

Build-a-Phage

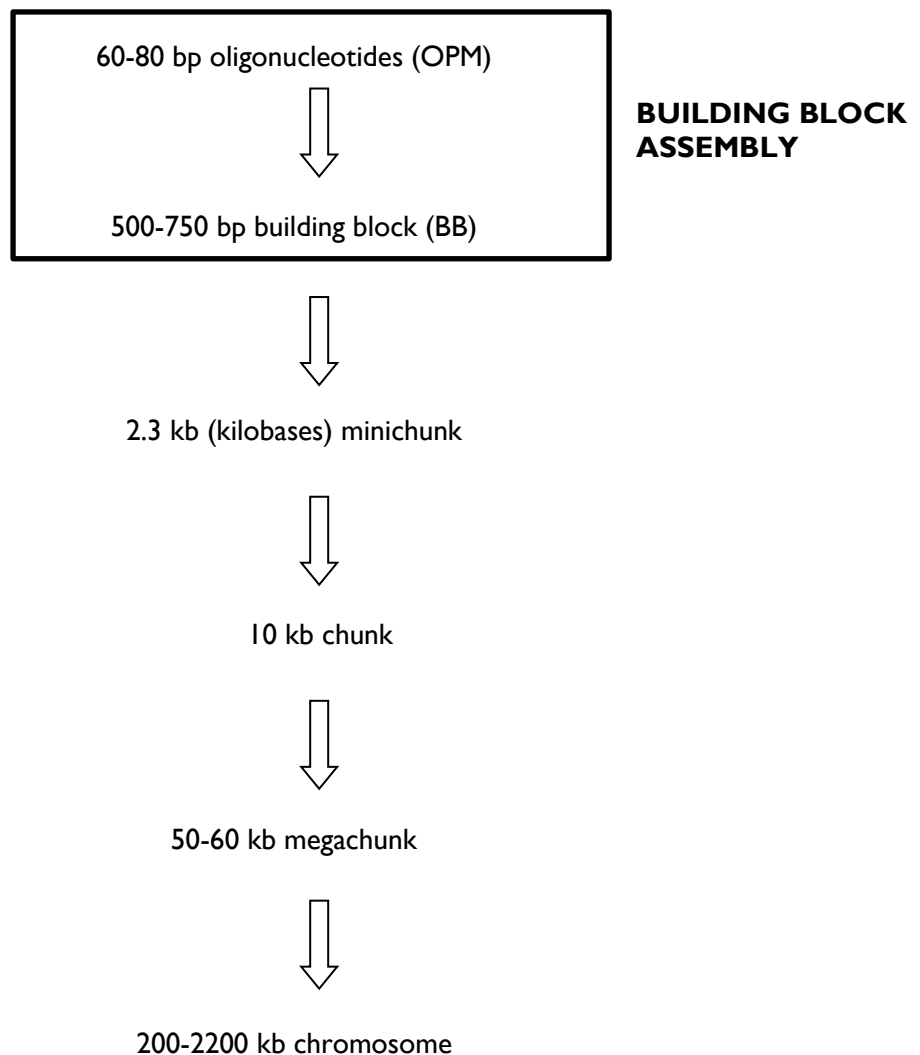
Lab Manual

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SYNTHETIC GENOME ASSEMBLY

This workflow was initially designed for the Synthetic Yeast Project (www.syntheticyeast.org) in which yeast chromosomes are assembled from oligonucleotides. While synthesis of those chromosomes is complete and we have now moved on to the synthesis of other genomes, such as phages, **we think it is still valuable to have students go through the process of building block assembly during the initial phase of the course.** Students can synthesize either a portion of the synthetic yeast genome that has already been completed (for example a portion of chromosome III which we can provide), or they can assemble a model gene such as GFP or RFP. Performing this initial synthesis prior to assembly of the phage genes gives students practice with some of the technically and conceptually difficult portions of the course (templateless and finish PCR).



BUILDING BLOCK ASSEMBLY

Step 1: Oligo design

The goal of building block synthesis is to assemble a “building block (BB)” of ~750 bp. Individual oligos are then ordered from commercial suppliers and serve as the template for synthesis.

Step 2: Templateless PCR

Templateless PCR is performed to form a larger construct (the building block, or BB) from mixtures of oligonucleotides.

Step 3: Finish PCR and Gel electrophoresis

Finish PCR is performed to amplify the full-length building block that is produced in small quantities in the templateless PCR. The success of the finish PCR is gauged by running a portion of the finish PCR reaction on a gel.

Step 4: Ligation and Bacterial Transformation

Building blocks that are of the correct size are cloned into a genetic cloning vector by Gibson assembly (traditional restriction digest and ligation could also be used) and used to transform bacteria. Bacteria are transformed with the cloned construct to create a population of clones for sequencing.

Step 5: Colony screening PCR

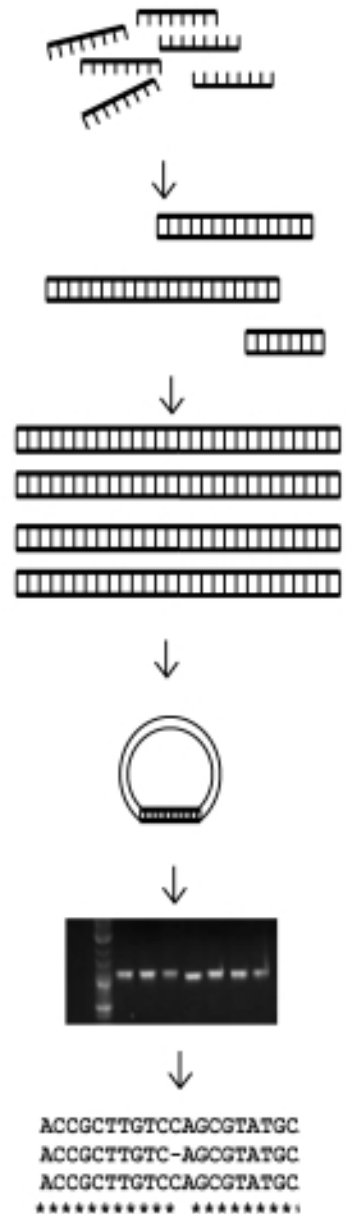
We verify that each bacterial clone has a DNA insert of the correct size by performing PCR and gel electrophoresis.

Step 6: DNA Sequence Analysis

Bacterial clones are sequenced to make sure that there are no sequence errors. Once the DNA has been sequenced, we compare the DNA sequence of each clone to the desired sequence to identify those without sequence errors (we call these “perfect clones”). This completes BB synthesis.

Step 7: Troubleshooting PCR

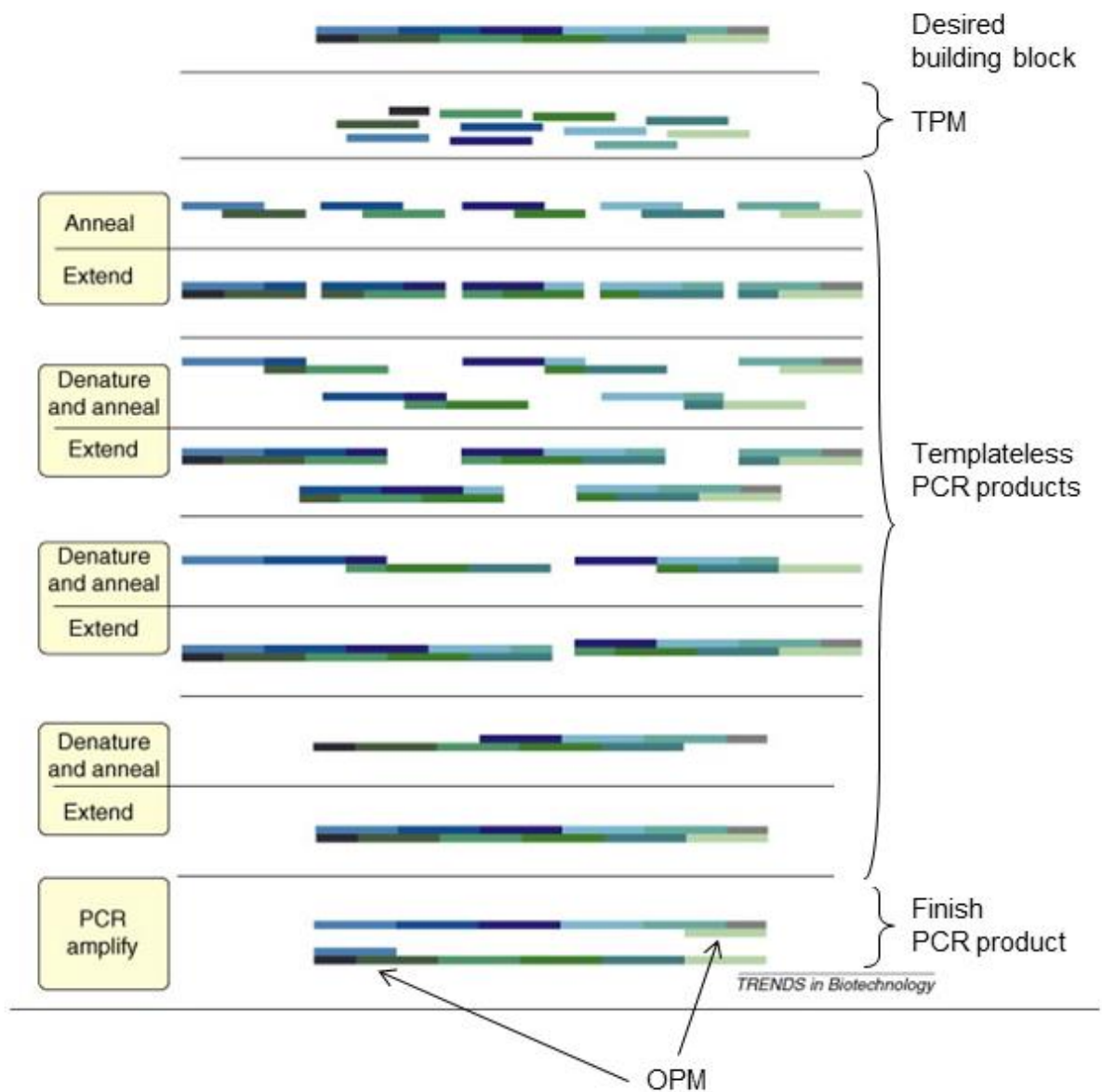
Since many BBs will not assemble correctly the first time, we change the templateless PCR and finish PCR reaction conditions to enable assembly of each building block.



Templateless PCR

The goal of templateless PCR is to combine a mixture of short single-stranded DNA oligonucleotides (the TPM) and assemble them into a longer double-stranded DNA sequence, which we term a building block (BB). This building block DNA may have a sequence that is completely new and may not have ever previously existed in nature.

During the templateless PCR reaction, the oligos are denatured, annealed, and extended through multiple rounds of PCR until finally a small amount of the full-length BB (approximately 500-750 bp) is synthesized.



Reaction Setup

- Before you set up reactions, make sure you reserve a spot in the PCR machine. You will need to sign up for 3 spaces in the PCR machine-label each with your initials and either “BB”, “PC” or “NC”.
- Keep dNTPs and Herculase enzyme **ON ICE AT ALL TIMES!**
- Note for instructors: because students have difficulty pipetting small volumes, I found that they would waste tremendous amounts of enzyme when it was given to them undiluted, so I dilute the Herculase enzyme before giving it to students (they should use the equivalent of 0.25 ul per 25 ul reaction).
- Note for instructors: The positive control can be any premixed template and primer pair that you know to work well.

