTG, then you will need to perform colony PCR on additional colonies from your #1B plate to make a total of 6 tested colonies. For example, if you have 2 glowing colonies on your #1A plate, test these 2 and then test 4 additional colonies from the #1B plate. If you had no glowing colonies on your #1A plate with IPTG, then you will need to perform colony PCR on 6 colonies from your #1B plate. You will use your J04450 plasmid as a positive control. The negative control will have no template DNA. Use the P1 (RFP-insert forward) and P6 (R-vector reverse) primers for your colony PCR reaction below. Only the correctly re-engineered plasmids should amplify with these primers since P6 is specific to R0011 and not R0010. So these primers will distinguish between the re-engineered plasmid and R0011 and J04450 used in the first PCR reactions as template DNA.

### Making Overnight Cultures Protocol

1. Label seven 15 mL Falcon tubes with your team letter and #1-6 for the number of the colony you will be testing and #7 is your blank (negative control to test for contamination in the media).
2. To practice sterile technique before you pipet media into your Falcon tubes (next step), remove the cap from the LB+Amp media bottle and **quickly** run the underside of the cap over the Bunsen burner **without burning yourself**, then set the cap down face up on the bench. Next hold the mouth of the bottle to the Bunsen burner while quickly rotating the bottle on all sides and be careful not to burn yourself on this step also. Flaming the bottle mouth creates an outward convection current that deters microbes from falling into the open mouth and kills any microbes that are on the mouth of the bottle itself.
3. Fill each of the Falcon tubes with 6 mL of LB+Amp liquid media. Unfortunately we don't have enough transfer pipettes for each team, so you will need to do this by setting your P1000 to 1000 mL and transfer this amount into each Falcon tube 6 times to get 6 mL total.

- You will use these culture tubes in the next protocol, so set them aside for now.

### Colony PCR Protocol

- Write down what colony PCR reactions you will be performing with specific numbers. For example, if you are testing 6 colonies on plate #1B, your reactions will be #1-6: colonies from plate #1B, #7: positive control (using the appropriate plasmid stated above), #8: negative control (no template DNA).
- Make a PCR mastermix from the Fermentas 2X Master Mix in a 1.5 mL tube. For one colony PCR reaction, you will add 5  $\mu$ l 2X Master Mix, 4.6  $\mu$ l of water, 0.2  $\mu$ l of the forward primer, and 0.2  $\mu$ l of the reverse primer. Multiply these amounts by however many reactions you are performing plus one (to allow for extra volume). For example, if you are performing 8 reactions (6 colonies plus the positive and negative control), you will add the following PCR reagents in the 9X column to your 1.5 mL mastermix tube.

PCR Reagent	1X	9X
2X Master Mix	5 $\mu$ l	45 $\mu$ l
Water	4.6 $\mu$ l	41.4 $\mu$ l
Forward primer	0.2 $\mu$ l	1.8 $\mu$ l
Reverse primer	0.2 $\mu$ l	1.8 $\mu$ l
<b>Total</b>	<b>10 <math>\mu</math>l</b>	<b>90 <math>\mu</math>l</b>

- Vortex your mastermix tube and add 10  $\mu$ l to each of your 8 PCR tubes. There will hopefully be a little leftover if you pipetted correctly.
- Close the caps and label them with your team letter and #1-8.
- Get the plate(s) you want to use for colony PCR (see above), either the #1A or 1B plate, or both.
- Bring your culture tubes over next to you.
- Put a clean pipet tip on your pipette and touch the tip on colony #1 (closer to the center of the colony if possible), then put the pipet tip in your #1 colony PCR reaction and gently wiggle the pipet tip to dislodge any cells. Then do not throw away the pipet tip! Instead put the same pipet tip in your #1 culture tube and again wiggle the pipet tip (this time not gently) to dislodge the remaining cells.
- Repeat for colonies #2-6.
- Add 1  $\mu$ l of the positive control plasmid to your #7 PCR tube. Nothing goes in the #8 tube.
- Put the PCR tubes in the fridge until an instructor can put them in the thermocycler. The same program will be used as in the first lab protocol.
- Put the culture tubes in the shaking incubator set at 37°C.
- Clean up! Make sure everything is put or thrown away in the Biohazard waste bin, your bench has been wiped down with ethanol, and team boxes with samples are put back in the -20°C freezer.

## V. COLONY PCR RESULTS, MAKING GLYCEROL STOCKS, AND MINIPREPS

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Next we want to see which colony PCR reactions worked, save the cultures that we want for future use by making a glycerol stock, and then isolating the plasmids from these cultures. We will know which colony PCR reactions worked because they have the expected size bands after running them on a gel.

### **Materials and Equipment**

See “Agarose Gel Electrophoresis” section in Lab 1

Cryotubes

40% Glycerol (80% Glycerol diluted 1:1 in molecular grade water)

