Synthetic Genome Assembly

This workflow was initially designed for the Synthetic Yeast Project ([www.syntheticyeast.org](http://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, **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 gives students practice with some of the technically and conceptually difficult portions of the course (templateless and finish PCR).

**BUILDING BLOCK ASSEMBLY**

60-80 bp oligonucleotides (OPM)

500-750 bp building block (BB)

2.3 kb (kilobases) minichunk

10 kb chunk

50-60 kb megachunk

200-2200 kb chromosome

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.

# Oligo Mixes

Typically, 12-18 oligos are used to synthesize each building block (BB). These oligos are ordered individually and are delivered to us in 96 well plates. Each 96-well plate can contain oligos to make up to 4-5 complete building blocks.

For each building block that you are assigned, you will need to combine these 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. Next week, you will 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.

# Templateless primer mix (TPM)

The templateless primer mix (TPM) contains the 12-18 oligos that comprise the building block. All primers must be present at a concentration of 300nM in the TPM (a dilution of 1/20). 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 BB name, 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.

In some cases, there may be more than 20 oligos that comprise a building block. Mix 10 ul of each oligo and add no water. Even though the concentration will be slightly more dilute than 300 nM this does not seem to have any effect on reaction efficiency.

# 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 -20oC when not in use.

1. Label your tube with: the BB name, 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

**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!**

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 |
| 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:**

94oC, 3 minutes

**5 cycles:**

94oC, 30 seconds

69oC, 30 seconds

72oC, 1 minute

**5 cycles:**

94oC, 30 seconds

65oC, 30 seconds

72oC, 1 minute

**20 cycles:**

94oC, 30 seconds

61oC, 30 seconds

72oC, 1 minute

**1 cycle:**

72oC, 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 |
| 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:**

 94oC, 3 minutes

**25 cycles:**

 94oC, 30 seconds

 55oC, 30 seconds

 72oC, 1 minute

**1 cycle:**

 72oC, 3 minutes

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://52.25.194.81/watermarker/>). Enter the sequence of your gene into the genomic sequence box. Switch the organism *to Mycobacterium smegmatis* and click “Generate Watermarks”.

1. 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?
2. 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: Expected size of PCR product:

End position:

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:

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(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/*.*](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-](http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=246196) [bin/showcodon.cgi?species=246196](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.

1. 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).

1. 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.

1. 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 on oligonucleotide to serve as a primer for DNA synthesis.

1. 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.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| 5’ | 3’ | 5’ | 3’ |  |
| CT | AGA CCC GCT | CAT | GCA TGT CCA ATG CAT |  |
|  | GGG CGA TGA | CAC GTA | CGT TAC GTA CAC GTC | TGA |

## Step 4: Optimizing oligonucleotide melting temperatures.

1. 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 Tm in their overlap regions.

1. Why might it be important to have a uniform Tm 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).

1. What is the length of your building block? How many oligos are required to synthesize the sequence?
2. 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)?
3. 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.

1. What about the oligos has changed? How would this affect the stability (Tm) of the double stranded DNA?
2. 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.

Yeast Transformation

# The plasmid DNA which induces the Scramble system will be transformed into competent yeast cells. This plasmid will be selected using a selectable (auxotophic) marker on the plasmid. Yeast cells containing the selectable marker will be able to grow on media lacking an amino acid.