1. You will be performing 3 PCR reactions (1 building block (BB) plus one positive control reaction (PC) and one negative control reaction (NC).

2. Combine the reagents listed below into three different PCR tubes on ice (these are the very small tubes).

Reagent	Volume per 25ul reaction
2.5 mM dNTPs	2.5ul
5X buffer	5 ul
Diluted Herculase enzyme	15 ul
Total	22.5 ul

3. To the first tube, add 2.5 ul from the tube marked TPM.

4. To the second tube, add 2.5 ul from the tube marked PC. This will be your positive control reaction.

5. To the third tube, add 2.5 ul of water. This will be your negative control reaction.

6. Cap your tubes and **make sure that they are sealed tightly** so that the liquid will not evaporate. Flick the tubes gently to mix.

7. Place your tubes in the PCR machine in the positions for which you signed up. Make sure that you have recorded which sample (BB, PC, or NC) is in each position in the PCR machine.

Reaction Conditions:

1 cycle:

94°C, 3 minutes

5 cycles:

94°C, 30 seconds

69°C, 30 seconds

72°C, 1 minute

5 cycles:

94°C, 30 seconds

65°C, 30 seconds

72°C, 1 minute

20 cycles:

94°C, 30 seconds

61°C, 30 seconds

72°C, 1 minute

1 cycle:

72°C, 3 minutes

Finish PCR

We just used templateless PCR to assemble a small amount of the full-length building block (BB) from oligonucleotides. However, templateless PCR produces very little of the full-length BB. Because templateless PCR is so inefficient at synthesizing the full-length BB, it also creates many smaller DNA fragments that are intermediates in building block synthesis. Therefore, the templateless PCR that we performed resulted in a mixture of DNA products: a small amount of the full-length BB is present among other DNA products of varying sizes.

The goal of finish PCR is to exponentially amplify the building block sequence so that most of the DNA present in the mixture following finish PCR will be the full-length BB. This is accomplished by using the outer primer mix (OPM), which contains two oligos to amplify only full-length DNA. The OPM primers bind to either end of the building block (which now serves as a template), enabling only BBs that are full-length (and therefore contain both ends of the BB sequence) to be amplified by PCR.

Reaction Setup

- Before you set up reactions, make sure you reserve a spot in the PCR machine. You will need to sign up for 3 spaces in the PCR machine-label each with your initials and either “BB”, “PC” or “NC”.
- Keep dNTPs and Herculase enzyme **ON ICE AT ALL TIMES!**

1. Obtain your templateless PCR reactions from the PCR machine.

2. You will no longer need your PC and NC reactions today. To store them, transfer each to a separate tube by pipetting the liquid into a 1.7 ml tube. Label each tube with:

- Your initials
- “TPCR”
- Either “PC” or “NC”

Bring these tubes to the box up front to be frozen for next week, when we will run it on a gel-if the PC reaction shows a band of DNA, it will indicate that you properly set up your TPCR reactions.

2. To your templateless PCR reaction that is labeled “BB”, add 175 ul of water, cap the tube and invert to mix. We will use this diluted templateless PCR reaction in step 4 below.

You will be performing 3 PCR reactions (one building block (BB) plus one **NEW** positive control reaction (PC) and one **NEW** negative control reaction (NC)).

3. Combine all reagents listed below into 3 different PCR tubes (the very small tubes).

Reagent	Vol/25ul reaction
2.5 mM dNTPs	2.5ul
5x Buffer	5 ul
Diluted Herculase enzyme	13 ul
OPM	2.5 ul
Total	23 ul

4. To the first tube, add 2 ul of the templateless PCR reaction from step 2 above. Once this is done, discard the tube containing the remainder of the templateless PCR reaction.

5. To the second tube (PC), add 2 ul from the tube labeled PC.

6. To the third tube (NC), add 2 ul of water.

7. Cap your tubes and **make sure that they are sealed tightly** so that the liquid will not evaporate. Place your tubes in the PCR machine in the position for which you signed up. Make sure that you have recorded which sample (BB, PC or NC) is in each position in the PCR machine.

Reaction Conditions:

1 cycle:

94°C, 3 minutes

25 cycles:

94°C, 30 seconds

55°C, 30 seconds

72°C, 1 minute

1 cycle:

72°C, 3 minutes

PCR assignment

1. (6 pts) Attach a printout of your gel. Label each lane to indicate what sample was run in each lane.

2. (12 pts) Complete the table below.

PCR product	Are you expecting a PCR product?	If so, what is the expected size (bp)?	Actual size (bp; estimate from the gel picture)
TPCR PC			
TPCR NC			
FPCR PC			
FPCR NC			
BB			

3. (3 pts) The positive control (PC) consists of a plasmid and two primers that have been successfully amplified in the past. Why do you include this control when running a templateless or finish PCR reaction?

4. (3 pts) The negative control (NC) contains only DNA primers and water. Why do you include this control when running a templateless or finish PCR reaction?

5. (6 pts) The only PCR reaction that we did not run on a gel is the BB PCR reaction after templateless PCR but before finish PCR. What would you expect to see if you ran this PCR reaction on a gel? Therefore, why is there little point in running the templateless PCR BB product on a gel?

6. (6 pts) After DNA fragments have been separated by gel electrophoresis, how does the dye enable us to visualize the DNA (which is normally clear and colorless)? Why do we need to use both loading dye and also ethidium bromide?

7. (6 pts) When we set up the templateless PCR, we add all of the oligonucleotides to the reaction (the TPM), but when we set up the finish PCR we only add two of the primers. Why? Is finish PCR a template-dependent or template-independent PCR and what is the difference between the two in terms of what we are trying to accomplish with each reaction?

8. (6 pts) Look at the reaction conditions for the templateless PCR. Notice that the reaction begins at an annealing temperature of 69 degrees and then uses annealing temperatures of 65 and 61 degrees. In contrast, the finish PCR uses only one annealing temperature that is much lower (55 degrees). Why do the annealing temperatures differ and how does this enable the goals of each PCR reaction?

9. (6 pts) If you run your building block DNA on a gel after finish PCR and you get no PCR product, what are two things that you could change (in terms of the reaction conditions) to improve your results to get a PCR product?

10. (6 pts) When performing PCR, you want to maximize the yield of specific product and minimize the amount of nonspecific product that can result from primers binding to non-identical regions of DNA. If your PCR is not specific enough and you get more than one PCR product, what are two things that you could change (in terms of reaction conditions) to improve your results to get only one PCR product?

Gel Electrophoresis

So far, we have performed two steps of synthetic gene assembly to create a building block: (1) templateless PCR to assemble oligos into a small amount of full-length building block, and (2) finish PCR to amplify the full-length BB so that it becomes the most abundant DNA following PCR.

Now, we need to check how well each of these steps worked by running our PCR products (and controls!) on an agarose gel to verify whether we have assembled a significant amount of the full-length BB. Agarose gel electrophoresis will separate DNAs based upon their size. Therefore, if assembly of our BBs has been successful, we expect to see one DNA band whose size should approximate that of our desired BB. You can determine the size of each of your DNAs by comparing the size of your band to the size of the DNA marker (the 2-Log DNA ladder), which you will run on the gel with your DNAs.

Remember, we also have 4 control reactions (the PC and NC controls from the templateless PCR and the PC and NC controls from the finish PCR).

Pouring a Gel:

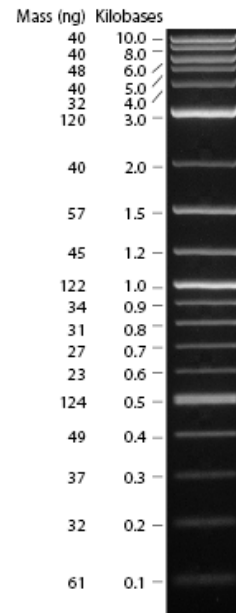
1. Weigh out 0.5 g of agarose on a piece of weigh paper. Transfer to an Erlenmeyer flask. Add 50 ml of 1x TAE.
2. Place the flask on a hot plate and heat until the agarose is completely transparent and colorless. Using a rubber heat mitt, periodically swirl the flask to ensure that the agarose is melting evenly. If there is any cloudiness or particulates, then place the agarose back on the hot plate for additional time until clear (check frequently).
3. Remove the flask of clear agarose and allow it to cool. This will take about 10 min. If you can hold your hand on the flask for 20 sec, then the agarose is cool enough to pour. (Warning: if poured while too hot, the agarose will leak and warp the plastic gel tray.)
4. While the agarose is cooling, place the gel tray into the gel box and add the black blockers and a comb.
5. When the agarose is cool, bring it to the hood to add 1 ul of ethidium bromide to the melted agarose (**CAUTION: ethidium bromide is a mutagen. Wear gloves and a lab coat at all times when handling gels and gel equipment!**).
6. Swirl the agarose to incorporate the ethidium bromide and pour the agarose into the gel tray –your gel should be about 1 cm thick.
7. Allow at least 20 minutes for the gel to solidify. Once solid, carefully remove the comb and the black blockers, and place the solidified gel (still on the tray) into the gel box so that the wells are oriented on the same side as the black electrode.
8. Add enough 1x TAE buffer to completely cover the gel by about 1 cm (~250 ml).

Preparing your samples:

1. Transfer each of your PCR products from the small PCR tubes to a new larger tube. Label each tube with either "BB", "FPCR PC", or "FPCR NC".
2. Obtain your TPCR PC and TPCR NC tubes that were stored in the freezer from last week. You should have 5 total samples now.
3. On a piece of parafilm, spot out 2 ul of 6x DNA loading dye with a P20 pipet. You should have 5 spots (one for each of your PCR products). Wait until your gel has started to solidify before beginning this step since the spots will dry out after ~10 minutes.
4. Add 5 ul of water to each spot of dye.
5. Add 5 ul of the PCR product.

Running a Gel:

1. Into the first lane of the gel load 5 ul of the 2-Log DNA ladder (mixed with water and dye).
2. Into lanes 2-5, load 10 ul of each of your PCR products (mixed with water and dye).
3. Place gel lid with electrodes on gel box. REMEMBER DNA RUNS FROM NEGATIVE (black) TO POSITIVE (red). CHECK YOUR LEADS: BLACK SHOULD BE AT THE TOP, RED SHOULD BE AT THE BOTTOM!
5. Set voltage to 100V. You should see bubbles coming up from the wire electrode.
6. After a few minutes, check to make sure everything is going smoothly. Run gel approximately 30 minutes or until the dye is 2/3 of the way down the gel.
7. Take a picture of your gel.
8. Use the DNA ladder (a picture is on the next page) to approximate the size of each of your building blocks. Make sure you check that your building block is the correct size - do not assume that because you have a band, it is the correct product!

