June 5, 2017
Meeting Agenda:
Sterile technique, taught by Tisa, and first day inside the lab

Attendance (rough)
1. Seth Martinez
2. Kira Dzedzy
3. Ioannis (Yianni) Zerefos
4. Kathleen Wright
5. Madlen Can
6. Carissa Walkosak
7. Dan Owens
8. Kelsey Murray

June 6, 2017
Meeting Agenda:
Plasmid Construct Discussion

Attendance (rough)
1. Kathleen Wright
2. Sara Leung
3. Maddie Callahan
4. Madlen Can
5. Kira Dzedzy
6. Ioannis (Yianni) Zerefos

June 7, 2017
Emma Oskar and Sam one on one meeting.

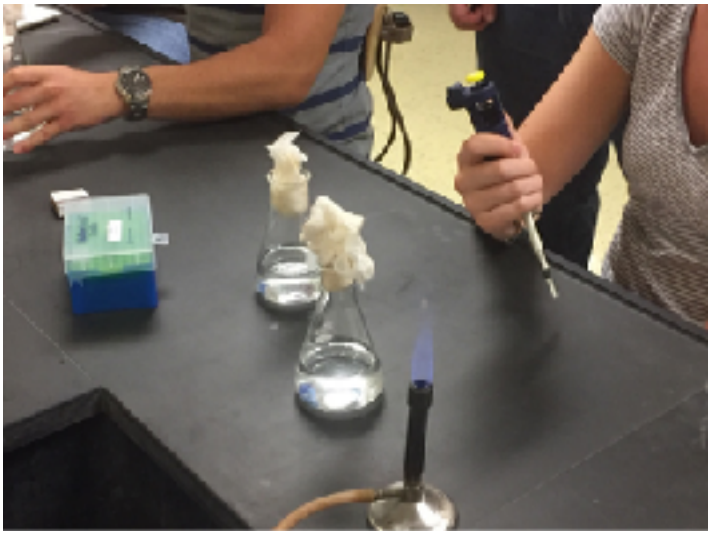
June 9, 2017
Meeting Agenda:
Sterile technique, Second Session

Attendance
1. Seth Martinez
2. Sara Leung
3. Madlen Can
4. Kira Dzedzy
5. Ioannis (Yianni) Zerefos
6. Maddie Callahan

June 15,2017

One of our first meetings as University of Delaware's iGem team members this summer was to go over safety in the lab. Sam one of our team's leaders thoroughly explained micro pipetting and using aseptic technique to transfer bacterial cultures from agar growth plates, a process we will later use in our experiments. Aseptic technique, as Sam explained, is a sanitary way of moving bacterial so that their environments don't become contaminated by foreign substances. Even clean air carries microbes!

Figure 1 shows eager team members listening in the lab (space provided by the University of Delaware) to Sam's lecture on aseptic technique. This is where much of our experimenting will occur for our project.



In Figure 2 a team member demonstrates the proper way to handle goggles normally worn in the lab were not necessary for this demonstration as no harmful chemicals were used, just water for demonstration purposes.

Figure 3 is the work space of a group member showing the proper way to clean up one's space after working in the lab. Notice that it was wiped down and all beakers are tightly closed. Also the Bunsen burner is detached from the gas nozzle and turned off.

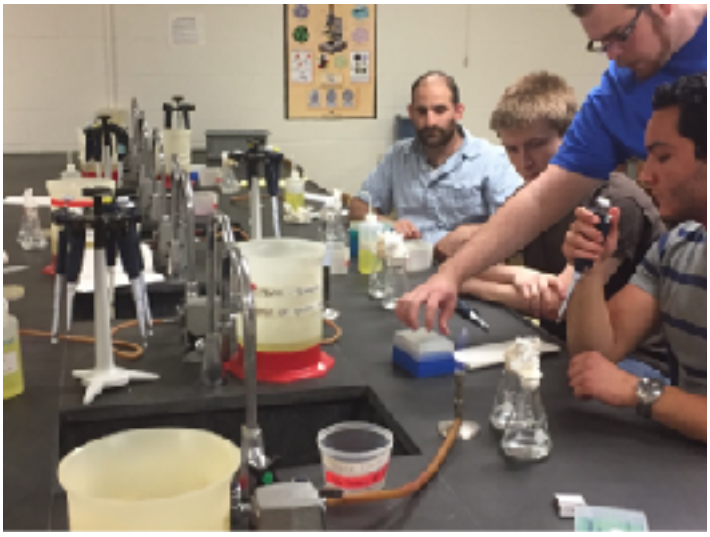


Figure 4 shows Sam explaining to fellow team members the importance of keeping the box that houses sterile pipetting tips. He explained that the least amount of air exposure is essential to ensure contaminates don't interfere with experimenting.

June 23, 2017

Meeting Agenda:
Plate Streaking Practice

Attendance

1. Kira
2. Yianni
3. Madlen
4. Sarah

June 29,2017

Meeting Agenda:
Our team met to go over proper streaking technique. This means that we learned how to properly transfer a bacteria culture from one petri dish to another using sterile loops. The point of transferring cultures from one petri dish to another is in order to grow new cultures from one similar culture to obtain a greater volume for experimenting on. In addition, we learned that we do not want too many clusters of bacteria growing on top of each other because it would make it hard to identify individual cultures.

Figure 1 is a petri dish after some days of incubation that shows bacterial growth. Notice how the colonies grow further apart on the left compared to the heavily streaked right most quadrant.

Figure 2 is a black board drawing that a student made to help other team members understand the proper way of streaking. Quadrant 1 (top left of petri dish) will have the most condensed amount of bacteria while quadrant 4 will have the most diluted and spaced out colonies.

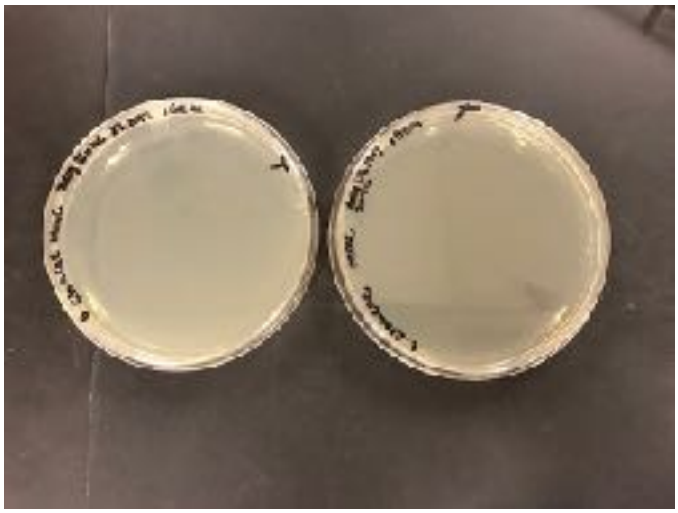


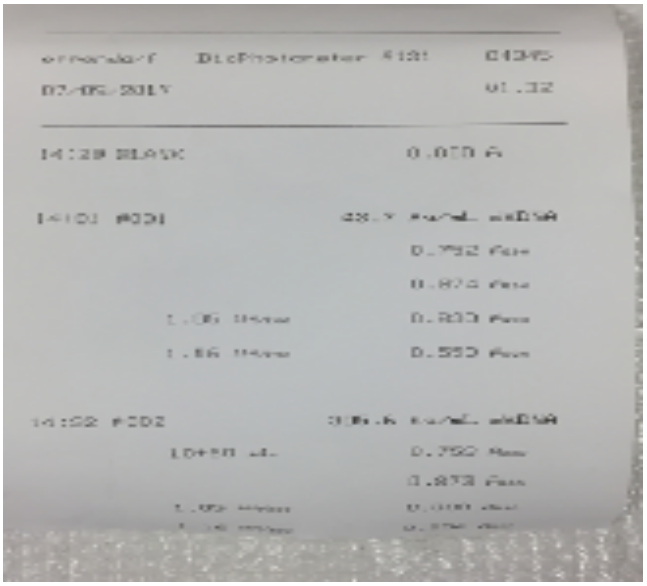
Figure 3: These are properly labeled petri dishes before they have been streaked with bacterial cultures.

Figure 4: A team member demonstrates the proper way to handle a sterile loop when transferring bacteria. It is essential that nothing touch the loop in order to ovoid contamination of the petri dish.

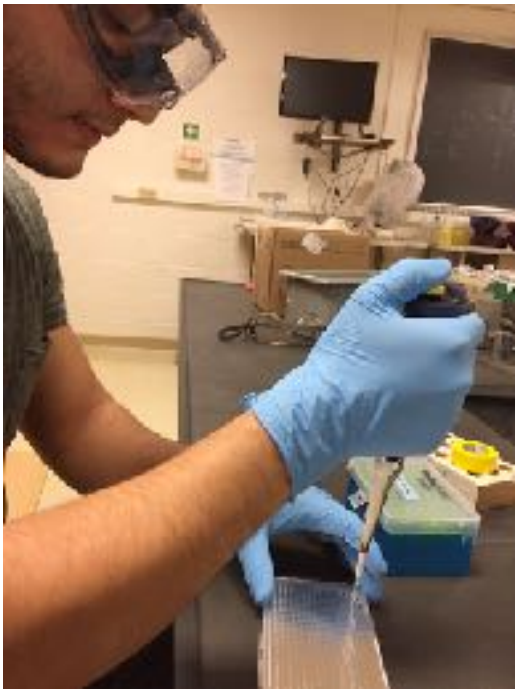
Figure 5: Here is the team today all working hard and wearing goggles, gloves, and long pants, the proper safety attire.

July 9, 2017

Today's meeting focused on resuspending the DNA sequences obtained from iGem, and measuring the concentrations to verify them. Following iGem recommendation we resuspended the DNA in 10 µl of DI water, before adding 50 additional µls to the solution. We used a spectrophotometer to analyze the concentration of this 60 µl solution, with the following results:



The results of this preliminary test showed a concentration below what was expected, to be sure and eliminate any error, further trials were run in an experiment. In the first section, three wells were resuspended and each moved into three centrifuge tubes before being diluted to a total volume of 60 µls.



Above: Resuspension of DNA in 10 µl of DI

In the second section, six total wells were resuspended and moved into three centrifuge tubes at two wells apiece, each tube was diluted to a total volume of 120 µls. And in the third section a similar procedure was carried out, simply resuspending and adding three wells worth of DNA to each tube before diluting each tube to a total volume of 180 µls. A diagram of the wells taken and the corresponding results of the experiment can be seen below (Note: The second of the 120 µl tubes should be lower in concentration, accounting for a minor error):
(here post the photos of the design from the board and of the long results printout sheet)

Figure 1: This is a package of dehydrated DNA cells which is what we resuspended in DI water.