Vortex

Refrigerated Centrifuge

Overnight cultures in LB+Amp media

Qiagen Miniprep Kit

Microcentrifuge

### **Colony PCR Gel Protocol**

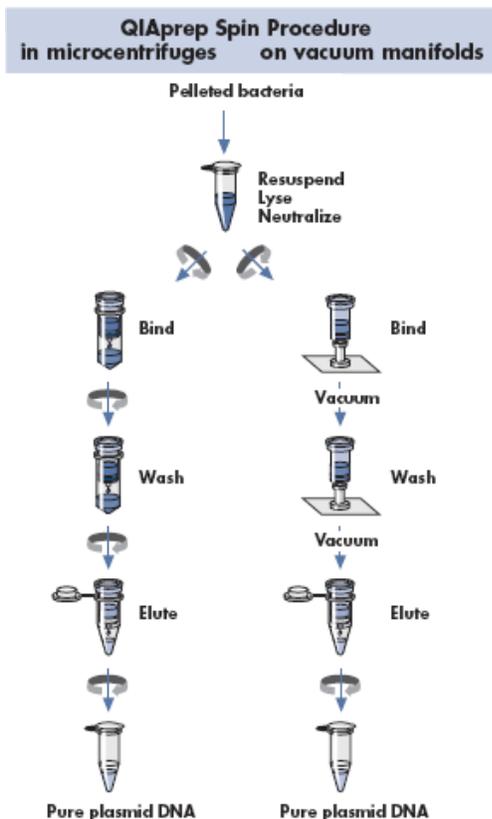
Run a gel on your colony PCR products from yesterday. Do everything the same as described in Lab 1 except that you will load the entire 10 ul PCR product into the gel with 10 ul of 2X loading buffer. Analyze the gel results and determine whether there are any colonies that give you the expected PCR product size. Save glycerol stocks and perform minipreps of the overnight cultures made from these colonies in using the following protocols.

### **Making Glycerol Stocks Protocol**

1. Vortex overnight cultures that you want to make glycerol stocks of.
2. Add 750  $\mu$ l of culture and 750  $\mu$ l of 40% glycerol to a cryotube.
3. Vortex.
4. Put immediately in the  $-80^{\circ}\text{C}$  freezer for future use.

### **Minipreps Protocol**

(Taken from the Qiagen Miniprep Kit Protocol with a Microcentrifuge)



1. Put remaining culture in the refrigerated centrifuge set at 4°C and spin at 5400 X g for 10 minutes.
2. Slowly pour supernatant into waste container (50 mL Falcon tube works well) and remove remaining supernatant with a P200 pipette set to 200 µl.
3. Resuspend pelleted bacterial cells in 250 µl Buffer P1 and transfer to a **labeled** microcentrifuge tube. Ensure that RNase A has been added to Buffer P1. No cell clumps should be visible after resuspension of the pellet. If LyseBlue reagent has been added to Buffer P1, vigorously shake the buffer bottle to ensure LyseBlue particles are completely dissolved. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.
4. Add 250 µl Buffer P2 and mix thoroughly by inverting the tube 4–6 times. Mix gently by inverting the tube. Do not vortex, as this will result in shearing of genomic DNA. If necessary, continue inverting the tube until the solution becomes viscous and slightly clear. Do not allow the lysis reaction to proceed for more than 5 min. If LyseBlue has been added to Buffer P1 the cell suspension will turn blue after addition of Buffer P2. Mixing should result in a homogeneously colored suspension. If the suspension contains localized colorless regions or if brownish cell clumps are still visible, continue mixing the solution until a homogeneously colored suspension is achieved.
5. Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube 4–6 times. To avoid localized precipitation, mix the solution thoroughly, immediately after addition of Buffer N3. Large culture volumes (e.g. ≥5 ml) may require inverting up to 10 times. The solution should become cloudy. If LyseBlue reagent has been used, the suspension should be mixed until all trace of blue has gone and the suspension is colorless. A homogeneous colorless suspension indicates that the SDS has been effectively precipitated.

6. Centrifuge for 10 min at 13,000 rpm (~17,900 x g) in a table-top microcentrifuge. A compact white pellet will form.
7. Apply the supernatants from step 4 to the QIAprep spin column by decanting or pipetting.
8. Centrifuge for 30–60 s. Discard the flow-through.
9. Wash the QIAprep spin column by adding 0.5 ml Buffer PB and centrifuging for 30–60 s. Discard the flow-through. This step is necessary to remove trace nuclease activity when using endA+ strains such as the JM series, HB101 and its derivatives, or any wild-type strain, which have high levels of nuclease activity or high carbohydrate content. Host strains such as XL-1 Blue and DH5 $\alpha$ <sup>TM</sup> do not require this additional wash step.
10. Wash QIAprep spin column by adding 0.75 ml Buffer PE and centrifuging for 30–60 s.
11. Discard the flow-through, and centrifuge for an additional 1 min to remove residual wash buffer. Important: Residual wash buffer will not be completely removed unless the flow-through is discarded before this additional centrifugation. Residual ethanol from Buffer PE may inhibit subsequent enzymatic reactions.
12. Place the QIAprep column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50  $\mu$ l Buffer EB (10 mM Tris-Cl, pH 8.5) or **water (this gives better results downstream)** to the center of each QIAprep spin column, let stand for 1 min, and centrifuge for 1 min.
13. Measure concentration with Nanodrop and store at -20°C.

## VI. DNA SEQUENCING

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In order to know for sure we have the correct engineered plasmid, we need to sequence it. This is also a check to see if there are unexpected mutations in our plasmid. These mutations can be the result of either PCR errors or replication of the plasmid after culturing the bacteria with this plasmid.

### Materials and Equipment

Purified plasmids (minipreps)  
Forward primer  
Reverse primer  
1.5 mL tubes

### Protocol

1. Fill out UW DNA sequencing form online and print out two copies (one for you and one for the sequencing facility).
2. Label the appropriate number of 1.5 mL tubes with the sample name as indicated on the sequencing form.
3. Add 1200 +/- 300 ng miniprep plasmid (4  $\mu$ l works well if about 300 ng/ $\mu$ l) with 8 pmol of the appropriate primer (1  $\mu$ l if 8 pmol/ $\mu$ l), and the remaining volume with water to 12  $\mu$ l (7  $\mu$ l in the example given here).
4. When all your samples are prepared, give them to the sequencing facility with your form.
5. Clean up! Make sure everything is put or thrown away in the Biohazard waste bin, your bench has been wiped down with ethanol, and team boxes with samples are put back in the -20°C freezer.