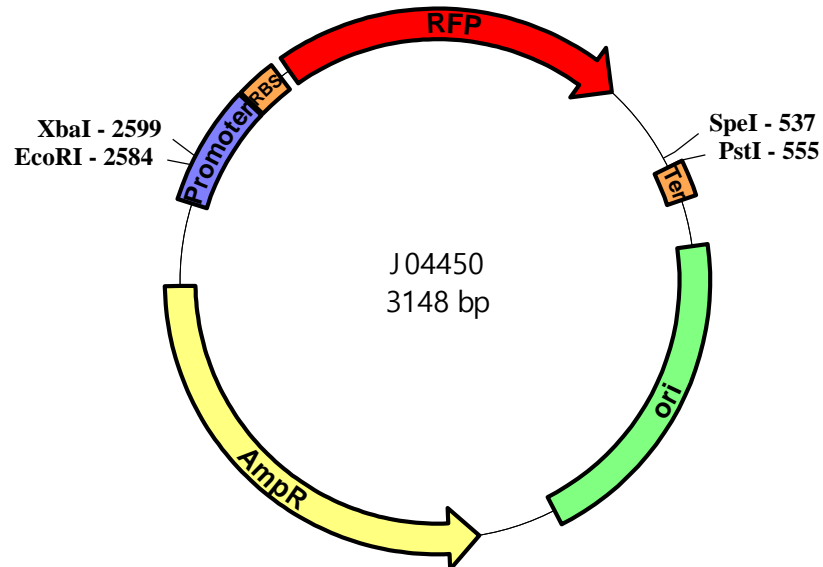


Gibson Assembly & Bacterial Transformation

Following the production of our DNA building block (which codes for the Red Fluorescent Protein-RFP) by templateless and finish PCR and verification that most of this DNA is the correct size by gel electrophoresis, we now must clone this building block into a plasmid vector. This cloning step is important for two reasons. First, it allows us to separate the population of DNA molecules into individual DNA molecules, thereby enabling us to screen the individual molecules to identify those that have no errors in the DNA sequence. Second, cloning allows us to create a permanent frozen bacterial stock of each plasmid so that we may store each building block.

Our building block PCR product (the RFP gene) will be cloned into a plasmid called J04450 which has the following features:

- Bacterial origin of replication (ori)
- Multiple cloning site (MCS)
- Selectable marker (ampicillin resistance gene)
- Bacterial promoter
- Ribosome binding site (RBS)
- Transcription terminator (Ter)



Gibson Assembly

Remember that the Gibson Assembly Master Mix contains enzymes and therefore **should be kept on ice at all times!**

1. Label a PCR tube (the tiny tubes) with your initials.
2. In that tube (**on ice**), combine:

Vector	5 ul
Building block insert	5 ul
Gibson Assembly Master Mix	10 ul

2. Place tube in the PCR machine and incubate at 50C for 15 minutes. (During this step, you can complete steps 1 and 2 under Bacterial Transformation below).
3. Remove tube from PCR machine and place on ice.

Bacterial Transformation

Once the vector and DNA insert are joined together with the Gibson Assembly Master Mix, the resulting recombinant DNA molecule is transformed into bacteria. Only a small number of bacterial cells in the population will become transformed. We can detect transformed cells because the plasmid DNA contains an antibiotic resistance gene; bacterial cells that become transformed will become resistant to the antibiotic (in this case, ampicillin). Ampicillin acts as a selective agent, allowing only those cells that contain a plasmid to grow.

E. coli that efficiently take up foreign DNA are said to be *competent*, and the method that they use to take up DNA is called *transformation*. Bacterial cells can be made competent by growing them at log phase, harvesting them from the growth media and then treating with calcium chloride. (It has been suggested that the Ca^{2+} neutralizes the negative charge of the phosphate backbone of the DNA. This neutralization prevents the DNA from being repulsed by the negatively charged phospholipids on the cell membrane, allowing the DNA to more efficiently enter the cell.) These competent bacteria can be stored at -80°C and subsequently used for transformation.

Competent bacteria take up DNA when the membrane is permeabilized, typically by heating quickly to 42°C . It is a tricky process because bacteria must be kept cold right until the heat shock step. **Taking the bacterial cells off of ice even briefly can lead to a dramatic decrease in transformation efficiency.**

It is important to use sterile technique throughout the transformation procedure. Keep the lids on your Petri dishes as much as possible and keep the foil top on the beaker of microcentrifuge tubes. Remove the cap as briefly as possible when pipetting the media. **Please remember to wash your hands with soap before leaving the lab!**

This is the easiest step to "mess up" in the entire workflow. Cells must be kept cold at all times! As soon as they thaw, they start dying. The more time passes (up to the point where LB is added) the more they die and the fewer transformants you'll get. Chill labeled tubes, ligation mixes, etc., on ice prior to mixing of cells and DNA (ligation mix).

1. Get three LB/Amp plates and label them "BB", "PC", and "NC". Put the plates in the 37°C incubator to warm up.
2. Label three microcentrifuge tubes (the larger size tubes) "BB", "PC", and "NC". Close the caps and place these empty tubes on ice.
3. Add 2.0ul of the Gibson Assembly reaction to the "BB" tube. Place tube back on ice.
4. Add 2.0ul of the positive control DNA to the "PC" tube. Place tube back on ice.
5. Bring your tubes (**on ice**) up to the front to have 25ul of thawed competent cells transferred into each of your microcentrifuge tubes.
6. Incubate all tubes on ice for 30 minutes.
7. **Bring your ice bucket and a timer to the water bath**, and heat shock your tubes by placing at 42°C for 30 seconds. **Timing is critical here!**
8. Immediately (ie, **bring your ice bucket to the water bath**) place heat shocked cells back on ice for 2 minutes.
9. Add 350 ul room temperature SOC medium to each transformation reaction and then put in a microcentrifuge rack at room temperature.
10. Put the rack with tubes in the 37°C incubator for 1 hr.
11. Pipette 100 ul from each tube onto corresponding LB/Amp plates (only remove the tops from the plates long enough to add the cells). Spread bacteria by adding 5-6 sterile glass beads, shaking the plates with beads and then pouring beads into the waste.
12. Allow the liquid to soak into the plates. Once the plates appear dry, incubate the plates overnight at 37°C (When you are incubating or storing plates, they should be stored upside down to prevent condensation from dripping onto them).

Bacterial Transformation Assignment

1. Complete the table below:

	Number of white colonies	Number of pink colonies	Total number of colonies	Percent pink colonies
Building block				
Positive control				
Negative control				

2. When performing Gibson assembly, why is it crucial that the vector and insert contain the same sequences at each of their ends? What would happen if you tried to use Gibson assembly to join a vector and insert that did not have complementary DNA ends?
3. When cloning using restriction enzymes, we must very carefully pick which enzymes we use based on the sequence of our insert DNA. How is Gibson assembly more flexible, allowing you to clone an insert regardless of its sequence in contrast to traditional cloning with restriction enzymes?
4. Is plasmid J04450 a cloning vector or an expression vector? What sequences on the vector allow you to tell what type of vector it is?
5. How would you be able to tell if your **Gibson assembly reaction** didn't work (what differences would you expect in the number and/or color of bacterial colonies on your three plates)?

6. How would you be able to tell if your **bacterial transformation** didn't work (what differences would you expect in the number and/or color of bacterial colonies on your three plates)?

7. It would have been easy to take the building block PCR product and sequence it directly without first cloning it into a plasmid. Why is it important to perform cloning (putting the PCR product into a plasmid and transforming it into bacteria) before performing DNA sequencing to identify one DNA molecule with an error-free version of the RFP sequence?

8. When you pick bacterial colonies for DNA sequencing, it is crucial to pick only one colony and not to accidentally combine two colonies that are next to each other on the bacterial plate. Why would it be a problem if you did accidentally combine two colonies together (think about your answer to question 6 above)?

The following questions pertain to the bacterial transformation that we will perform later in the semester, when we will combine 3-4 building blocks of phage DNA (rather than cloning the RFP gene). These 3-4 building blocks will be combined with a plasmid vector in the hopes that the building blocks will all join together with the plasmid.

9. When we perform bacterial transformation later in the semester, we will use petri plates containing X-Gal. What is the role of X-Gal in the bacterial plates? On plates with X-Gal, what color cells contain plasmids with a DNA insert and why?

10. What is the difference between a selection and a screen? Is ampicillin used in a selection or a screen? Is X-gal used in a selection or a screen?

Colony Screening PCR

Note to instructors:

- **If you do not have time to inoculate cultures and grow them before the day you are performing CS-PCR, you can inoculate bacteria into 10 ul of LB+Amp and use these cultures immediately for CS-PCR. After setting up the PCR reactions, the plates can be incubated at 37C to allow the cultures to grow.**
- **Both the Promega GoTaq and the NEB OneTaq master mixes work well for this procedure.**

We now have our successful finish PCR products (building blocks) cloned into the vector. While the predominant product in your PCR reaction was the full-length BB, there were likely also other shorter DNA molecules present as well. Before we spend the money to sequence these clones, we want to be sure that they contain a DNA insert that is the correct size. We can accomplish this by PCR. We will use the plasmid with our insert as a template and we will amplify that insert to make sure that it is the correct size. We can amplify any insert, regardless of sequence, by using PCR primers that anneal to the vector on either side of the PCR product insert. Remember that the expected size of your PCR product will include everything between the 5' end of the forward primer binding site and the 3' end of the reverse primer binding site.

Innoculating cultures for colony screening PCR:

1. Aliquot 100 ul of LB+Amp broth into the wells of a sterile 96 well plate (with a lid).
2. Use a wooden toothpick to pick one white or light blue colony (make sure that the colony is well isolated from neighboring colonies.)
3. Grow the cultures overnight at 37C for 18-24 hours (plates may be stationary, and shaking is not necessary).
4. This culture will be used as template for PCR.

Colony screening PCR Reaction Setup:

5. Prepare a master mix for all PCRs (10 CS-PCR reactions plus one positive and one negative control).

Reagent	Stock Concentration	Vol/20ul reaction	Master Mix (x15)
Forward vector primer	20uM	1.0 ul	
Reverse vector primer	20uM	1.0 ul	
Water		7.0 ul	
GoTaq Master Mix	2X	10.0 ul	

6. Aliquot 19 ul per well into PCR tubes. Add 1.0 ul of either the bacterial culture or the positive control.
7. Put your PCR reactions into the PCR machine and begin the PCR reaction.

Reaction Conditions:

94°C, 4 minutes

30 cycles:

94°C, 30 seconds

55°C, 30 seconds

72°C, 1 minute

72°C, 3 minutes

8. Save your 96-well plate of bacteria until we analyze the PCR reactions. If the PCR reactions indicate that we have an insert of the correct size, then we can come back to these cultures to regrow the bacterial cells with the plasmid to send the plasmid for DNA sequencing of the insert.

Colony Screening PCR Part 2

Pouring a Gel:

1. Weigh out 0.5 g of agarose on a piece of weigh paper. Transfer to an Erlenmeyer flask with 50 ml of 1x TAE.
2. Place the flask on a hot plate and heat until the agarose melts. Using a rubber heat mitt, periodically swirl the flask to ensure that the agarose is melting evenly.
3. Continue heating until agarose is completely transparent and colorless. If there is any cloudiness or particulates, then place it back on the hot plate for additional time until clear (check frequently).
4. Remove the bottle of clear agarose and allow it to cool. This will take about 10 min. If you can hold your hand on the bottle for 20 sec, then the agarose is cool enough to pour. (Warning: if poured while too hot, the agarose will warp the plastic gel tray.)
5. Place the gel tray into the gel box and insert the appropriate comb.
6. Bring the agarose to the instructor to add 1 ul of ethidium bromide to the melted agarose (CAUTION: ethidium bromide is a mutagen. Wear gloves at all times when handling gels!).
7. Pour the agarose into the gel tray –your gel should be about 1 cm thick.