Figure 2: Sarah and Yianni work together.

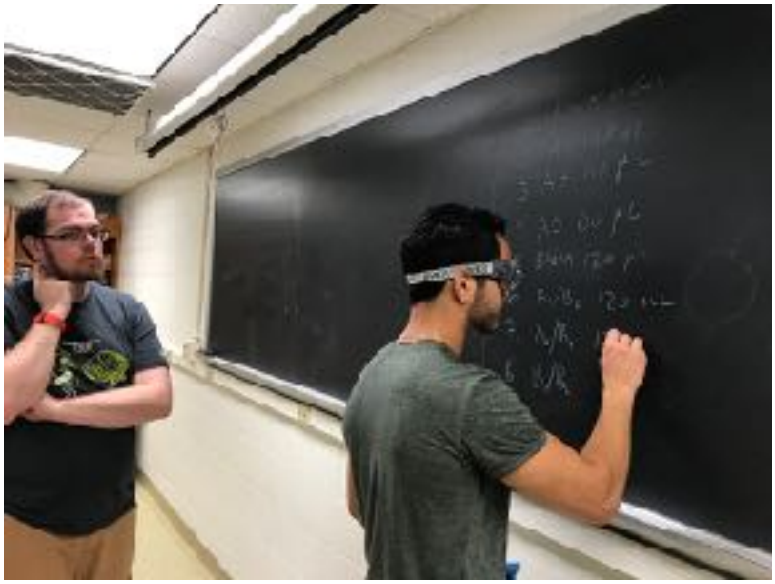


Figure 3: Yianni writes on the board while Sam dictates our data so that the whole team is coordinated.

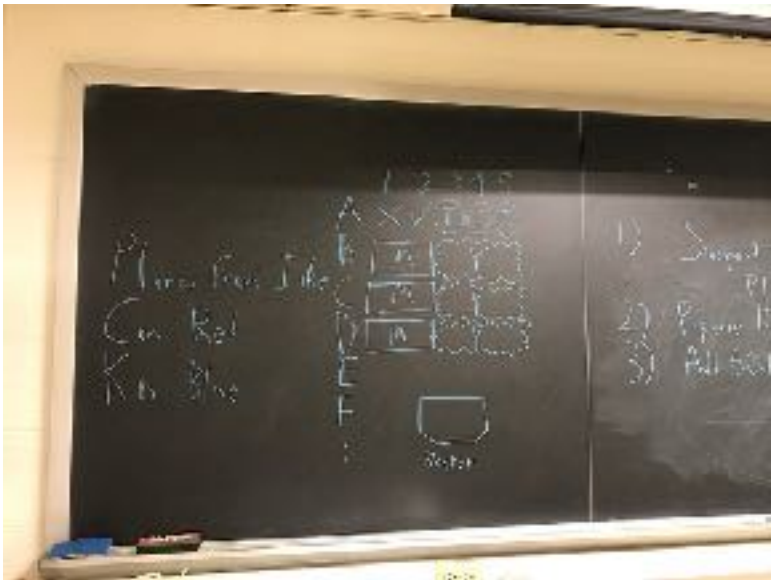


Figure 4: This is a diagram Sam made so that team members knew which wells they were extracting NA from and how much they were resuspending in

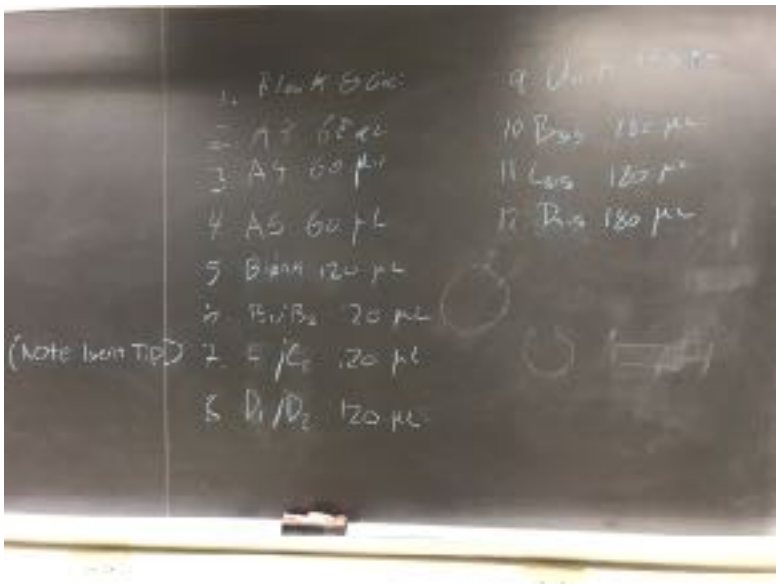


Figure 5: On the board is written the volumes and coordinates for the wells that the DNA was extracted from so that when the tests went though the Spectrophotometer we knew which data was for which test.

Figure 6: Maddie is bummed that when she was extracting DNA she pressed too hard on the bottom of the well with a pipette so that the tip

Figure 7: Ian and Sam use a Spectrophotometer to measure concentrations of resuspended DNA.

Figure 8: The spectrophotometer that we use in lab.

Figure 9: These are the results from the Spectrophotometer for our DNA concentrations (see Figure 5 to tell which test number is for which concentration).

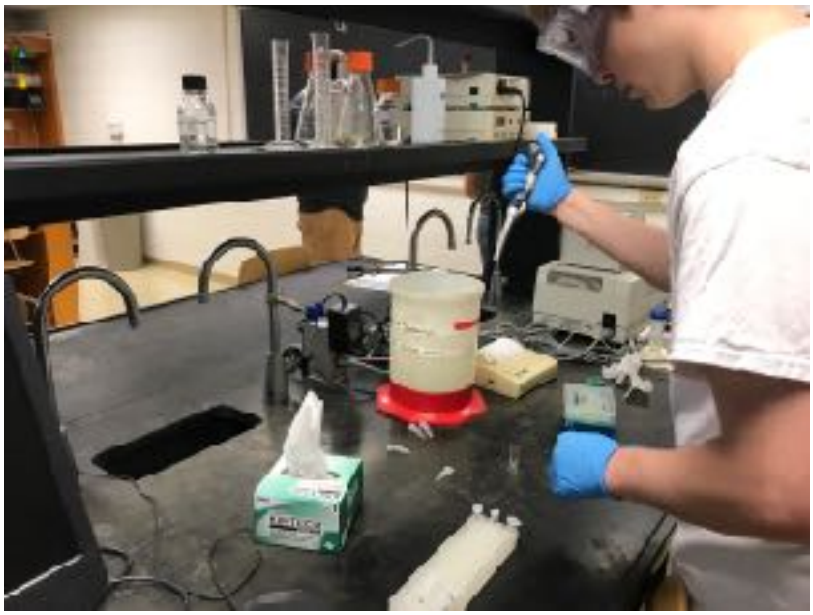


Figure 10: Ian properly disposes of used pipette tips when cleaning up.

July 10, 2017

Attendance

1. Sam
2. Kira
3. Maddie
4. Sarah
5. Yianni
6. Seth
7. Ian
8. Erin

Today during our meeting we discussed and worked on logistics. We worked on the team t shirt, wiki and logistics for the Mid Atlantic meet up. We also looked at the results for the transformations that we ran yesterday.

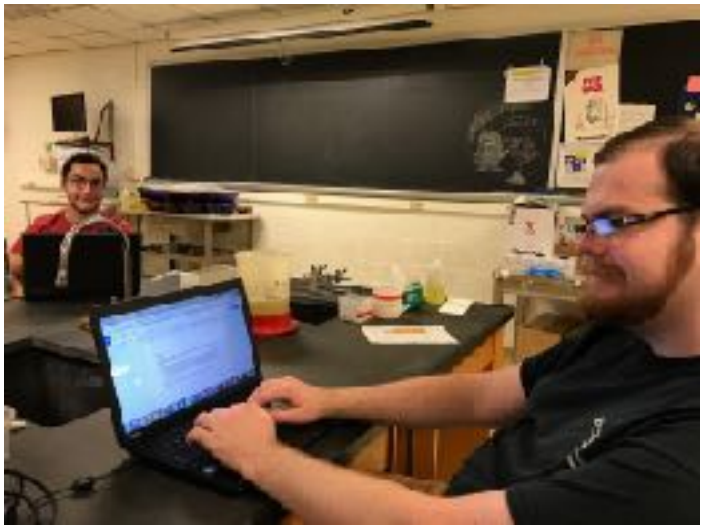


Figure 1: Sam looks in good spirits while working on the google doc.

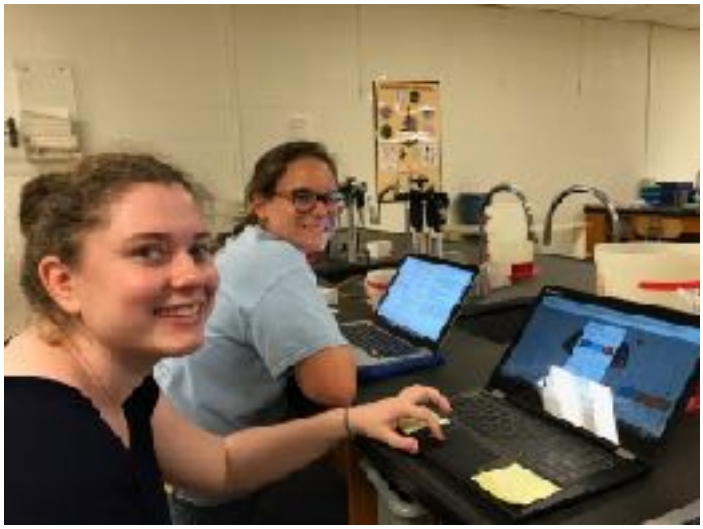


Figure 2: Team members Sarah and Kira work hard together on organizing for the website and preparing for lab.

Figure 3: This is where we house some of our bacteria cultures when we want to incubate.

Figure 4: Before going into the lab team members read through the protocol for the day.

Figure 5: Team members work side by side to efficiently label and streak petri dishes for our bacteria cultures.