1. Obtain one tube of yeast cells. Spin these down in the microcentrifuge for 1 min at full speed.
2. Remove the liquid (called the supernatant; you can toss it with the pipet tip into the biohazard trash. Resuspend the cells in 1 ml of sterile water. Spin cells down in the microcentrifuge for 1 min at full speed.
3. Remove the liquid (you can toss it with the pipet tip into the biohazard trash. Resuspend the cells in 1 ml of 0.1M LiAc (lithium acetate). Spin cells down in the microcentrifuge for 1 min at full speed.
4. Remove the liquid (you can toss it with the pipet tip into the biohazard trash. Resuspend the cells in 1 ml of 0.1M LiAc (lithium acetate).
5. Obtain four sterile microcentrifuge tubes (one for the assembly transformation, one for the positive control, and one for the negative control). Add 100 ul of yeast competent cells from step 4 to each tube. Label these tubes “CRE”, “NO CRE”, “PC”, and “NC”.
6. Centrifuge the yeast cells at full speed for 1 minute. Remove the liquid (called the supernatant; you can toss it with the pipet tip into the biohazard trash.
7. Prepare a yeast transformation master mix. You have a tube containing 960 ul of 50% polyethylene glycol (PEG). To this tube add 144 ul of 1.0M lithium acetate (LiAc) and 40 ul of single-stranded herring sperm DNA. Mix very well by vortexing for 10 seconds.
8. Aliquot (distribute) 286 uL of yeast transformation mix into each of the three microcentrifuge tubes containing the yeast cell pellets from step 6. Pipet up and down very well to mix the yeast cells.
9. To the “PC” tube, add 25 uL from the provided PC DNA tube (this is 1 ug of plasmid DNA).
10. To the “NC” tube, add 25 uL of sterile water.
11. To the “CRE” tube, add 25 ul of provided plasmid DNA (plasmid pLM161).
12. To the “NO CRE” tube, add 25 ul of provided plasmid DNA (plasmid pRS416).
13. Vortex the tubes for 10 seconds to thoroughly mix the DNA with the transformation mix and yeast cells.
14. Incubate in a 42°C water bath for 20 minutes. During this time, obtain 3 SC-Ura dropout plates. Label one “PC”, one “NC”, and one “EXP”.
15. After the heat shock, centrifuge the tubes at top speed for 30 seconds.
16. Remove the supernatant with a P1000 pipette set to 1000 ul.
17. Add 300 μL of sterile water to each tube. Gently pipette to resuspend the pellet.
18. From each tube, transfer 250 ul of the transformation product onto your appropriately labeled SC-Ura dropout plates. Add ~5-7 sterile large glass beads to each plate, shake the plates and toss the glass beads into the waste container.
19. Once the plates are no longer wet, incubate the plates at 30°C for 2 days (remember to turn the plates upside down).
20. Pick 2 new SC-Ura plates, label them “CRE” and “NO CRE” and with your initials. Parafilm and leave in the incubator with your transformation plates

Scramble: Inducing the Scramble system

Your instructor has inoculated a liquid culture of your yeast strain containing the Cre plasmid (pRS413-CreEBD). The plasmid contains the Cre gene (remember that Cre induces the Scramble system) under the control of the estradiol promoter. Adding estradiol to the cells will induce the expression of Cre and therefore the Scramble system.

1. Measure the optical density of the yeast culture, which gives you an estimate of the cell density.
	1. Transfer 1 ml of media to a cuvette. Place in the spectrophotometer and blank the instrument (this only needs to be done once for the class).
	2. Transfer 1 ml of your yeast culture to a cuvette. Place in spectrophotometer and record the optical density.
2. Use the formula C1V1=C2V2 to determine the volume of cells equal to an OD of 0.1

(Concentration (OD) as measured on spec)(Volume to be determined) = (0.1) (25 ml)

Volume to be determined = \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ ml

1. Into a sterile flask, add:
	1. The volume of cells that you calculated above
	2. 25 ml of liquid media
	3. 5 ul of beta-estradiol (1 uM final concentration).
2. Incubate the culture with shaking for 2-2.5 hours. (The cells are Scrambling now!)
3. Transfer 100 ul of the cell culture to a microcentrifuge tube.
4. Spin down the cells for 1 min at full speed. Remove the supernatant. Resuspend the culture in 1 ml sterile water.
5. Repeat step 7 two more times, ending with the cells resuspended in 1 ml sterile water.
6. Onto two selective plates, add 100 ul water. Into this drop, add 10 ul of cells from step 7. Spread the cells on the plates with glass beads.
7. Discard beads and when the liquid has absorbed into the plates, incubate the plates upside down at 30C for 2 days.

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.

# F:\Phage plate.jpgStep 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.

PCR purification of Phage BBs

The next step after finish PCR 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. 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 microcentifuge 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.
1. 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
1. 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

1. 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.
2. 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.

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).

1. 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
2. Pipet up and down 10X to completely mix the contents of the tube.
3. Aliquot (distribute) 20 ul per tube of the mix into 8 PCR tubes (the very small ones).
4. Into PCR tube 1, add 1 ul from phage tube 1.
5. Repeat step #4 for phage tubes #2-6.
6. Into PCR tube #7, add 1 ul from tube PC.
7. Into PCR tube #8, add 1 ul of sterile water.
8. 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:**

**1 cycle:**

98oC, 10 seconds

55oC, 30 seconds

72oC, 30 seconds

72oC, 10 minutes

1. 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.

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 ul of phage buffer into each tube (keep tubes closed at all times!)
3. Take 10 ul from your phage stock tube (pipet up and down 3 times before removing the 10 ul) and transfer to the 10-1 tube. Invert the tube 10 times to mix well.
4. Take 10 ul from your 10-1 tube (pipet up and down 3 times before removing the 10 ul) and transfer to the tube10-2. 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 ul of the diluted phages to these new tubes (10 ul from the old 10-3 tube to the new 10-3 tube, etc.). Keep the new tubes with 10 ul and discard the old set of tubes in the biohazard trash.
8. To each tube, add 300 μl 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.