8. Allow at least 20 minutes for the gel to solidify. Once solid, remove the comb(s), and place the solidified gel (still on the tray) into the gel box with 1x TAE buffer. The buffer should completely cover the gel.

Preparing your samples:

To each sample, add 4 ul of 6X DNA loading dye (add the dye directly into the PCR tubes).

Running a Gel:

1. Load 9 ul of your samples and 5ul of 2-Log DNA ladder.
2. Place gel lid with electrodes on gel box.
3. REMEMBER DNA RUNS FROM NEGATIVE (black) TO POSITIVE (red). CHECK YOUR LEADS: BLACK SHOULD BE AT THE TOP, RED SHOULD BE AT THE BOTTOM!
4. Set voltage to 100V.
5. Run gel approximately 20 to 30 minutes, then check dye front to make sure you don't run your samples off the gel. (You should always check a few minutes into each run just to make sure everything is going smoothly.)
6. Take a picture of your gel. Make sure you check what the expected size of your building blocks are-do not assume that because you have a band, it is the correct product!

DNA Sequence Analysis

Now that we've transformed our building blocks into bacteria, we need to sequence the inserts to verify that they contain a building block without any sequence errors. Today we will learn to analyze DNA sequences to identify "perfect clones".

When sequencing data is sent to us, we receive both a text file containing the sequence of the DNA and also the data from the sequencing machine in the form of a color-coded electropherogram. The electropherogram, or trace, represents the data obtained from sequencing detector, with the height of each peak representing the strength of the signal. We can therefore see the quality of the sequencing data that was obtained as well as investigate any ambiguities in the sequence.

Now we need to determine if our clones contain a sequence that perfectly matches the building block or if they have DNA sequence errors. To accomplish this, we use a bioinformatics tool called Clustal W.

Go to Moodle and download the zip file "Sequence Analysis Files" under the Lab section.

Go to the Clustal W homepage (www.ebi.ac.uk/Tools/clustalw2/index.html). Input the title of your sequence and then input the reference DNA building block sequence. The line before the BB sequence must contain >Name.of.Sequence (no spaces). Skip a line and input the forward sequencing reaction, preceded by >Name.of.Sequence.For. Click Align.

Clustal W gives you a scores table indicating the pairwise alignment similarity score (out of 100). It also provides a DNA alignment. Residues that are identical in the two sequences marked with a *.

Go to the Sequence Manipulation Suite (http://www.bioinformatics.org/sms2/rev_comp.html), make sure that you are on the Reverse Complement page and input the reverse sequencing read. Click Submit. Cut and paste this sequence into Clustal W preceded by >Name.of.Sequence.Rev.

You should now see your reference building block sequence aligned to both the forward and reverse sequencing reads. Residues that are identical in all the sequences marked with a *. For those residues that are not identical, **a mutation exists if the forward and reverse sequencing read agree and differ from the reference sequence.**

DNA Sequence Analysis Assignment

1. Many of our building blocks will contain errors in their DNA sequence. What are the two most common sources of error when making synthetic DNA building blocks?

2. The signal at the end of the electropherogram is not as strong as at the beginning; the peaks are much shorter and broader and become difficult to distinguish from one another. What is the reason that the strength of the signal tapers off? Sometimes instead of an A, T, C or G, the electropherogram will indicate N as the nucleotide. What does this N represent and why do they occur more frequently at the end of a sequencing read?

3. Our building block is ~700 bp long. Looking at the electropherogram, would one sequencing reaction be sufficient to accurately determine the sequence of our clones? Why or why not? How does the use of two sequencing reads ensure that we will get good sequencing data across the entire building block insert?

4. When you align your sequencing read to the reference DNA building block sequence using ClustalW why does the alignment extend past the end of the building block sequence? What does that extra sequence represent?

Analysis of mutations in building block sequences

5. Analyze the sequencing files for Analysis 1, 2 and 3 in the Assignment folder. Determine the nature and position of all mutations in the sequenced clone (ie deletion of A at nucleotide 140, transition of C to T at 34, etc.). Remember, if your forward and reverse reads do not agree, one of the sequences is probably HIGHER quality than the other at every individual discrepant base (it's more likely the ends close to the sequencing primer are more reliable than the ends farther away from the sequencing primer). **When numbering, use the nucleotide number of the BB.**

Only record a mutation if the forward and reverse reads agree with each other and disagree with the building block sequence.

	Name of building block	Mutations present in sequence
Analysis 1		
Analysis 2		
Analysis 3 (You only need to do 3-1 for this part)		

6. For each alignment, click View Alignment File, print the sequence alignment and circle all mutations (as defined above), and **submit this with your assignment.**

Error rates in building block synthesis

7. For analysis 3, there are 4 different clones of the same building block. Identify all of the mutations in those 4 sequences. **Only record a “mutation” if the forward and reverse reads agree with each other and disagree with the building block sequence. Also, only record a mutation if it falls within the bounds of the building block sequence.**

Sequencing ID	Name of Building block	Length of BB (nt)	# insertions	# deletions	# transitions	# transversions	Total # mutations
Analysis 3-1							
Analysis 3-2							
Analysis 3-3							
Analysis 3-4							

8. Total # of mutations in all 4 sequences combined: _____

Total # nucleotides sequenced (ie size of your BB x 4): _____

9. We would like to know the overall error rate for creation of our building blocks (we call this value α) since this information will help us to determine the efficiency of our method and protocols. The error rate can be calculated as follows:

$$\alpha = (\text{Total \# mutations found}) / (\text{Total \# building block nucleotides sequenced}).$$

$$\text{For example: } 13 \text{ mutations in 4 clones of a } 750 \text{ bp BB, then } \alpha = 13 / (4 * 750) = 0.001$$

From the information in question 8 the building block you analyzed, $\alpha =$ _____

10. The probability of a perfect clone (p_c) is calculated by $p_c = e^{-\alpha L}$ (L is the length of the BB).

What is the probability that any one of your clones is perfect? _____

However, what we really need to know is how many clones we need to send for sequencing. We want to send as few clones as possible (sequencing is expensive!).

If the probability of having one perfect clone in the batch is (p_b) is $p_b = 1 - (1 - p_c)^B$ (B is the number of clones in a batch).

What is the chance that a batch of 12 sequences will have a perfect clone? _____

PCR Troubleshooting

- I. What was the problem (if any) with your original finish PCR reaction for RFP synthesis?

What was your hypothesis for why your PCR did not work?

What will you change to try to get your finish PCR to work (annealing temp changes, extension temp changes, additional rounds of PCR, etc.)?

What was the problem (if any) with your Gibson Assembly and bacterial transformation?

What was your hypothesis for why your Gibson Assembly and bacterial transformation did not work?

What will you change to try to get your Gibson Assembly and bacterial transformation to work?

SEMI-SYNTHETIC PHAGE ASSEMBLY

Step 1: Oligo design using Watermarker program and oligo mixes

The goal of building block synthesis is to assemble a “building block (BB)” of ~750 bp from oligonucleotides. In this case, the BB will be one gene from a phage genome. We use software to insert a watermark that identifies the BB as being synthetic DNA. We then divide the BB into single-stranded oligos that are 60-80 bp long which are ordered from commercial suppliers

Step 2: Templateless PCR and Finish PCR

Templateless PCR is performed to form a larger construct (the building block, or BB) from mixtures of oligonucleotides. Finish PCR is performed to amplify the full-length building block that is produced in small quantities in the templateless PCR.

Step 3: Gel electrophoresis

The success of the finish PCR is gauged by running a portion of the finish PCR reaction on a gel.

Step 4: Purification of PCR product

Building blocks that are of the correct size are purified using a spin column kit for use in electroporation with the wild-type phage genome.

Step 5: Electroporation of Bacteria with Synthetic Gene & Phage DNA

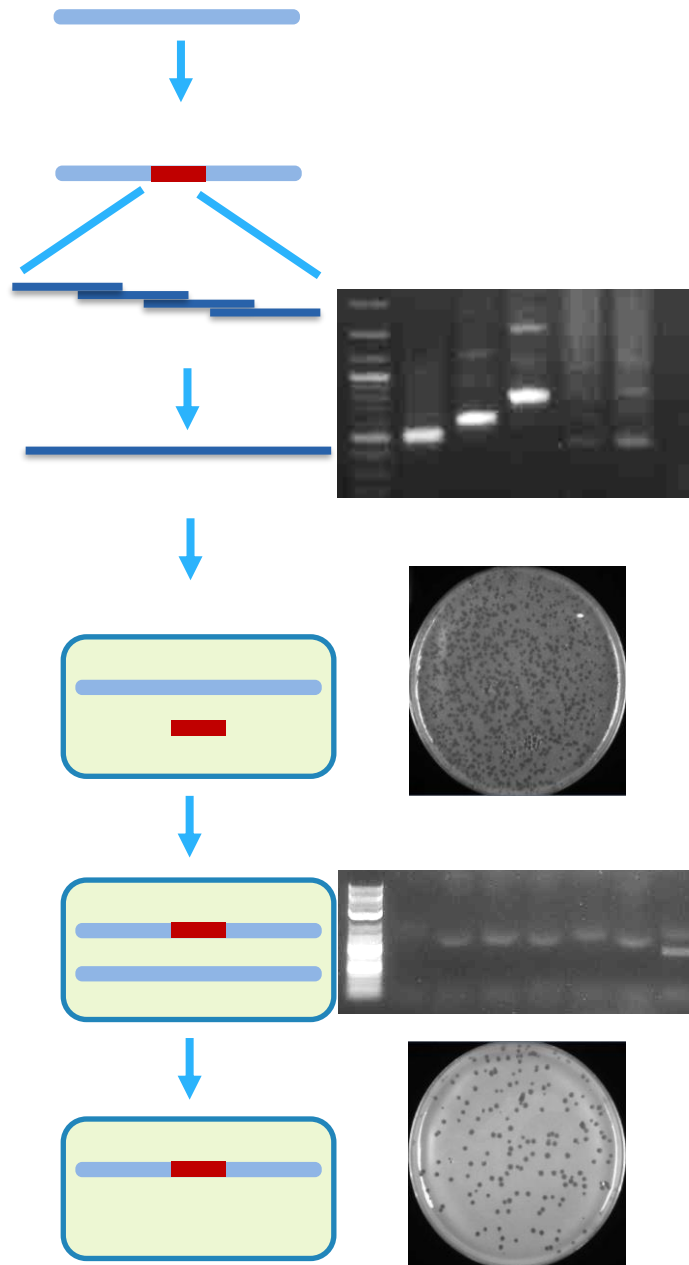
Electroporation is used to insert both the wild-type phage genome and the synthetic gene into bacterial cells. In these cells, the two DNAs will recombine, creating a semi-synthetic phage which will create plaques when the cells are plated.

Step 6: PCR screening of phage plaques

Several plaques from each plate are selected and screened with primers that anneal to the watermark sequences. The presence of a PCR product should indicate the presence of synthetic DNA within a functional phage genome.

Step 7: Dilution of semi-synthetic phage to determine titer

Once semi-synthetic phages are obtained, they can be diluted and plated to enable isolation of a pure phage clone and to determine the titer of the semi-synthetic phage relative to a wild-type phage.