July 11, 2017

Meeting Agenda:
6pm today inoculations and plates were removed from incubator
8pm inoculations into incubator (Ian has record of which plates had counts that team members did, which ones moved and which ones in past for today's work)
9:30pm July 10th inoculation put into shaking incubator

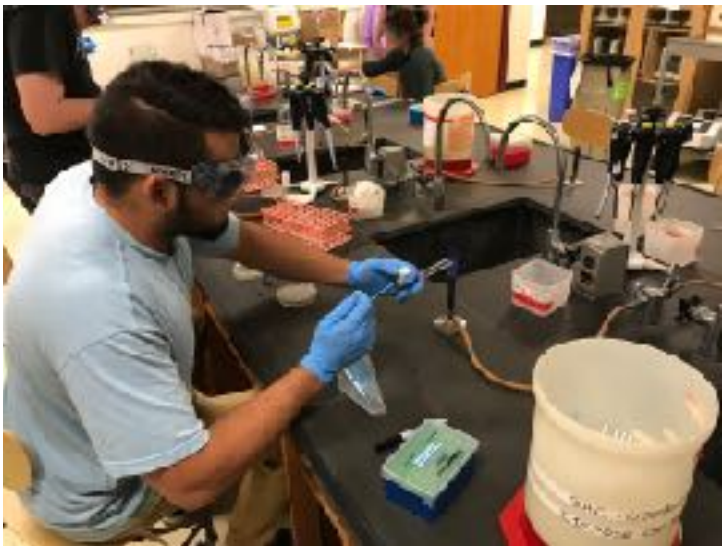


Figure 1: Seth uses proper safety and sterile technique to streak a plate with bacteria.

Figure 2: Ian, Seth, and Sarah smile while working together!

Figure 3: Erin looks focused while labeling a petri dish.

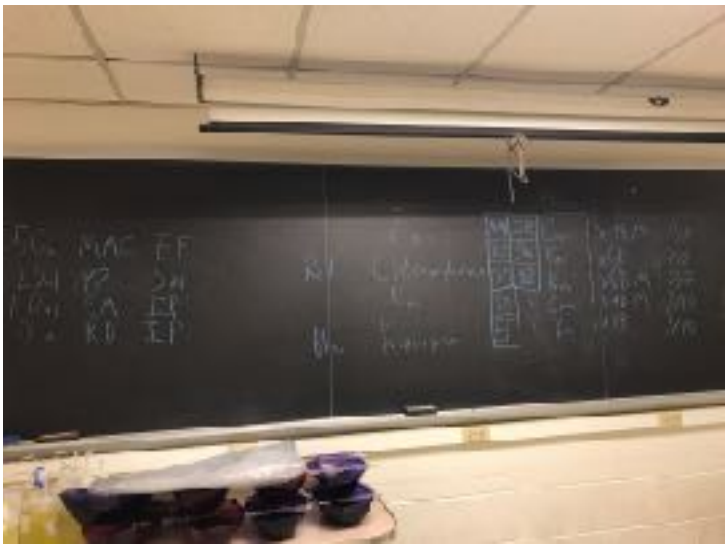


Figure 4: The board that Sam drew up to keep teammates organized when plating bacteria.

July 27, 2017

Meeting Agenda:

Today some team members met with Adam to go over the following week's meeting schedule.

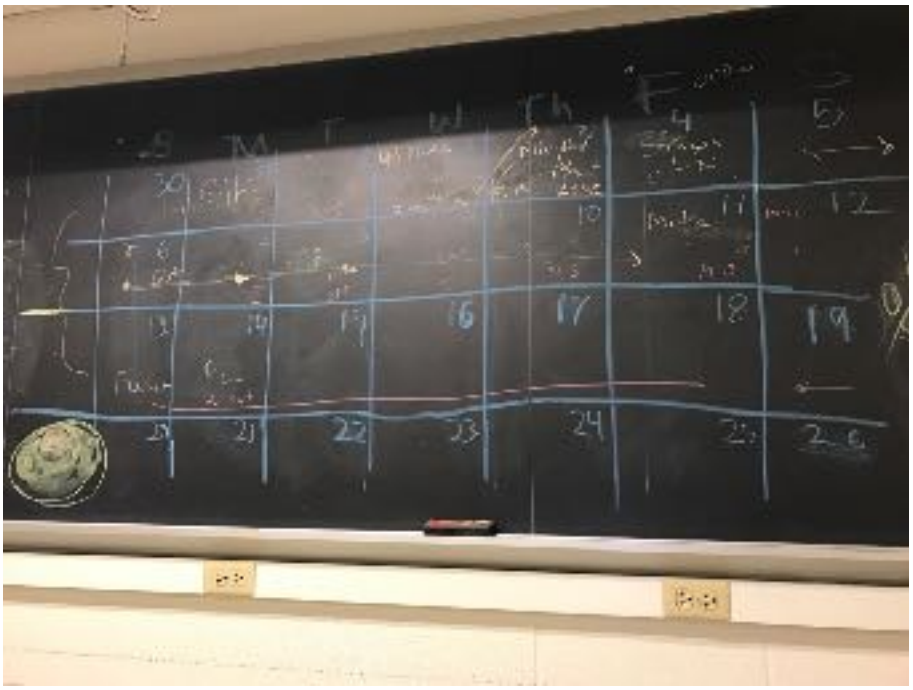
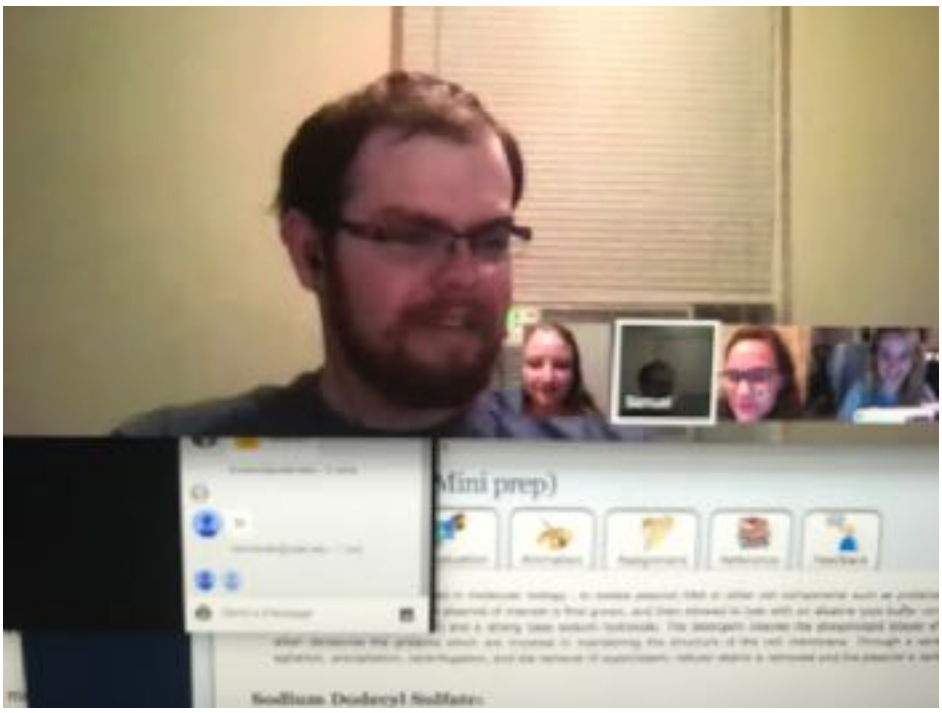


Figure 1: This is our working calendar that Adam sketched out on the black board so that all students could give their input on the meeting agendas and timing.

July 28, 2017

Meeting Agenda:
Review our presentation for the meetup.
Today Maddie, Jessica, Sarah, and Sam scheduled a video chat to finalize our presentation and receive advice from team leader Sam. A link to our Mid Atlantic Meetup presentation can be found here:
https://docs.google.com/presentation/d/1DhsYRuCD6L1dt75r2AW40DX4j4GzAIinG5J4l2IjH7o/edit?usp=sharing



July 29, 2017

Meeting Agenda:
Today members of the team drove down to UVA to present our research and ideas for the future. As a group we got to connect with other schools in attendance which were:
1.William and Mary
2.University of Maryland
3.UNCA
4.UVA
5.University of Georgia
6.University of Delaware
We learned from and were inspired by projects presented by other teams and got to talk to judges of the Jamboree as well as hear from past iGem team members who have started businesses based off of their iGem projects.

July 31, 2017

Meeting Agenda:
Gibson Assembly using G-blocks and suspended DNA and Transformation
Today we spend a long day in the lab. A brief summary of the following protocols was that first we labeled reaction tubes, then added 100 microliters of TE buffer to G-Block vials. Then we centrifuged at 5,900rpm for 10 seconds and solubilized at 50 degrees Celsius to make a solution of DNA and TE buffer. We completed transformation for reach of the 7 bacteriocins that we chose to test (Curvacin A, PelB-Pediocin, Sakacin QC, Acidocin AD, Sakacin P, CvaAB) and then preformed Gibson assembly. Then we streaked the bacteria that we hope accepted our plasmid onto petri dishes. And incubated for 48 hours until our next meeting.

As a team we followed this protocol for DNA transformation except for the following two variations:

- We added 5 minutes to the reaction time (20 vs 15 mins at 50 degrees C)
- We did not vortex our master mix

In addition, this is a schedule of the Gibson Assembly protocol that we preformed. Any thing in green is an adjustment we made because we were running behind initially scheduled timing.

University of Delaware iGEM team
Laboratory Instructions for Gibson Assembly and Transformation
Monday July 31, 2017

Overview:

The objective of these procedures is to assemble plasmid constructs, each containing a single bacteriocin or accessory gene and use these constructs to transform competent E.Coli. The double stranded DNA provided to us by IDT includes the following 13 components:

CvaA/B bp 1 - 2140	ABC Transporter	1000ng**	CvaAB
CvaA/B bp 2116 - 4257	ABC Transporter	1000ng**	CvaAB
Curvacin A	Bacteriocin	1000ng**	CurA
Enterocin A	Bacteriocin	1000ng**	EntA
Pediocin PA-1	Bacteriocin	1000ng**	PedPA1
Sakacin P	Bacteriocin	1000ng**	SakP
pSB1C3 linearized vector	Chloramphenicol resistant plasmid	1000ng**	pSB1C3
pSB1K3 linearized vector	Kanamycin resistant plasmid	1000ng**	pSB1K3
Col_5_Acidocin_Ad	Bacteriocin	1000ng**	AcdAD
Col_5_Lactocin_705 beta	Bacteriocin*	1000ng**	LasBeta
Col_5_Lactocin_705 alpha	Bacteriocin*	1000ng**	LasAlpha
Col_5_Sakacin_QC	Bacteriocin	1000ng**	SakQC
PelB_PediocinPA1	Bacteriocin	1000ng**	PBPedPA1
*Individual components of a 2 part system **Nominal mass			

Except for the two Lactocins, all of the above bacteriocin and transport genes will be assembled into constructs. Bacteriocins will be ligated into a chloramphenicol resistant backbone. The ABC transporter will be ligated into a Kanamycin resistant backbone. Following assembly, the reaction products will be used to transform competent e.coli. Transformed cells will be plated on selective media and incubated for 48 hours. The final product of this day's work will be a set of master plates for a total of 8 transformed cell lines. Each plate will be labeled with the date, gene symbol, plasmid, dilution level and operator's initials. The lactocins may be used at a later date.