Oligo Design and Watermarking

Watermarking

When we create synthetic DNA (such as synthetic phage genes), we need to mark that DNA as synthetic by inserting watermark sequences. The Watermarker program recodes the DNA sequence that you enter so that the amino acids are preserved but the nucleotides are changed. This will allow PCR primers to be created that anneal to the synthetic DNA but not the wild-type phage DNA. These primers will be used to verify that the synthetic gene has been incorporated into a phage genome. Our goal when watermarking is to change the nucleotide sequence by at least 33%.

1. How are watermarks used to differentiate between native and synthetic DNA? Specifically, what experimental procedure is performed and how does it help us to distinguish native and synthetic DNA? Why is more than one watermark sequence needed? Why do we try to change the nucleotide sequence by at least 33%?

Go to the Watermarker program (<http://watermarker.loyola.edu/>). Enter the sequence of your gene into the genomic sequence box. Switch the organism to *Mycobacterium smegmatis* and click "Generate Watermarks".

2. Did your initial attempt generate any watermarks? What parameters will you change to generate watermarks and why are these conditions more likely to be successful in producing watermark sequences?

3. Once you have successfully generated watermarks, record:

Sequence of Left Watermark: _____

Start position of left watermark: _____ End position: _____

Sequence of Right Watermark: _____

Start position of right watermark: _____ End position: _____

Expected size of PCR product: _____

Melting temp of left watermark: _____ Melting temp of right watermark: _____

Percent changed left watermark: _____ Percent changed right watermark: _____

Oligo Design

Our goal is to build a synthetic gene from oligonucleotides. The GeneDesign program allows us to take the sequence of our synthetic gene and break it up into the optional set of overlapping nucleotides for assembly into a synthetic gene.

The GeneDesign program allows us to make additional modifications to the gene. One of these is **codon optimization**, which is a strategy of using the most common codons to encode each amino acid in the hope of maximizing protein production. In performing codon optimization, we do not want to change the sequence of amino acids, but we want to change which codons encode the amino acids. This is termed **recoding the genome**.

To recode the genome, we must obtain the DNA sequence of the building block (synthetic gene) that we will be synthesizing. We can derive a DNA sequence from the protein sequence by “reverse translating” the protein to obtain a nucleotide sequence (note that for any protein sequence one can obtain multiple different DNA sequences depending upon which codons you choose to use).

For this exercise you can use the sequence of your synthetic gene or you can use this amino acid sequence for practice:

MAKINGSYNTHETICGENESTHISISMYFAVRITEGENEWHATISYRS

(we are using the one letter abbreviation for amino acids as shown in the table below)

Abbreviation	Amino acid
A	alanine
C	cysteine
D	aspartic acid
E	glutamic acid
F	phenylalanine
G	glycine
H	histidine
I	isoleucine
K	lysine
L	leucine
M	methionine
N	asparagine
P	proline
Q	glutamine
R	arginine
S	serine
T	threonine
V	valine
W	tryptophan
Y	tyrosine
X	stop codon

To design our oligonucleotides, we will use a program called Gene Design, available at <http://54.235.254.95/gd/>

Codon optimization

(This step is optional; if you simply want to break your gene into oligonucleotides, go to “Building Block Design (constant length overlap)” from the GeneDesign home page and pick up at “Creating oligos” below. **Note that codon optimization will likely change any watermark sequences**-you will need to manually reinsert these before ordering the oligos.

Step 1: Reverse Translate to obtain a nucleotide sequence

Click on “Design a Gene”. Enter the amino acid sequence of your building block (using the single letter amino acid code). Click on “Reverse Translate”. You should get a nucleotide sequence as an output from the program.

Step 2: Recoding the genome for codon optimization

Synonymous codons are codons that encode the same amino acid. We can recode the genome by substituting synonymous codons for the original codons. Organisms tend to favor certain synonymous codons over others (these are called preferred and non-preferred codons), a phenomenon we term **codon bias**. Preferred codons are associated with more efficient translation while non-preferred codons are associated with less efficient translation. If we use all preferred codons when recoding the genome, we are “optimizing the genome”.

Click “Back” on your browser. Notice that at the bottom you can select the organism that you are working on. This table will then show you the most preferred codons for each of the 6 organisms (H. sapiens, S. cerevisiae, E. coli, B. subtilis, D. melanogaster and C. elegans). You can manually enter the preferred codons for any species-for M. smegmatis, the codon usage table can be found here to identify the most preferred codons: <http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=246196>

1. For which amino acids is the same codon preferentially used in all 6 species? How many differences are there in preferred codon usage between S. cerevisiae and C. elegans?

Once you have selected your organism, click on “reverse translate” to obtain a nucleotide sequence. The nucleotide sequence that you are given now uses the preferred codons of your organism to encode your building block. This potentially alters the nucleotide sequence without altering the protein sequence.

2. When thinking about gene expression, what is one advantage to using the preferred codons to encode your synthetic gene(s)? By changing the DNA sequence, what are some potential problems that we may encounter at the level of the RNA?

Step 3: Other options for recoding the genome

At the bottom of the web page, click on “Codon Juggling”. This will give you several different versions of the sequence: an optimized version, a most different sequence, etc. Scroll to the bottom of the page and notice that all of the sequences encode the same amino acids.

Notice that for each of the nucleotide sequences that you are given the %GC is very similar. The program is designed to maintain the GC content of the DNA when selecting which codon to use for each amino acid (in order to obtain the proper GC content, the most preferred codon is not always the one that is used at each position).

3. Why might we want to control the %GC in each building block? What properties of the DNA sequences would be affected by a change in the %GC?

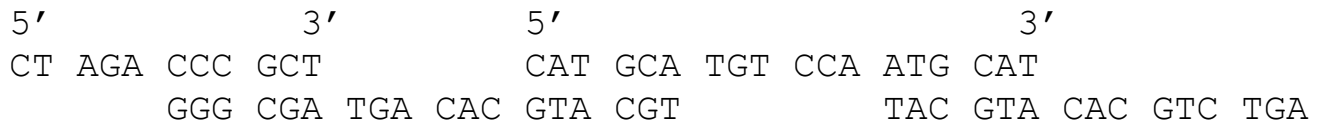
Creating oligos

Now that we have our nucleotide sequence, we need to have DNA chemically synthesized. However, only single stranded DNA can be chemically synthesized. Rather than ordering one piece of DNA that is the length of our building block (750 bp), we order our DNA as oligonucleotides of approximately 60 bp.

4. If we ordered one piece of DNA that was 750 bases long from the synthesis company, what percent of the DNA molecules would be perfect (without any synthesis errors) if the efficiency of synthesis were 99.5% for each step of oligo synthesis? If we ordered a piece of DNA that was 60 bases long, what percent of the DNA molecules would be perfect if the efficiency of synthesis were 99.5% for each step of oligo synthesis?

We order our oligonucleotides as single-stranded DNA and then use enzymes to synthesize the complementary strand, thereby producing double-stranded DNA. We design our oligos to partially overlap one another (we term these “gapped oligos”). This allows the end of one oligonucleotide to serve as a primer for DNA synthesis.

- Each oligonucleotide can only be extended in one direction. Why? On the figure below depicting overlapping oligos, fill in the missing DNA nucleotides and use an arrow to indicate the direction in which each section of DNA is synthesized.



Step 4: Optimizing oligonucleotide melting temperatures.

- A building block is typically comprised of 12-18 oligonucleotides. How does the use of oligos that overlap one another ensure that the oligos assemble in the correct order to form the building block?

One of the reasons that we need the Gene Design program is that the program will adjust the length of the oligos to design oligos that have the same T_m in their overlap regions.

- Why might it be important to have a uniform T_m when assembling oligos into a building block?

Click “Back” on your browser and then click “BB design (sequence overlap)”. Make sure the settings are at “Target oligo length” of 80 bp and “overlap melting temperature” of 56deg. Make sure that “generate gapped oligos” is selected. Click “Design oligos”. Notice that in the white box at the top you see four DNA strands: the top and bottom strands are the two complete strands of the building block and in between them you see the overlapping oligos that can be used to synthesize the building block. At the bottom of the page, you should see all of your oligos that are needed to make this building block listed (all written in the 5’ to 3’ direction).

8. What is the length of your building block? How many oligos are required to synthesize the sequence?

9. Add up the lengths of all of your oligos. What is the total length of all the oligos together? Why does this differ from the overall length of the (building block (think about how the oligos assemble)?

10. What is the average length of the oligos and what is the range of oligo lengths? Why aren’t all of the oligos the same size (why have some been lengthened and others shortened)?

Hit the “Back” button on your browser. Change the “Overlap melting temperature” to 64 degrees.

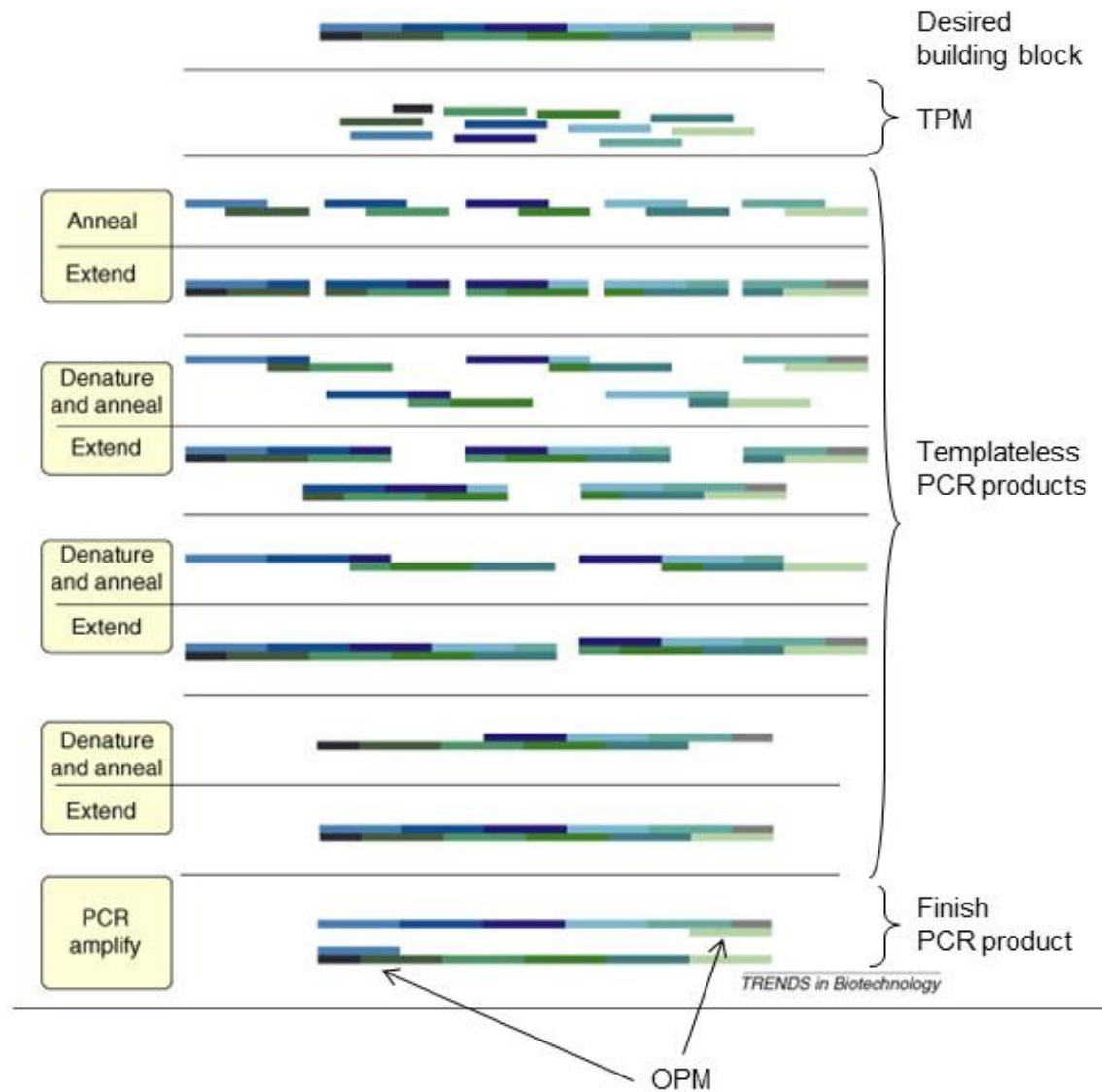
11. What about the oligos has changed? How would this affect the stability (T_m) of the double stranded DNA?

12. What would be the advantage to using longer oligos for gene synthesis? What would be the advantage to using smaller oligos for gene synthesis? (Think about your answers about error rates above).

Click “Assembly oligos (FASTA format)” and submit the list of oligos for one building block along with your assignment. Repeat for your remaining building blocks.

Creating Oligo Mixes

For your phage building block, you will need to combine 12-18 oligos together to create a templateless primer mix (TPM). This TPM will be used in a templateless PCR reaction (TPCR), which will anneal the oligos together and extend them to make longer DNA sequences, including only a small amount of the full-length building block sequence. You will then use the outer primer mix (OPM) to amplify the full-length BB in a finish PCR reaction (FPCR) so that the predominant DNA following FPCR is your desired full-length BB. Oligos are ordered individually and are delivered to us in 96 well plates.



Templateless primer mix (TPM)

The templateless primer mix (TPM) contains the oligos that comprise the building block. All primers must be present at a concentration of 300nM in the TPM (a dilution of 1/20 from their current concentration of 6 uM). The oligos should be combined in a 1.7ml microcentrifuge tube, and primer mixes should be stored at -20°C when not in use.

1. Label your tube with: the name of the phage BB, the type of oligo mix (TPM), and the primer concentration (300 nM)
2. To the tube, add 10 ul of each of the TPM oligos
3. Add H₂O to get a final volume of 200ul.
4. Mix thoroughly and keep the tube on ice.

Outer primer mix (OPM)

For each building block, the outer primer mix (OPM) contains **only** the first and last oligos. The outer primers must be present at a concentration of 3uM in the OPM (the oligos must both be diluted by 1/2). Again, these dilutions should be done in a 1.7ml microcentrifuge tube, and primer mixes should be stored at -20°C when not in use.

1. Label your tube with: the name of the phage BB, the type of oligo mix (OPM), and the primer concentration (3 uM)
2. To the tube, add 10 ul of each of the OPM oligos
3. Mix thoroughly
4. Put this primer mix into the box marked “OPM mixes” to be stored until next week

Templateless PCR

- Before you set up reactions, make sure you reserve a spot in the PCR machine. You will need to sign up for 3 spaces in the PCR machine-label each with your initials and either “PH”, “PC” or “NC”.
- Keep dNTPs and Herculase enzyme **ON ICE AT ALL TIMES!**
- Note for instructors: because students have difficulty pipetting small volumes, I found that they would waste tremendous amounts of enzyme when it was given to them undiluted, so I dilute the Herculase enzyme in buffer before giving it to students (they should use the equivalent of 0.25 ul per 25 ul reaction).
- Note for instructors: The positive control can be any premixed template and primer pair that you know to work well.

1. You will be performing 3 PCR reactions (1 phage building block (PH), a positive control reaction (PC) and a negative control reaction (NC).

2. Combine the reagents listed below into three different PCR tubes on ice.

Reagent	Volume per 25ul reaction
1.25 mM dNTPs	5ul
5X buffer	5 ul
Diluted Herculase enzyme	12.5 ul
Total	22.5 ul

3. To the first tube, add 2.5 ul from the tube marked TPM.

4. To the second tube, add 2.5 ul from the tube marked PC. This will be your positive control reaction.

5. To the third tube, add 2.5 ul of water. This will be your negative control reaction.

6. Cap your tubes and **make sure that they are sealed tightly** so that the liquid will not evaporate. Flick the tubes gently to mix.

7. Place your tubes in the PCR machine in the positions for which you signed up. Make sure that you have recorded which sample (PH, PC, or NC) is in each position in the PCR machine.

Reaction Conditions:

1 cycle:

94°C, 3 minutes

5 cycles:

94°C, 30 seconds

69°C, 30 seconds

72°C, 1 minute

5 cycles:

94°C, 30 seconds

65°C, 30 seconds

72°C, 1 minute

20 cycles:

94°C, 30 seconds

61°C, 30 seconds

72°C, 1 minute

1 cycle:

72°C, 3 minutes

Finish PCR

We just used templateless PCR to assemble a small amount of the full-length building block (PH) from oligonucleotides. However, templateless PCR produces very little of the full-length BB. Because templateless PCR is so inefficient at synthesizing the full-length BB, it also creates many smaller DNA fragments that are intermediates in building block synthesis. Therefore, the templateless PCR that we performed resulted in a mixture of DNA products: a small amount of the full-length BB is present among other DNA products of varying sizes.

The goal of finish PCR is to exponentially amplify the building block sequence so that most of the DNA present in the mixture following finish PCR will be the full-length BB. This is accomplished by using the outer primer mix (OPM), which contains two oligos to amplify only full-length DNA. The OPM primers bind to either end of the building block (which now serves as a template), enabling only BBs that are full-length (and therefore contain both ends of the BB sequence) to be amplified by PCR.

Reaction Setup

- Before you set up reactions, make sure you reserve a spot in the PCR machine. You will need to sign up for 3 spaces in the PCR machine-label each with your initials and either “PH”, “PC” or “NC”.
- Keep dNTPs and Herculase enzyme **ON ICE AT ALL TIMES!**

1. Obtain your templateless PCR reactions from the PCR machine.

2. You will no longer need your PC and NC reactions today. To store them, transfer each to a separate tube by pipetting the liquid into a 1.7 ml tube. Label each tube with:

- Your initials
- “TPCR”
- Either “PC” or “NC”

Bring these tubes to the box up front to be frozen for next week, when we will run it on a gel-if the PC reaction shows a band of DNA, it will indicate that you properly set up your TPCR reactions.

2. To your templateless PCR reaction that is labeled “PH”, add 175 ul of water, cap the tube and invert to mix. We will use this diluted templateless PCR reaction in step 4 below.

You will be performing 3 PCR reactions (one phage building block (PH), one **NEW** positive control reaction (PC) and one **NEW** negative control reaction (NC)).

3. Combine all reagents listed below into 4 different PCR tubes (the very small tubes).

Reagent	Vol/25ul reaction
1.25 mM dNTPs	5 ul
5x Buffer	5 ul
Diluted Herculase enzyme	10.5 ul
OPM	2.5 ul
Total	23 ul

4. To the first tube (PH), add 2 ul of the PH templateless PCR reaction from step 2 above. Once this is done, discard the tube containing the remainder of the templateless PCR reaction.

5. To the third tube (PC), add 2 ul from the tube labeled PC.

6. To the fourth tube (NC), add 2 ul of water.

7. Cap your tubes and **make sure that they are sealed tightly** so that the liquid will not evaporate. Place your tubes in the PCR machine in the position for which you signed up. Make sure that you have recorded which sample (PH, PC or NC) is in each position in the PCR machine.

Reaction Conditions:

1 cycle:

94°C, 3 minutes

25 cycles:

94°C, 30 seconds

55°C, 30 seconds

72°C, 1 minute

1 cycle:

72°C, 3 minutes

Gel Electrophoresis

So far, we have performed two steps of synthetic gene assembly to create a building block: (1) templateless PCR to assemble oligos into a small amount of full-length building block, and (2) finish PCR to amplify the full-length BB so that it becomes the most abundant DNA following PCR.

Now, we need to check how well each of these steps worked by running our PCR products (and controls!) on an agarose gel to verify whether we have assembled a significant amount of the full-length BB. Agarose gel electrophoresis will separate DNAs based upon their size. Therefore, if assembly of our BBs has been successful, we expect to see one DNA band whose size should approximate that of our desired BB. You can determine the size of each of your DNAs by comparing the size of your band to the size of the DNA marker (the 2-Log DNA ladder), which you will run on the gel with your DNAs.

Remember, we also have 4 control reactions (the PC and NC controls from the templateless PCR and the PC and NC controls from the finish PCR).

Pouring a Gel:

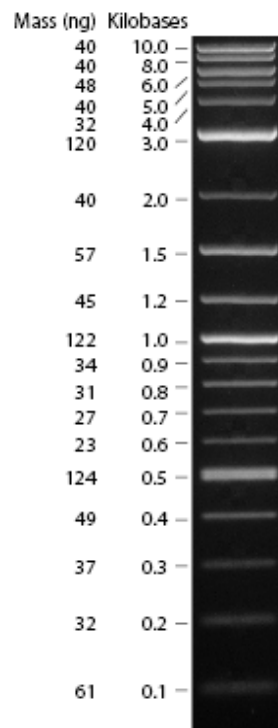
1. Weigh out 0.5 g of agarose on a piece of weigh paper. Transfer to an Erlenmeyer flask. Add 50 ml of 1x TAE.
2. Place the flask on a hot plate and heat until the agarose is completely transparent and colorless. Using a rubber heat mitt, periodically swirl the flask to ensure that the agarose is melting evenly. If there is any cloudiness or particulates, then place the agarose back on the hot plate for additional time until clear (check frequently).
3. Remove the flask of clear agarose and allow it to cool. This will take about 10 min. If you can hold your hand on the flask for 20 sec, then the agarose is cool enough to pour. (Warning: if poured while too hot, the agarose will leak and warp the plastic gel tray.)
4. While the agarose is cooling, place the gel tray into the gel box and add the black blockers and a comb.
5. When the agarose is cool, bring it to the hood to add 1 ul of ethidium bromide to the melted agarose (**CAUTION: ethidium bromide is a mutagen. Wear gloves and a lab coat at all times when handling gels and gel equipment!**).
6. Swirl the agarose to incorporate the ethidium bromide and pour the agarose into the gel tray – your gel should be about 1 cm thick.
7. Allow at least 20 minutes for the gel to solidify. Once solid, carefully remove the comb and the black blockers, and place the solidified gel (still on the tray) into the gel box so that the wells are oriented on the same side as the black electrode.
8. Add enough 1x TAE buffer to completely cover the gel by about 1 cm (~250 ml).

Preparing your samples:

7. Transfer each of your PCR products from the small PCR tubes to a new larger tube. Label each tube with either "PH BB", "FPCR PC", or "FPCR NC".
8. Obtain your TPCR PC and TPCR NC tubes that were stored in the freezer from last week. You should have 6 total samples now.
9. On a piece of parafilm, spot out 2 ul of 6x DNA loading dye with a P20 pipet. You should have 5 spots (one for each of your PCR products). Wait until your gel has started to solidify before beginning this step since the spots will dry out after ~10 minutes.
10. Add 5 ul of water to each spot of dye.
11. Add 5 ul of the PCR product.