Materials and Equipment for G-Block re-suspension

- TE Buffer 10 mM Tris, 1mM EDTA, pH 8
- Microcentrifuge, Vortexer
- Water bath heated to 50 degrees C

Materials and Equipment for Gibson Assembly:

- Microfuge tubes
- Gibson HiFI Kit reagents (Master Mix)
- Wet Ice
- Water Bath at 50 degrees C (and floaty tube holders)

Materials and Equipment for Transformations

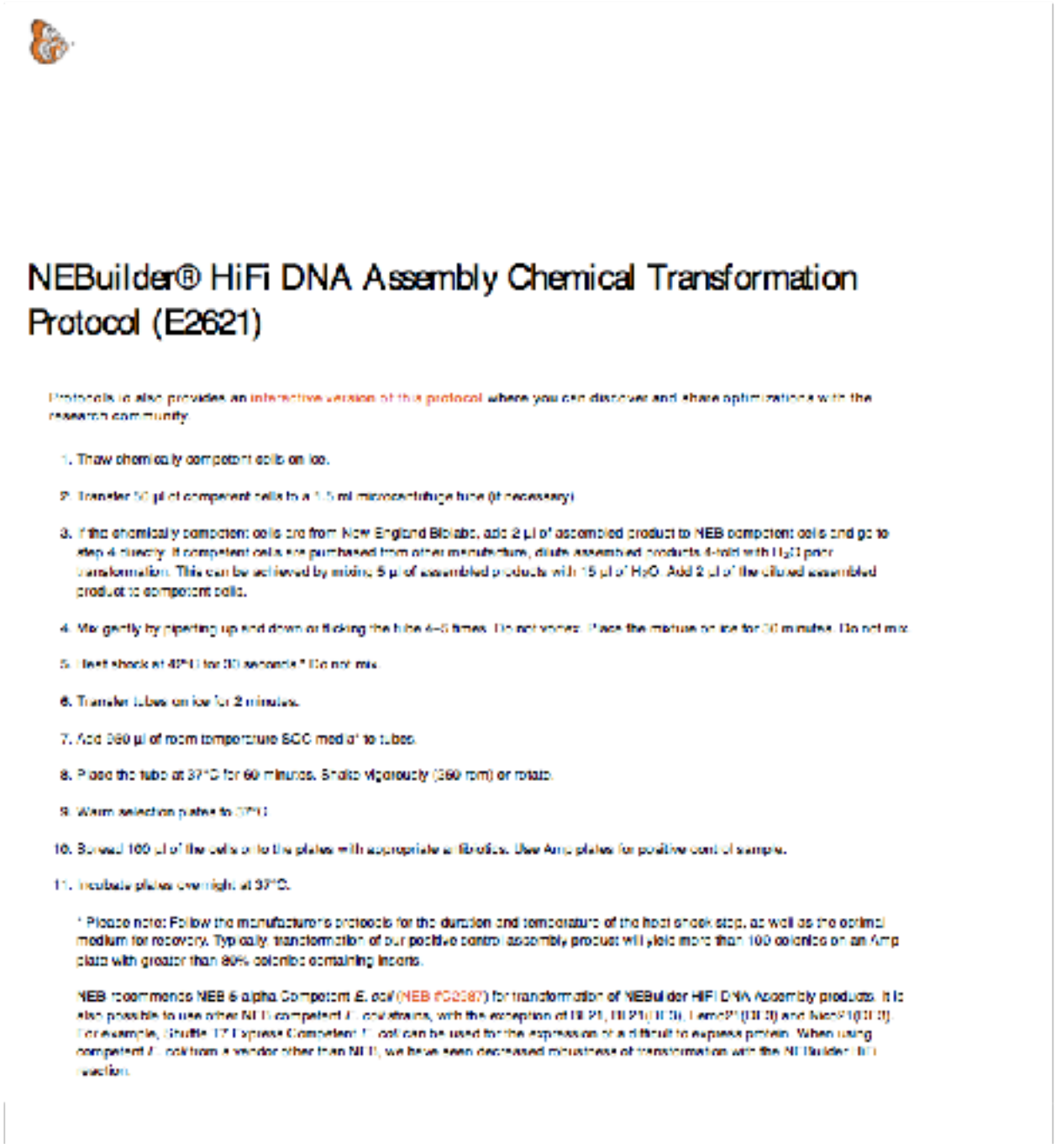
- Microfuge tubes -- labeled appropriately
- Competent Cells -- thawed on ice within 20 minutes of use
- Water Bath at 42 degrees C (and floaty tube holders)
- Shaking Incubator
- Dilution tubes, prepared in advance with 450 uL of SOC

General Procedure
5:30 pm -- 6:30 pm

1. Gather all required materials, turn on water baths and fetch ice

2. If Necessary, prepare TE Buffer.
3. Calculate molarities

NEBuilder® HiFi DNA Assembly Chemical Trans... https://www.neb.com/protocols/2015/02/17/nebui...



NEBuilder® HiFi DNA Assembly Chemical Transformation Protocol (E2621)

Protocols.io also provides an interactive version of this protocol where you can discover and share optimizations with the research community.

1. Thaw chemically competent cells on ice.
2. Transfer 50 µl of competent cells to a 1.5 ml microcentrifuge tube (if necessary).
3. If the chemically competent cells are from New England Biolabs, add 2 µl of assembled product to NEB competent cells and go to step 4 directly. If competent cells are purchased from other manufacturers, dilute assembled products 4-fold with H_2O prior transformation. This can be achieved by mixing 5 µl of assembled products with 15 µl of H_2O. Add 2 µl of the diluted assembled product to competent cells.
4. Mix gently by pipetting up and down or flicking the tube 4–5 times. Do not vortex. Place the mixture on ice for 30 minutes. Do not mix.
5. Heat shock at 42°C for 30 seconds*. Do not mix.
6. Transfer tubes on ice for 2 minutes.
7. Add 950 µl of room temperature SOC medium to tubes.
8. Place the tube at 37°C for 60 minutes. Shake vigorously (250 rpm) or rotate.
9. Warm selection plates to 37°C.
10. Spread 100 µl of the cells prior to the plates with appropriate antibiotics. Use Amp plates for positive control samples.
11. Incubate plates overnight at 37°C.

* Please note: Follow the manufacturer's protocols for the duration and temperature of the heat shock step, as well as the optimal medium for recovery. Typically transformation of our positive control assembly product will yield more than 100 colonies on an Amp plate with greater than 80% colonies containing inserts.

NEB recommends NEB 5-alpha Competent *E. coli* (NEB #C2987) for transformation of NEBuilder HiFi DNA Assembly products. It is also possible to use other NEB competent *E. coli* strains, with the exception of JM 101, JM 109 (JM 83), Lemo21(DE3) and Mach1(DE3). For example, Shuffle T7 Express Competent *E. coli* can be used for the expression of a difficult to express protein. When using competent *E. coli* from a vendor other than NEB, we have seen decreased robustness of transformation with the NEBuilder HiFi reaction.

for gibson reactions, double checked by a second operator

4. Label 8 microfuge tubes for Gibson Reactions:
   a. Prefix RX
   b. Symbol, eg SakP, PedPA1 etc. . .
5. Label 8 microfuge tubes for transformation, and set aside.
6. Re-suspend GBlocks following procedure on package insert.
   a. Add 100 uL TE buffer, vortex, incubate 50 C for 20 minutes
   b. Vortex again and do a quick spin to collect all liquid at the bottom of the vial
7. Check DNA concentration on the spectrophotometer
   a. Dilute 10 uL of each resuspended G-Block in 90 uL TE, measure absorbance at 260nm and 320 nm

6:30 pm -- 8:00 pm

8. Set up Gibson reactions on ice, Taking care not to contaminate microfuge tubes
a. For Bacteriocins:
i.5 uL Bacteriocin + 5 uL pSB1C3 Vector + 10 uL master mix
b. For CvaAB
i.6 uL CvaAB-1 + 6uL CvaAB-2 + 3 uL pSB1K3 Vector + 10 uL master mix
9. Incubate Reactions at 50 C for 15 to 60 minutes
a. Start time: 7:03 pm End Time: 7:23

8 :00 pm -- 10:00 pm

9. 10 minutes prior to the end of Gibson Assembly, fetch competent cells from -80 (on ice)
10. Transfer Competent cells ~ 50uL to sterile microfuge tubes
11. Follow Incubation procedure as described in NEB Protocol
a. 30 minutes on ice. Start time: End Time:
b. 30 second heat shock at 42 C
c. Add 950 uL SOC
d. Shake and incubate for 60 minutes at 37 C, 250 rpm

12. At the end of incubation, prepare a 10x dilution of each transformation mixture
a. 50uL transformation mixture in 450 uL SOC
13. Prepare the following master plates for each trasnformation
a. 100 uL of transformation mixture, spread on plate
b. 100 uL of the 10 x dilution spread on selective plate
c. Streak a loop full of the transformatio mixture
14. Incubate plates inverted at 37 C for ~ 48 hours.