Running a Gel:

1. Into the first lane of the gel load 5 ul of the 2-Log DNA ladder (mixed with water and dye).
2. Into lanes 2-5, load 10 ul of each of your PCR products (mixed with water and dye).
3. Place gel lid with electrodes on gel box. REMEMBER DNA RUNS FROM NEGATIVE (black) TO POSITIVE (red). CHECK YOUR LEADS: BLACK SHOULD BE AT THE TOP, RED SHOULD BE AT THE BOTTOM!
5. Set voltage to 100V. You should see bubbles coming up from the wire electrode.
6. After a few minutes, check to make sure everything is going smoothly. Run gel approximately 30 minutes or until the dye is 2/3 of the way down the gel.
7. Take a picture of your gel.
8. Use the DNA ladder (a picture is on the next page) to approximate the size of each of your building blocks. Make sure you check that your building correct size - do not assume that because you have a correct product!



block is the
band, it is the

PCR purification

The next step after this is to combine the phage building block with the rest of the phage genome. Before we can join these DNAs together, we must remove the old buffers and proteins from the PCR reaction using a purification kit. (This protocol uses Promega's Wizard SV PCR purification kit, but any equivalent kit can be substituted). We will purify the phage gene that you just amplified (you can throw out your PC and NC reactions).

1. Add 80 ul of water to your PH gene and mix by pipetting.
2. Add 100 ul of Membrane Binding Solution and pipet up and down several times to mix.
3. Obtain a spin column/collection tube and label with your initials. Add the liquid from step 2 to the top of the column. Let the tube sit for 1 min.
4. Spin in centrifuge for 1 min (be sure the centrifuge is balanced with another tube on the opposite side).
5. Remove the spin column and pour out the liquid from the bottom collection tube. Put the spin column back in the collection tube.
6. Add 700 ul of Membrane Wash Solution to the top of the column.
7. Spin in centrifuge for 1 min (be sure the centrifuge is balanced with another tube on the opposite side).
8. Remove the spin column and pour out the liquid from the bottom collection tube
9. Add 500 ul of Membrane Wash Solution to the top of the column.
10. Spin in centrifuge for 1 min (be sure the centrifuge is balanced).
11. Remove the spin column and pour out the liquid from the bottom collection tube
12. Without adding any additional liquid, spin the empty tube in centrifuge for 1 min (be sure the centrifuge is balanced).
13. Obtain a 1.7 ml microcentrifuge tube and label with your initials and "PH".
14. Discard the collection tube and transfer the column to the new microcentrifuge tube.
15. Add 30 ul of nuclease-free water to the column. The microcentrifuge tube cap will not close over the spin column, just leave the tube open.
16. Wait 1 min and then spin the tube and column in centrifuge for 1 min.
17. Throw out column. The liquid in the bottom of the microcentrifuge tube is your purified DNA.

Electroporation of Bacteria with Synthetic Gene & Phage DNA

Note for instructors: These protocols are based on those from Graham Hatfull's lab. The originals are available here:

http://phagesdb.org/media/workflow/protocols/pdfs/BRED_Main_Protocol_2.2013.pdf and here: <http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0003957>

Now that we have created synthetic genes, we need to combine that synthetic gene with the rest of the phage genome. We will do this by combining the native phage genome with the synthetic gene inside the host cell (the bacteria *Mycobacterium smegmatis*). Within the bacterial cell, the native phage genome and the synthetic gene will undergo the process of recombination, which will join them into a semi-synthetic phage genome.

If it is infectious, the semi-synthetic phage genome will burst the initial host bacterial cell, causing it to rupture and release phages which will then infect neighboring bacterial cells. These neighboring cells will then be ruptured, and the process will repeat, resulting in the formation of a plaque, a region where all of the bacterial cells have been burst.

Today we will introduce the native phage genome and synthetic gene into the bacterial cells and then plate those cells onto a petri dish. If the semi-synthetic phage that is formed is infectious, we will see plaques on the petri dishes next week. The method that we will use to introduce the phage genome and synthetic gene into bacteria is called electroporation; rather than using heat to get the DNA into the bacteria (as we did a few weeks ago), we will use a short pulse of electricity.

Preparation of Electro-competent Cells:

1. Measure the optical density of the *M. smegmatis* overnight culture in the spectrophotometer. The value should be between 0.4 and 0.8
2. Transfer 1.5 ml of the culture into a sterile microcentrifuge tube (use the P1000 pipet set to 750 ul twice). Incubate on ice for 30 min.
3. Spin the cells down at 5000 rpm for 10 min in the cold room.
4. Remove the liquid with a pipet tip and resuspend the cells in 800 ul 10% sterile ice cold glycerol. Pipet up and down until no clumps remain.
5. Repeat step 4.
6. Repeat step 4, but resuspend in 400 ul 10% sterile ice cold glycerol.
7. Repeat step 4, but resuspend in 200 ul 10% sterile ice cold glycerol.

Electroporation:

1. Label two tubes (+DNA and -DNA) and place them on ice so that they become cold.
2. Transfer 100 ul of cells to each of the two empty tubes.

3. To the first tube (+DNA), add 1 ul of the phage DNA and 300 ng of the synthetic gene building block (the PH that you purified last week). To the second tube (-DNA), add 4 ul of water. Incubate on ice for 10 min.

5. During the 10 min incubation:

- Obtain 2 cuvettes and place on ice so that they become cold.
- Obtain one tube of 7H9 media with ADC
- Obtain two Pasteur pipettes

6. After the 10 min incubation:

- Transfer the DNA and cells from the first tube into the first cuvette (on ice!)
- Wipe the cuvette with a Kimwipe to remove any wetness
- Transfer the cuvette into the black cuvette holder and insert into electroporation machine
- Press the PULSE button twice to deliver the pulse of electricity
- Pick up the Pasteur pipette with liquid. As soon as the machine beeps, pull out the cuvette holder and immediately add the 7H9 media with ADC on top of the cells

Transfer the mixture of 7H9 media without ADC and cells to a glass test tube and place in 37 degree incubator for 1 hour

7. 15 minutes before the incubation is done, prepare the top agar. Melt the MBTA (Middlebrook top agar) on a hot plate until just melted and allow to cool for 10 min.

8. Once the one hour incubation time is done, add the following to a new tube tube (add in the order listed):

- 10 ul of cells from the glass test tube shaking at 37C
- 300 ul of *Mycobacteria smegmatis* cells
- 1.5 ml 7H9 media without ADC
- 2.5 ml MBTA

Vortex gently and pour the mixture into a petri dish. Swirl gently to distribute the mixture evenly in the plate. Allow to cool for ~10 minutes and then incubate at 37 degrees.

Electroporation of Bacteria with Phage DNA Assignment

1. What were the results of your TPCR and FPCR assembly of the phage gene? How could you change the conditions to improve the results (if necessary)?

2. Fill in the chart with the results of your electroporation below:

Sample	Number of plaques
+DNA	
-DNA	

3. Before we used our DNA for electroporation, we were careful to purify the DNA using a column purification kit. One of the reasons was to remove excess salt that was present in the PCR buffer. Why is it crucial to remove all the salt when using electroporation to introduce DNA into cells?
4. Look back at the previous bacterial transformation lab protocol that we performed earlier in the semester. What are two ways in which that protocol is similar to this electroporation protocol? What are two ways in which they are different?
5. How does a plaque assay allow you to determine the number of infectious phages present?
6. Last time we performed transformation, we plated cells directly on to the top of the petri dish. This time we used top agar to plate the cells. Top agar has a lower agar concentration than the regular agar in petri dishes. When working with phages and performing plaque assays, why would this be advantageous?

7. Our FPCR products (our synthetic genes) contain codon optimized versions of the synthetic gene. What do we mean by codon optimizing a gene? Why might we use different codons depending on which species our gene will be expressed in?

8. What is the reason that synthetic biologists might codon optimize a gene that they are working with in the lab?

9. What is one possible negative unforeseen consequence of codon optimizing a gene?

10. Our next step will be to screen our semi-synthetic phage using PCR and then to dilute our phages so that we are sure that each plaque is generated from a single phage particle? Why is this step necessary if during electroporation we were able to successfully create the semi-synthetic phage?

Picking Plaques

When phages successfully propagate among bacteria growing in a lawn, plaques are formed. These are areas of clearing where phages have infected and lysed cells. Plaques can range in size, turbidity, and morphology. Often, it is necessary to collect a sample of the phages that have made a particular plaque (or putative plaque) to perform further experiments with them (such as testing their relative infectivity). This protocol describes how to “pick” the plaque and obtain a liquid sample of phage.

Reaction setup:

1. Distribute 100 ul of phage buffer into 6 different microcentrifuge tubes. Label these tubes 1-6.
2. Place a new sterile pipet tip on the end of your pipet. Pick one plaque on the plate and touch the center of the plaque once with the tip. The idea is to penetrate the top agar layer, but not go all the way through to the bottom agar. The angle of the pipette tip is not critical, although with small plaques, approaching the plaque perpendicularly seems to work best.
3. Move the tip to the prepared tube of Phage Buffer, lower the point under the level of the liquid, and shake moderately from side to side to release phages into the buffer. It's also a good idea to pipet up and down several times to “wash” any phages from inside the tip into the buffer.
4. Repeat for tubes 2-6, picking a fresh plaque for each tube. Allow to sit at room temperature for 30 minutes
5. Parafilm your bacterial plates from last week and store in the fridge until we analyze the PCR reactions.

PCR Screening of Phage Plaques

Now that we have selected several plaques and isolated the phages, we need to check whether these phages have the synthetic gene or the native gene. The synthetic gene contains two watermark sequences within the synthetic gene which mark the DNA sequence as being synthetic. We will therefore perform PCR with primers that bind to these watermark sequences. If the synthetic sequence is present, the primers will bind and we will get a DNA product. If the primers do not bind because the synthetic sequence is absent then we will not get a DNA product amplified. When screening, we want to start with several plaques to maximize the likelihood that we have at least one that is correct (contains the synthetic gene). We will therefore be setting up 8 PCR reactions (6 different plaques plus positive and negative controls).

9. In a large microcentrifuge tube **on ice**, combine the following components:

- 100 ul of 2X master mix (2X MM)
- 10 ul of primer WM1 (watermark 1)
- 10 ul of primer WM2 (watermark 2)
- 74 ul of sterile water
- 6 ul of DMSO

10. Pipet up and down 10X to completely mix the contents of the tube.

11. Aliquot (distribute) 20 ul per tube of the mix into 8 PCR tubes (the very small ones).

12. Into PCR tube 1, add 1 ul from phage tube 1.

13. Repeat step #4 for phage tubes #2-6.

14. Into PCR tube #7, add 1 ul from tube PC.

15. Into PCR tube #8, add 1 ul of sterile water.

16. Put your 8 PCR tubes into one column of the PCR machine and begin the PCR reaction.

Reaction Conditions:

1 cycle:

98°C, 30 seconds

35 cycles:

98°C, 10 seconds

55°C, 30 seconds

72°C, 30 seconds

1 cycle:

72°C, 10 minutes

17. Run PCR products on a 1% gel in TAE buffer.

If the PCR reactions indicate that we have successfully introduced the synthetic gene into the rest of the phage genome, we now have a stock of the phage sample (the plaque that you picked into phage buffer). If none are positive, we can pick more plaques and screen more phages for positives.