Work sheet for Gibson Reactions.

pSB1C3
OD260: **OD320:** **Actual Mass:** **Fragment fm/ng:**

pSB1K3
OD260: **OD320:** **Actual Mass:** **Fragment fm/ng:**

Curvacin A Reaction
OD260: **OD320:** **Actual Mass:** **Fragment fm/ng:**

Fragment	Volume Added	Actual Mass	Actual Moles
pSB1C3 dsDNA	5 uL	50ng	0.04pmol
Bacteriocin dsDNA	5 uL	50ng	0.075pmol

Enterocin A
OD260: **OD320:** **Actual Mass:** **Fragment fm/ng:**

Fragment	Volume Added	Actual Mass	Actual Moles
pSB1C3 dsDNA	5 uL	50ng	0.04pmol
Bactericon dsDNA	5 uL	50ng	0.074pmol

Pedicocin PA1

OD260: **OD320:** **Actual Mass:** **Fragment fm/ng:**

Fragment	Volume Added	Actual Mass	Actual Moles
pSB1C3 dsDNA	5 uL	50ng	0.04pmol
Bacteriocin dsDNA	5 uL	50ng	0.071pmol

h;;2

Sakacin P

OD260: **OD320:** **Actual Mass:** **Fragment fm/ng:**

Fragment	Volume Added	Actual Mass	Actual Moles
pSB1C3 dsDNA	5 uL	50ng	0.04pmol
Bacteriocin dsDNA	5 uL	50ng	0.0745pmol

Acidocin AD

OD260: **OD320:** **Actual Mass:** **Fragment fm/ng:**

Fragment	Volume Added	Actual Mass	Actual Moles
pSB1C3 dsDNA	4 uL	40ng	0.032pmol
Bacteriocin dsDNA	5 uL	50ng	0.0685pmol

Add 1 uL water

Sakacin QC

OD260: **OD320:** **Actual Mass:** **Fragment fm/ng:**

Fragment	Volume Added	Actual Mass	Actual Moles
pSB1C3 dsDNA	5 uL	50ng	
Bacteriocin dsDNA	5 uL	50ng	0.07pmol

PelB-Pediocin PA1

OD260: **OD320:** **Actual Mass:** **Fragment fm/ng:**

Fragment	Volume Added	Actual Mass	Actual Moles
pSB1C3 dsDNA	5 uL	50ng	0.04pmol
Bacteriocin dsDNA	5 uL	50ng	0.073pmol

CvaAB

OD260: **OD320:** **Actual Mass:** **Fragment fm/ng:**

Fragment	Volume Added	Actual Mass	Actual Moles
pSB1K3 dsDNA	3 uL	30 ng	0.0225 pmol
CvaAB-1	6 uL	60 ng	0.0456pmol

CvaAB-2	**6 uL**	**60 ng**	**0.0456pmol**

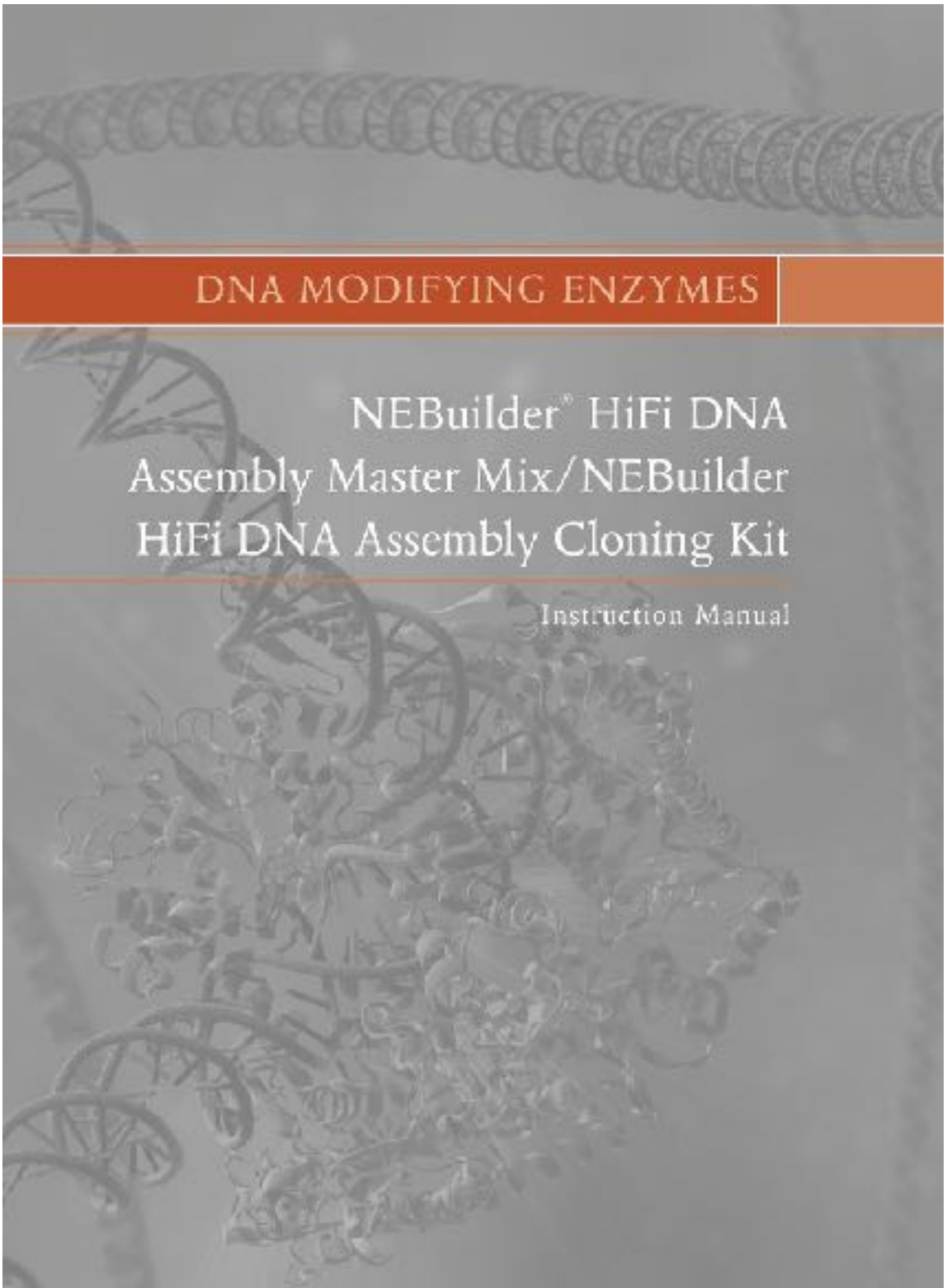
15 uL of Master mix

This is the protocol we followed for Gibson Assembly:

DNA MODIFYING ENZYMES

NEBuilder® HiFi DNA Assembly Master Mix/NEBuilder HiFi DNA Assembly Cloning Kit

Instruction Manual



NEB #E2621S/L/X,
NEB #E5520S

NEW ENGLAND BioLabs® inc.

This product is intended for research purposes only. This product is not intended to be used for therapeutic or diagnostic purposes in humans or animals.

Limited Warranty: The NEBuilder® HiFi DNA Assembly Master Mix and NEBuilder® HiFi DNA Assembly Cloning Kit are warranted to perform according to specifications stated on the certificate of analysis. No other warranty is made, whether express or implied, including any warranty of merchant ability or fitness for a particular purpose. This warranty limits NEB and its licensors' liability to only the price of the product. Neither NEB nor its licensors shall have any responsibility or liability for any special, incidental, indirect or consequential loss or damage whatsoever.

Table of Contents:

Components:

NEBuilder HiFi DNA Assembly Master Mix:

Important Note: Upon arrival, store the kit components at –20°C.

NEBuilder HiFi DNA Assembly Master Mix

Positive Control:
2 overlapping dsDNA fragments for control assembly

NEBuilder HiFi DNA Assembly Cloning Kit:

Important Note: Upon arrival, store the kit components at –80°C. Before use, thaw and vortex the master mix thoroughly and keep on ice. After first use, store the HiFi DNA Assembly Master Mix, SOC Outgrowth Medium and positive controls at –20°C. Store the competent cells at –80°C.

NEBuilder HiFi DNA Assembly Master Mix

NEB 5-alpha Competent *E. coli* (High Efficiency)

SOC Outgrowth Medium

Positive Controls:
2 overlapping dsDNA fragments for control assembly
pUC19 Control DNA for NEB 5-alpha Competent *E. coli*

Required Materials Not Included:

DNA Polymerase (for generating PCR products):

For generating PCR Products, we recommend Q5® High-Fidelity DNA Polymerase (NEB #M0491) or related products, such as Q5 Hot Start High-Fidelity DNA Polymerase (NEB #M0493) or Q5 Hot Start High-Fidelity 2X Master Mix (NEB #M0494).

LB (Luria-Bertani) plates with appropriate antibiotic.

For selection of transformed competent cells, we recommend LB plates with appropriate antibiotic.

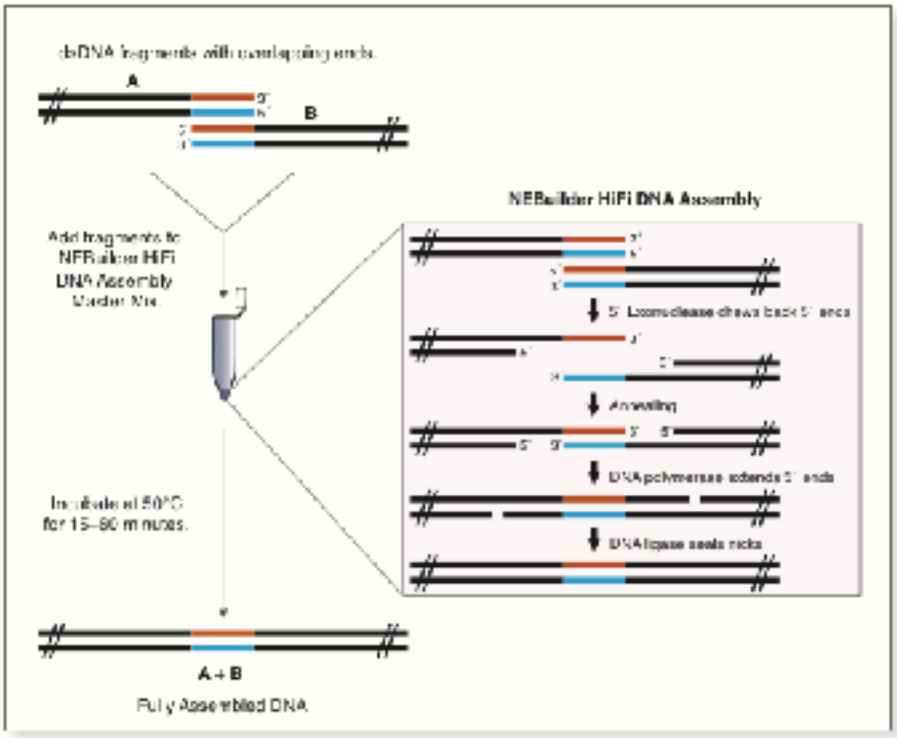
Introduction:

NEBuilder HiFi DNA Assembly Master Mix was developed to improve the efficiency and accuracy of DNA assembly. This method allows for seamless assembly of multiple DNA fragments, regardless of fragment length or end compatibility. This method has been used to assemble either single-stranded oligonucleotides or different sizes of DNA fragments with varied overlaps (15–80 bp). It has utility for the synthetic biology community, as well as those interested in one-step cloning of multiple fragments due to its ease of use, flexibility and simple master-mix format. The reaction includes different enzymes that work together in the same buffer (see Figure 1):

- The exonuclease creates single-stranded 3′ overhangs that facilitate the annealing of fragments that share complementarity at one end (the overlap region)
- The polymerase fills in gaps within each annealed fragment
- The DNA ligase seals nicks in the assembled DNA

The end result is a double-stranded fully sealed DNA molecule that can serve as template for PCR, RCA or a variety of other molecular biology applications, including direct transformation of *E coli*.

Figure 1: Overview of the NEBuilder HiFi DNA Assembly Method



Specification:

10 µl of 2X NEBuilder HiFi DNA Assembly Master Mix was incubated with 6 DNA fragments [4 fragments of 1,000 bp and one fragment of 1,152 bp with 80 bp overlap, and a vector of 3,373 bp (20 bp overlap), 0.05 pmol each] in a final volume of 20 µl at 50°C for 60 minutes. NEB 5-alpha Competent *E. coli* (NEB #C2987) were transformed with 2 µl of the assembled products according to the transformation protocol. Successfully assembled fragments produce an intact *lacZ* gene in the pACYC184 vector, and yield blue colonies on an IPTG/Xgal/Chloramphenicol plate when incubated overnight at 37°C after transformation. Greater than 100 blue colonies were observed when 1/10 of the outgrowth (500 µl) was spread on a plate.

Overview of NEBuilder HiFi DNA Assembly Master Mix/DNA Assembly Cloning Kit Workflow:

- Design primers to amplify fragments (and/or vector) with appropriate overlaps (see pages 4–10).
- Amplify fragments using a high-fidelity DNA polymerase.
- Prepare linearized vector by using a high-fidelity DNA polymerase or by restriction enzyme digestion.
- Determine concentration of fragments and linearized vector using agarose gel electrophoresis, a NanoDrop® instrument or other method.
- Add fragments and linearized vector to NEBuilder HiFi DNA Assembly Master Mix and incubate at 50°C for 15 minutes to 1 hour, depending on the number of fragments being assembled.
- Transform into NEB 5-alpha Competent *E. coli* (provided with cloning kit or purchased from NEB) or use directly in other applications.

Design and PCR of Fragments for DNA Assembly:

Note: We highly recommend using our web tool, NEBuilder Assembly Tool, available at nebuilder.neb.com, to design PCR primers with overlapping sequences between the adjacent DNA fragments and for their assembly into a cloning vector.

NEBuilder Assembly Tool is the fastest and easiest approach to obtaining ready-to-use sequences for overlapping primers. However, it does not give details about the primer-design workflow. In some cases, it might be appropriate to manually alter primer sequences in order to adapt them for the use in more complex assemblies, such as those that include site-specific mutagenesis. For this purpose, it is absolutely necessary to understand the general requirements and rules that apply to PCR primers used in conjunction with HiFi DNA Assembly Mix. The sections below offer step-by-step directions and recommendations for the manual design of primers for the assembly of two or more PCR fragments, as well as primer design for assembly of PCR fragments into a cloning vector prepared either by PCR or by restriction digestion.

Structure of the Overlapping Primers

PCR primers for use in HiFi DNA assembly must have two sequence components:

- an overlap sequence, required for the assembly of adjacent fragments;
- a gene-specific sequence, required for template priming during PCR.

The non-priming overlap sequence is added at the 5′ end of the primer. This sequence is homologous to the 5′-terminal sequence of the adjacent fragment to be assembled. The length of overlap sequence is dependent on the GC content of the sequences.

The priming gene-specific sequence is added at the 3′ end of the primer after the overlap sequence. The priming sequence should meet the criteria required for template annealing during PCR.

The Tm of the 3′ gene-specific sequence of the primer can be calculated using the Tm calculator found on the NEB website at tmcalculator.neb.com.

General Recommendations for Design of Overlapping Primers

To achieve efficient assembly of PCR fragments into a vector, we suggest using a 15–25 nt overlap with a Tm equal to or greater than 48°C (assuming A-T pair = 2°C and G-C pair = 4°C). To prevent errors in primer design it is highly recommended to first perform DNA fragment assembly *in silico* and create a final sequence file displaying both DNA strands (Fig. 2A, Step I). This virtual sequence may then be used as a template to design overlapping primers. Figure 2A shows the workflow for overlapping primer design by using an *in silico*-created DNA sequence file. First, mark the junctions between the adjacent fragments 1, 2 and 3 (Fig. 2A, Step II). Next, at or near each junction choose 15–25 nucleotide sequences to serve as the overlap region between the two adjacent fragments (Fig. 2A, Step III). For the best fit, in terms of length and Tm, the overlap sequence can be composed of nucleotides which belong to only one fragment (overlap shown in blue) or it can be split between the two adjacent fragments in any combination (overlap shown in orange). Mark the first 5′ and the last 3′ nucleotide of the overlap sequence on both DNA strands (boxed sequence). Finally, starting from the first 5′ nucleotide, copy the entire overlap sequence in the 5′ to 3′ direction and, if necessary, continue to add nucleotides to the 3′ end until the gene-specific priming sequence length is reached (Fig. 2A, Step IV). The reverse overlapping primer is designed following the same steps as described above but copying the sequence from the complementary DNA strand in the 5′ to 3′ direction. Keep in mind that the two primers sharing the same overlap sequence are always used in separate PCR reactions, each in combination with the primer which primes the complementary sequence on the opposite end of the respective DNA fragment (Fig. 2A, Step V).

Figure 2A: Primer design using an *in silico* created final DNA sequence file.

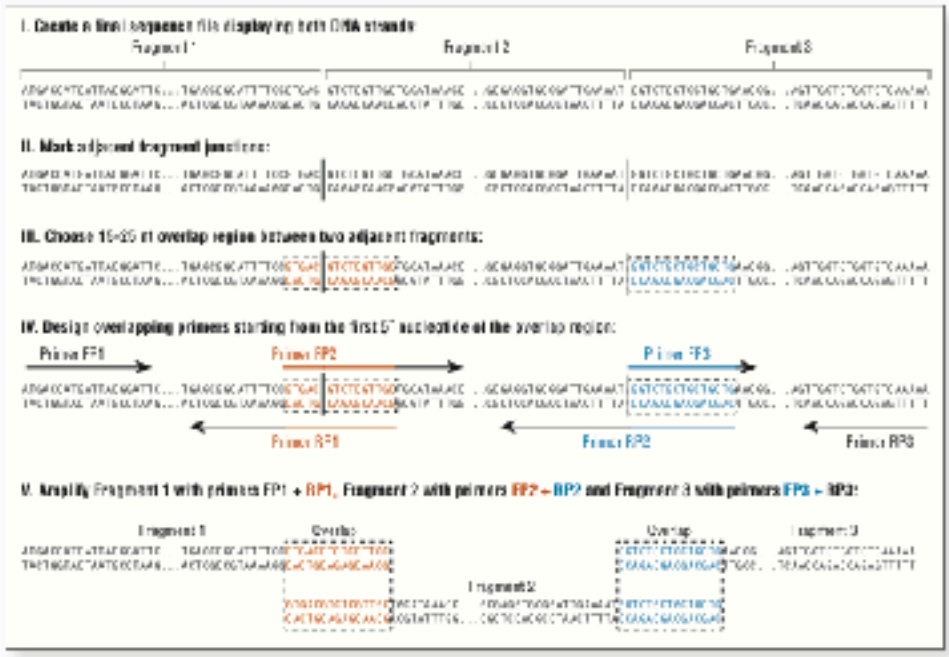
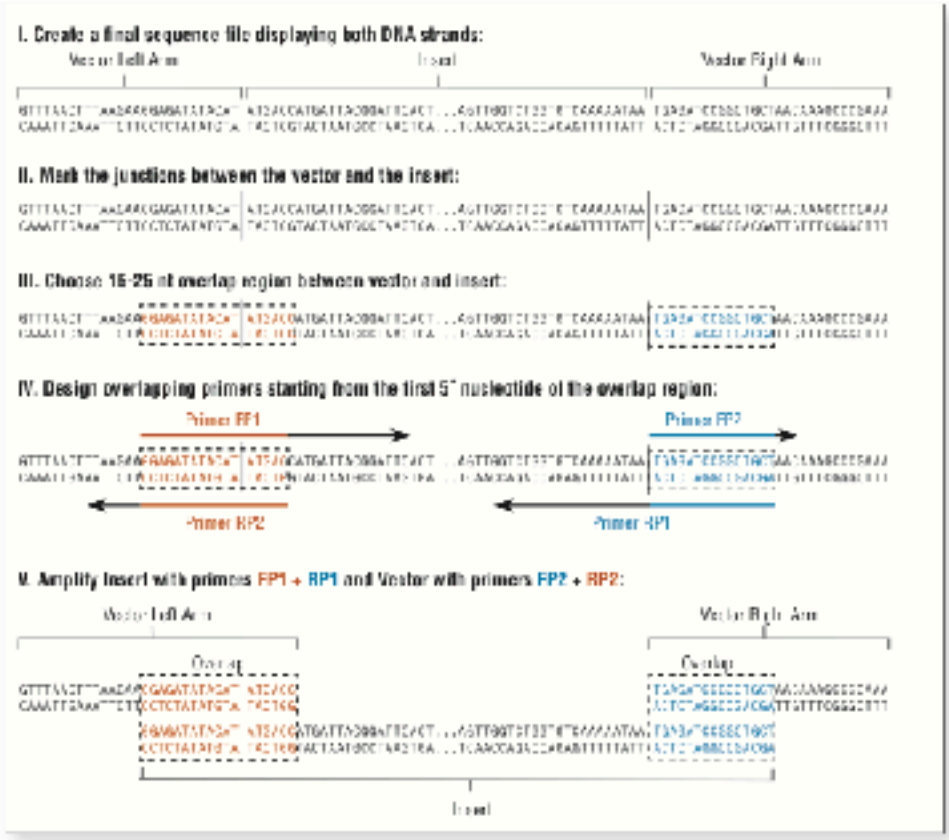


Figure 2B: Primer design for PCR-generated vector and insert using an *in silico* created final DNA sequence file.