Creation and Screening of semi-synthetic phages

4. Results of your electroporation and plaque assay. Describe and interpret your results and describe any alterations to the protocol that you would make next time.

5. Results of your PCR screening. Fill out the table below, **attach a picture of your gel with each lane labeled.**

Sample	PC	NC	Plaque 1	Plaque 2	Plaque 3	Plaque 4	Plaque 5	Plaque 6
Expected size of PCR product								
Actual size of PCR product								

6. Interpret the results of your PCR screening. Describe any alterations to the protocol that you would make next time.

Genome Databases

Once a genome is sequenced, that data must be deposited in a databank and then annotated. Annotation provides functional information about the location of genes and regulatory elements. This information is cataloged along with data relating to phenotypes and interactions between genes.

7. Sequence Retrieval

The first thing that we must do is to retrieve the sequence of your gene from the database. Go to the NCBI Nucleotide database at <http://www.ncbi.nlm.nih.gov/nucleotide> . Enter the name of our organism: Mycobacterium phage Giles. For this genome, what is the:

Accession number: _____ Genome length: _____

You will see that there are several different submissions for this genome data. For the original submission, what is the:

Molecule type (mol_type): _____

Isolation source: _____

Country and location: _____

Collection date: _____

Collected and identified by: _____

How many genes are in this genome (you may need to scroll down): _____

8. Characterization of your gene

Scroll down and find your gene. Click on the Gene ID number to open a new page. What is the lineage of your gene: _____

What is the length of your gene? _____

What are its sequence coordinates in the phage genome? _____

9. DNA alignment

Under “NCBI Reference Sequences (RefSeq)”, click on FASTA to get the nucleotide sequence of your gene. On the right toolbar, click Run BLAST to look for alignments in the database. We are using the BLAST algorithm (Basic Local Alignment Search Tool) to quickly find the sequence(s) that closely match our query sequence (your building block DNA). You should get a colored graph representing the quality of alignment generated by BLAST which ranges from dark blue (poor alignment) to red (very good alignment). BLAST provides statistics to help determine how good your alignment is. The e-value (the expect value) represents the probability that the alignment you produced could occur by random chance.

Gene	
How many hits does your query retrieve? What are they?	
For each hit, what is the probability the alignment would occur by random chance?	
What is the percent sequence identity between the search result and your query sequence?	

10. Go back and switch the Program Selection at the bottom of the BLAST page from “Highly similar sequences (megablast)” to “Somewhat similar sequences (blastn)”. What does this do to the number of hits that you retrieve and their e-values? Why?

Dilution of semi-synthetic phage to determine titer

1. Obtain your phage stock tube from last week. Select one of the phage stocks that was positive by PCR.
2. Obtain 5 sterile microcentrifuge tubes and label them 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , and 10^{-5} . Add 90 μ l of phage buffer into each tube (keep tubes closed at all times!)
3. Take 10 μ l from your phage stock tube (pipet up and down 3 times before removing the 10 μ l) and transfer to the 10^{-1} tube. Invert the tube 10 times to mix well.
4. Take 10 μ l from your 10^{-1} tube (pipet up and down 3 times before removing the 10 μ l) and transfer to the tube 10^{-2} . Invert the tube 10 times to mix well.
5. Repeat step 4, transferring from the 10^{-2} tube to the 10^{-3} tube, etc. until you have all dilutions completed through 10^{-5} .
6. You are done with the 10^{-1} and 10^{-2} tubes, so you can discard them in the biohazard trash now.
7. Obtain 3 new microcentrifuge tubes. Label them 10^{-3} , 10^{-4} , and 10^{-5} . Transfer 10 μ l of the diluted phages to these new tubes (10 μ l from the old 10^{-3} tube to the new 10^{-3} tube, etc.). Keep the new tubes with 10 μ l and discard the old set of tubes in the biohazard trash.
8. To each tube, add 300 μ l of fresh *M. smegmatis* cells.
9. Allow tubes to sit at room temp for 30 minutes.
10. Obtain 3 7H10 plates and label them with your initials and 10^{-3} , 10^{-4} , and 10^{-5} .
11. After 25 minutes, melt your MBTA in the microwave, mixing frequently to keep it from boiling over.
12. Obtain 3 15 ml conical tubes. To each, add 2.5 ml of MBTA and 2.5 ml of 7H9.
13. One at a time, take one of your phage/ *M. smegmatis* cell tubes, transfer the entire volume of liquid to the MBTA/7H9 solution. Mix by vortexing gently and pour onto the surface of a 7H10 plate (make sure the label corresponds to the dilution that you are using). Swirl the plate gently to distribute.
14. Allow plates to harden for ~10 minutes on the bench and then incubate at 37C overnight. Be sure to invert your plates.

BUILD-A-PHAGE SUPPLY LIST

Supplies Week 1 (Templateless and Finish PCR):

- Sterile PCR tubes and sterile 1.7 ml microcentrifuge tubes
- PCR nucleotides (dNTPs) [NEB N0447L]. These need to be diluted 4-fold in water.
- 5X Herculase buffer
- Herculase II enzyme. I dilute this before giving to students, so the **protocols all call for diluted Herculase enzymes**. NEB's HotStart Phusion enzyme can also be used and seems to work as well for templateless and finish PCR.
- Sterile water
- PCR positive control (combination of any plasmid template and two primers that are known to work well)
- One freezer box with dividers.

Supplies Week 2 (Gel electrophoresis):

- Agarose
- Erlenmeyer flasks
- Hot plates or microwave
- Carboy of 1xTAE
- Ethidium bromide
- Agarose gel boxes with combs
- 6x DNA loading dye
- 2-Log DNA ladder (premixed with dye; NEB N0469S)
- Sterile 1.7 ml microcentrifuge tubes

Supplies Week 3 (Gibson Assembly and Transformation):

- Sterile PCR tubes
- Wizard Gel electrophoresis and PCR purification kit
- Sterile microcentrifuge tubes
- NEB Gibson Assembly Kit with competent cells
- Sterile SOC media
- LB+Amp plates
- Incubator set to 37C for plates
- Water bath set to 42C
- Sterile glass beads or other method to plate cells

Supplies Week 4 (DNA sequence analysis):

- Laptops or access to computer lab

Supplies Week 5 (Troubleshooting PCR):

- Sterile PCR tubes and sterile 1.7 ml microcentrifuge tubes
- PCR nucleotides (dNTPs) [NEB N0447L]. These need to be diluted 4-fold in water.
- 5X Herculase buffer
- Herculase II enzyme. I dilute this before giving to students, so the **protocols all call for diluted Herculase enzymes**. NEB's HotStart Phusion enzyme can also be used and seems to work as well for templateless and finish PCR.
- PCR positive control (combination of any plasmid template and two primers that are known to work well)
- One freezer box with dividers.

Supplies Week 6 (Troubleshooting PCR-Gel electrophoresis):

- Agarose
- Erlenmeyer flasks
- Hot plates or microwave
- Carboy of 1xTAE
- Ethidium bromide
- Agarose gel boxes with combs
- 6x DNA loading dye
- 2-Log DNA ladder (premixed with dye; NEB N0469S)
- Sterile 1.7 ml microcentrifuge tubes

Supplies Week 7 (Oligo Design and Creating Oligo Mixes):

- Laptops or access to computer lab
- Oligonucleotides for assembly of a synthetic gene(s), diluted to 6 μ M in 96-well plates

Supplies Week 8 (Templateless and Finish PCR):

- Sterile PCR tubes and sterile 1.7 ml microcentrifuge tubes
- PCR nucleotides (dNTPs) [NEB N0447L]. These need to be diluted 4-fold in water.
- 5X Herculase buffer
- Herculase II enzyme. I dilute this before giving to students, so the **protocols all call for diluted Herculase enzymes**. NEB's HotStart Phusion enzyme can also be used and seems to work as well for templateless and finish PCR.
- PCR positive control (combination of any plasmid template and two primers that are known to work well)
- Sterile water
- One freezer box with dividers.

Supplies Week 9 (Gel electrophoresis):

- Agarose
- Erlenmeyer flasks
- Hot plates or microwave
- Carboy of 1xTAE
- Ethidium bromide

- Agarose gel boxes with combs
- 6x DNA loading dye
- 2-Log DNA ladder (premixed with dye; NEB N0469S)
- Sterile 1.7 ml microcentrifuge tubes

Supplies Week 10 (PCR purification and Electroporation of Bacteria with Phage DNA):

- Promega Wizard SV Gel and PCR Cleanup kit (A9281; 50 preps)
- Electroporation apparatus
- Electroporation cuvettes (VWR #89047-206)
- Sterile 7H9 media WITH ADC and sterile 7H9 media WITHOUT ADC
- Bottles containing 40 ml sterile MBTA (recipe below)
- Sterile 5 ml pipets and pipet aids
- Microwave
- 7H10 plates
- Sterile glass test tubes with caps
- Wild-type phage DNA (we use Mycobacteriophage Giles)
- *Mycoplasm*a *smegmatis* (or other bacterial host) cells containing pJV53 plasmid; grown overnight in 7H9 media with 10%ADC (see these references for details on the strain and plasmid:
http://phagesdb.org/media/workflow/protocols/pdfs/BRED_Main_Protocol_2.2013.pdf
<http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0003957>)

Supplies Week 11 (Picking plaques and PCR screening):

- Phage buffer (recipe below)
- Sterile microcentrifuge tubes and racks
- PCR positive control (combination of any plasmid template and two primers that are known to work well)
- NEB OneTaq Hot Start 2X Master Mix
- Watermark primers

Supplies Week 12 (Gel electrophoresis for PCR screening and Diluting phages):

- Agarose
- Erlenmeyer flasks
- Hot plates or microwave
- Carboy of 1xTAE
- Ethidium bromide
- Agarose gel boxes with combs
- 6x DNA loading dye
- 2-Log DNA ladder (premixed with dye; NEB N0469S)
- Sterile 1.7 ml microcentrifuge tubes
- Sterile 7H9 liquid media
- 15 ml conical tubes or sterile test tubes with lids
- Sterile 5 ml pipets and pipet aids
- Bottles containing 40 ml sterile MBTA
- Microwave

- 7H10 plates
- Phage buffer
- Incubator set to 37C

Media recipes:

Phage buffer:

Recipe available at: http://phagesdb.org/media/workflow/protocols/pdfs/PHProtocol_PhageBuffer.pdf

Note that you must add CaCl₂ to 1 mM before using phage buffer.

7H10 plates:

Combine 19g 7H10 agar, 12.5 ml 40% glycerol, and 890 ml distilled water. Autoclave and cool to 55 degrees. Add 100 ml ADC, 10 ml of 0.1M CaCl₂, 1 ml of 50 mg/ml carbenicillin, and 10 mg/ml cycloheximide. Mix well and pour plates (makes ~40 plates).

MBTA:

Combine 4.7g 7H9 powder, 7g Bacto agar, and 900 ml distilled water. Autoclave, mix well, and distribute ~40 ml into each sterile glass bottle. Allow to cool and harden (be careful not to close the cap tightly or it will be difficult to reopen).

7H9 media:

Combine 2.4 g 7H9 powder, 2.5 ml of 40% glycerol, and 450 ml distilled water. Autoclave and allow to cool.

For 7H9 without ADC, add 15 ml of 0.1M CaCl₂.

For 7H9 with ADC, add 15 ml of 0.1M CaCl₂ and 50 ml ADC.