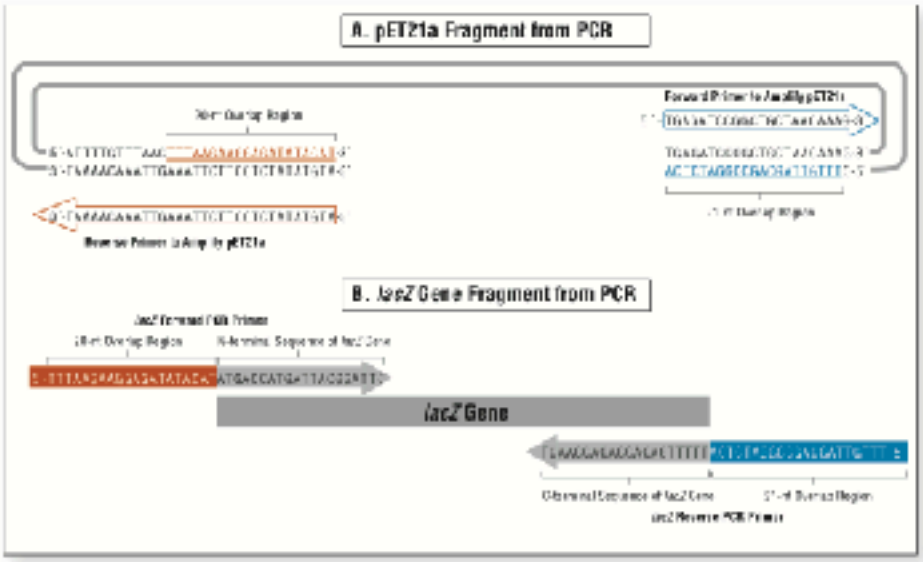
Primer Design for PCR-Generated Vector and Insert

For the purposes of primer design, the vector and the insert may be viewed as two PCR fragments that have to be assembled into a circular DNA molecule. This means that the primer design rules described above may also be applied for generation of the vector fragment and the insert fragment sharing overlapping ends. Use the *in silico*-created final sequence file as a template to design overlapping primers between the vector and the insert by accomplishing the same steps as described above, and as shown in Figure 2B.

If you intend to use a PCR-generated vector for one specific insertion, then the overlap sequence may be split between the vector and the insert in any combination to make shorter primers (Figure 2B, Step III, overlap shown in orange). However, if the same PCR-generated vector will be used for assembly of various inserts, then the entire overlap sequence must originate from the vector sequence and must be added to primers that will be used to amplify the insert (Figure 2B, Step III, overlap shown in blue). The latter case is also illustrated in Figure 3 for assembly of the *lacZ* gene into a pET21a vector. The pET21a forward primer (orange arrow) and the reverse primer (blue arrow) start at the position where the *lacZ* gene must be inserted. Both vector-specific primers completely match the vector sequence on the respective strands. This inverse PCR strategy yields a linear vector fragment. Generally, 10–100 pg of a vector is recommended as a template in the inverse PCR reaction.

To amplify the *lacZ* gene, both forward and reverse *lacZ*-specific priming sequences (gray) at their 5′ end are fused with the respective vector sequences to be used as overlap sequences in assembly with the vector. Within the *lacZ* Forward PCR primer, the overlap sequence (orange) is identical to the 20-nt terminal sequence on the top strand (orange) of the vector's left-arm (in the 5′→3′ direction). Within the *lacZ* Reverse PCR primer, the overlap sequence (blue) is identical to the 21-nt terminal sequence on the bottom strand (blue) of the vector's right-arm (in the 5′→3′ direction). The length of the overlap sequence is determined by the number of nucleotides needed to reach a Tm ≥ 48°C. If necessary, one may add additional nucleotides between the overlap sequence and the *lacZ*-specific sequence, for example, to introduce a unique restriction site.

Figure 3: Primer Design for Vector pET21a and *lacZ* Gene Assembly.



Primer Design for Assembly of Restriction Enzyme Digested Vector and PCR-Generated Insert

Restriction enzyme-treated vectors can have 5′ overhangs, 3′ overhangs or blunt ends. When vector is linearized by restriction digestion, the entire overlap sequence must originate from the vector sequence and must be added to primers that will be used to amplify the insert. The overlap region of the forward primer for the gene of interest (orange) should line up with the 3′ end of the overhang on the vector's left arm and extend back until the Tm ≥ 48°C (Fig.4A, Left side shown in a, b and c). This primer also includes gene-specific sequence at the 3′-end (gray). Keep in mind that the restriction site, which was used to digest the vector, will be lost in the assembled product. However, additional nucleotides may be added between the overlap region and gene-specific sequence region of inserted fragment to restore the pre-existing restriction site, to introduce a new restriction site, or keep the translation of the fusion protein in frame. A similar principle is applied to the design of the reverse primer for the gene of interest (Fig. 4A, Right side).

One of the unique features of the NEBuilder HiFi DNA Assembly Master Mix is the ability to remove both 3′ and 5′ end flap sequences upon fragment assembly. As shown in Figure 4A, panel d, additional 3′ and 5′ end flap sequences after a particular restriction enzyme digestion can be removed depending on the design of the insert sequence. This allows fragments generated by restriction enzyme digestion to assemble while eliminating the remaining restriction site sequences on both the 5′ and 3′ ends in the fragment junction.

Figure 4B shows primer design for assembly of the *lacZ* gene and pMAL-c5X, digested with NotI and SbfI. In this example, the forward primer of the gene has a "C" nucleotide (underlined, nearly invisible in actual figure) inserted between the 18-nt overlap and the N-terminal sequence of the *lacZ* gene to ensure the *lacZ* protein is in frame with the maltose binding protein.

Figure 4A: Assembly of Restriction Enzyme-Digested Vector and PCR-derived Insert

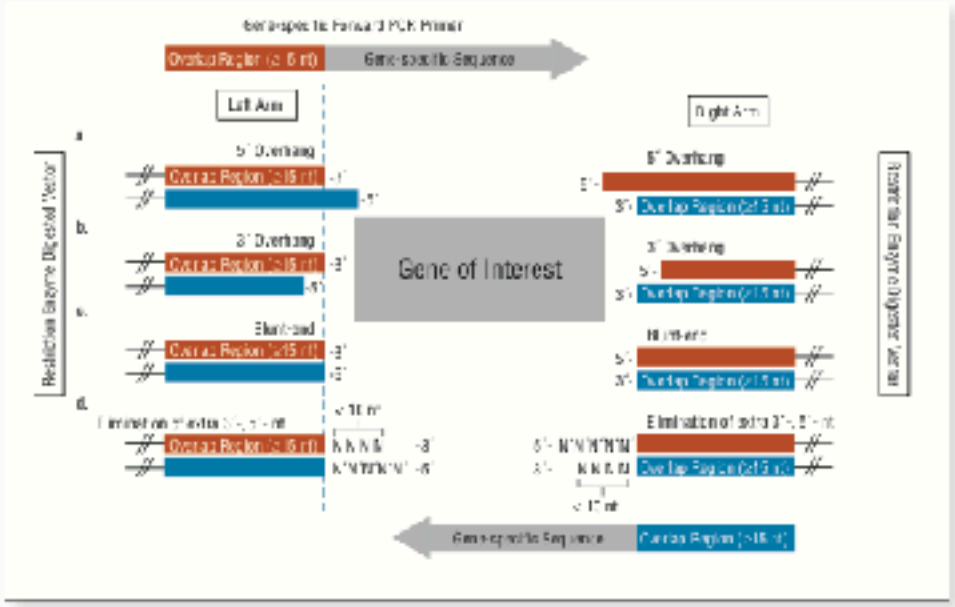
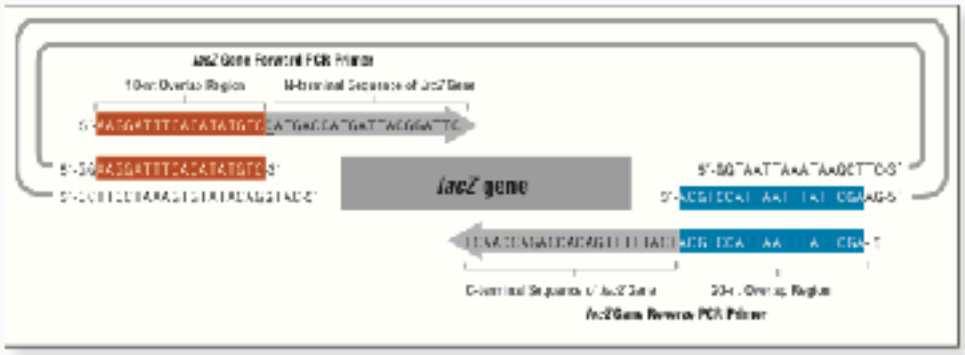


Figure 4B: Primer Design for *lacZ* Gene and NcoI/SbfI-cut pMAL-c5X Assembly



Useful Recommendations for Vector Digestion with Restriction Enzymes

In general, the cloning vector can be linearized by any restriction endonuclease or by any combination of two restriction endonucleases displaying unique site(s) at the desired locations within the vector sequence.

Note: Double digestion of vector DNA with two restriction endonucleases is the best approach to reduce the uncut vector background.

- Some restriction endonucleases cannot efficiently digest supercoiled DNA and thus may leave behind different amounts of uncut vector DNA. If not gel purified, the uncut vector is transformable, and will show up after transformation of the HiFi DNA assembly reaction, thereby, reducing the overall fraction of recombinant clones. The table "Cleavage of Supercoiled DNA" found at www.neb.com/tools-and-resources/selection-charts/ may be used as a reference for choosing the most suitable restriction endo-

nucleases and the number of activity units required for complete digestion of plasmid vector.

- Restriction endonucleases might have a reduced activity on plasmid DNA purified using various plasmid purification kits. In such cases, extended incubation time or increased enzyme concentration may be necessary to digest plasmid vector to completion (or as nearly as possible to completion). When applicable, NEB highly recommends using High-Fidelity (HF®) restriction endonucleases to avoid star activity, which may occur when digesting DNA for extended periods of time with elevated amounts of standard restriction endonuclease.
- Purification of restriction endonuclease-digested vector is not necessary unless the same restriction site is present in insert DNA. In such cases, either heat inactivate the restriction endonuclease or purify the linearized vector.

Useful Recommendations for PCR

NEB recommends using Q5 High-Fidelity DNA Polymerase (NEB #M0491) or related products (NEB #M0493 or NEB #M0494) to amplify fragments of interest prior to assembly. The use of this high-fidelity DNA polymerase yields PCR products with blunt ends, thereby reducing error rates at the fragment junctions.

- When using circular plasmid DNA as a template, it is important to use a minimal amount of DNA (e.g., 0.1–0.5 ng of plasmid template per 50 µl PCR reaction) in order to reduce the template background after transformation. If higher amounts of plasmid template must be used in the PCR reaction or higher amounts of PCR product must be used in the HiFi DNA assembly reaction, it is recommended to digest the PCR product with DpnI restriction endonuclease in order to destroy plasmid template before setting up the NEBuilder HiFi DNA assembly reaction (for protocol, see below).
- Verify PCR product purity and yield by gel electrophoresis. If non-specific DNA fragments are obtained, you will need to purify the target fragment from the agarose gel to ensure the correct product assembly is produced during the NEBuilder HiFi DNA assembly reaction.

- PCR product purification is not necessary as long as the product is > 90% pure. You can add unpurified PCR product directly from the PCR reaction into the assembly reaction, for up to 20% of the total reaction volume (e.g., PCR products should account for 4 µl, or less, in a 20 µl NEBuilder HiFi DNA assembly reaction). Larger volumes of unpurified PCR products could significantly inhibit both the assembly and the transformation. In such cases, it is recommended to column purify PCR products and, if necessary, to concentrate DNA by ethanol precipitation.

(Optional) DpnI Digestion Protocol

When higher amounts of plasmid template must be used in the PCR reaction, it is recommended that the PCR product be digested with DpnI (NEB #R0176) in order to destroy the plasmid template before setting up the assembly reaction. DpnI cleaves only *E. coli* Dam methylase-methylated plasmid DNA, but does not cleave the PCR product, since it is not methylated.

DpnI Digestion Protocol:

1. In a 10 µl reaction, mix 5–8 µl of PCR product with 1 µl of 10X CutSmart™ Buffer and 1 µl (20 units) of DpnI.
2. Incubate at 37°C for 30 minutes.
3. Heat-inactivate DpnI by incubating at 80°C for 20 minutes.
4. Proceed with the NEBuilder HiFi DNA Assembly Protocol, as described below.

NEBuilder HiFi DNA Assembly Reaction Protocol:

Optimal Quantities

NEB recommends a total of 0.03–0.2 pmols of DNA fragments when 1 or 2 fragments are being assembled into a vector, and 0.2–0.5 pmols of DNA fragments when 4–6 fragments are being assembled. Efficiency of assembly decreases as the number or length of fragments increases. To calculate the number of pmols of each fragment for optimal assembly, based on fragment length and weight, we recommend the following formula, or using the tool, NEBiocalculator (nebiocalculator.neb.com)

pmols = (weight in ng) x 1,000 / (base pairs x 650 daltons)

50 ng of 5000 bp dsDNA is about 0.015 pmols

50 ng of 500 bp dsDNA is about 0.15 pmols

The mass of each fragment can be measured using the NanoDrop instrument, absorbance at 260 nm or estimated from agarose gel electrophoresis followed by ethidium bromide staining.

HiFi DNA Assembly Protocol

1. Set up the following reaction on ice:

	Recommended Amount of Fragments Used for Assembly		
	2–3 Fragment Assembly*	4–6 Fragment Assembly**	Positive Control†
Recommended DNA Ratio	vector:insert = 1:2	vector:insert = 1:1	
Total Amount of Fragments	0.03–0.2 pmol* X µl	0.2–0.5 pmol** X µl	10 µl
NEBuilder HiFi DNA Assembly Master Mix	10 µl	10 µl	10 µl
Deionized H_2O	10-X µl	10-X µl	0
Total Volume	20 µl††	20 µl††	20 µl

* Optimized cloning efficiency is 50–100 ng of vector with 2-fold excess of inserts. Use 5 times more insert if size is less than 200 bp. Total volume of unpurified PCR fragments in the assembly reaction should not exceed 20%.

** To achieve optimal assembly efficiency, design ≥ 20 bp overlap regions between each fragment with equimolarity (suggested: 0.05 pmol each).

† Control reagents are provided for 5 experiments.

†† If greater numbers of fragments are assembled, increase the volume of the reaction, and use additional NEBuilder HiFi DNA Assembly Master Mix.

2. Incubate samples in a thermocycler at 50°C for 15 minutes (when 2 or 3 fragments are being assembled) or 60 minutes (when 4–6 fragments are being assembled). Following incubation, store samples on ice or at –20°C for subsequent transformation.

Note: Extended incubation up to 60 minutes may help to improve assembly efficiency in some cases (for further details see FAQ section on page 15).

3. Transform NEB 5-alpha Competent *E. coli* cells (provided in the cloning kit or purchased separately from NEB) with 2 µl of the assembled product, following the transformation protocol on page 12.

NEBuilder HiFi DNA Assembly Transformation Protocol

1. Thaw chemically-competent cells on ice.
2. Add 2 µl of the chilled assembled product to the competent cells. Mix gently by pipetting up and down or by flicking the tube 4–5 times. Do not vortex.
3. Place the mixture on ice for 30 minutes. Do not mix.
4. Heat shock at 42°C for 30 seconds. Do not mix.
5. Transfer tubes to ice for 2 minutes.
6. Add 950 µl of room-temperature SOC media to the tube.

7. Incubate the tube at 37°C for 60 minutes. Shake vigorously (250 rpm) or rotate.
8. Warm selection plates to 37°C.
9. Spread 100 µl of the cells onto the selection plates. **Use Amp plates for the positive control sample.**
10. Incubate overnight at 37°C.

Usage Notes:

To ensure the successful assembly and subsequent transformation of assembled DNAs, NEB recommends the following:

- *DNA*: PCR product purification is not necessary if the total volume of all PCR products is 20% or less of the assembly reaction volume. Higher volumes of PCR products may reduce the efficiency of high-fidelity DNA assembly and transformation due to the elevated carryover amounts of PCR reaction buffer and unused primers present in the PCR product. Column purification of PCR products may increase the efficiency of both high-fidelity DNA assembly and transformation by 2–10 fold and is highly recommended when performing assemblies of three or more PCR fragments or assembling longer than 5 kb fragments. Purified DNA for assembly can be dissolved in ddH_2O (Milli-Q® water or equivalent is preferable), TE or other dilution buffers.
- *Insert*: When directly assembling fragments into a cloning vector, the concentration of assembly fragments should be at least 2 times higher than the concentration of vector. **For assembly of 4 or more fragments into a vector, we recommend using an equimolar ratio of fragments.**
- *Transformation*: NEB 5-alpha Competent *E. coli* (High Efficiency, NEB #C2987) provided with the NEBuilder HiFi DNA Assembly Cloning Kit are recommended for use for assembled products of less than 20 kb. It is also possible to use other NEB competent *E. coli* strains, with the exception of BL21, BL21(DE3), Lemo21(DE3) and Nico21(DE3). For example, SHuffle® T7 Express Competent *E. coli* can be used for the expression of a difficult to express protein. When using competent *E. coli* from a vendor other than NEB, we have seen decreased robustness of transformation with high-fidelity DNA assembled products.
- *Electroporation*: Electroporation can increase transformation efficiency by several logs. When using the NEBuilder HiFi DNA Assembly Master Mix, use 1 µl of the assembled product for electroporation, and plate multiple dilutions.

Should you require the use of Electrocompetent cells, please use the following protocol:

Electrocompetent Cells Transformation Protocol:

1. Thaw electrocompetent cells on ice.
2. Transfer 50 µl of electrocompetent cells to a pre-chilled electroporation cuvette with 1 mm gap.
3. Add 1 µl of the assembly product to electrocompetent cells.
4. Mix gently by pipetting up and down.
5. Once DNA is added to the cells, electroporation can be carried out immediately. It is not necessary to incubate DNA with cells.
6. Add 950 µl of room-temperature SOC media to the cuvette immediately after electroporation.
7. Place the tube at 37°C for 60 minutes. Shake vigorously (250 rpm) or rotate.
8. Warm selection plates to 37°C.
9. Spread 100 µl of the cells onto the plates.
10. Incubate overnight at 37°C.

- *Biology:* Some DNA structures, including inverted and tandem repeats, are selected against by *E. coli*. Some recombinant proteins are not well tolerated by *E. coli* and can result in poor transformation or small colonies.

Frequently Asked Questions (FAQs):

What are the advantages of this method compared to traditional cloning methods?

NEBuilder HiFi DNA assembly allows insertion of one or more DNA fragments into virtually any position of the linearized vector and does not rely on the presence of restriction sites within a particular sequence. Therefore, the user has complete control over what is assembled and insertion of unwanted additional sequence, often used to facilitate the manipulation of multiple DNA sequences, can be avoided. Furthermore, the NEBuilder HiFi DNA assembly method is fast, relative to standard restriction enzyme-based cloning. Lastly, a greater number of DNA fragments can be joined in a single reaction with greater efficiency than conventional methods.

Are there any differences between NEBuilder HiFi DNA Assembly Master Mix and NEBuilder HiFi DNA Assembly Cloning Kit ?

The NEBuilder HiFi DNA Assembly Master Mix in both products is the same. The NEBuilder HiFi DNA Assembly Cloning Kit includes additional NEB 5-alpha chemically competent *E. coli*.

What is the difference between NEBuilder HiFi DNA Assembly Master Mix/DNA Assembly Cloning Kit and the current Gibson Assembly Master Mix/Cloning kit?

The NEBuilder HiFi DNA Assembly Master Mix utilizes a high-fidelity polymerase. While protocols for these kits are similar, the assembled products from NEBuilder HiFi DNA Assembly Master Mix and NEBuilder HiFi Cloning Kit will typically result in more colonies with higher accuracy. When large DNA (> 10 kb) or multiple fragments (4+) need to be assembled, increasing the overlap region to 30 bp improves the efficiency of assembly and transformation. There are also no licensing fee requirements from NEB with the NEBuilder products.

What is the largest single fragment that has been assembled with NEBuilder HiFi DNA Assembly Master Mix?

NEBuilder HiFi DNA Assembly Master Mix has been used to clone a 12 kb DNA fragment into a 7.4 kb plasmid in *E. coli*, totaling up to 19 kb in length. For assembled products greater than 10 kb, NEB recommends NEB 10-beta Competent *E. coli* (High Efficiency, NEB #C3019) or NEB 10-beta Electrocompetent *E. coli* (NEB #C3020).

How many fragments of DNA can be assembled in one reaction?

The number of DNA segments that can be assembled in one reaction is dependent on the length and sequence of the fragments. NEBuilder HiFi DNA Assembly Master Mix has been used to efficiently assemble up to eleven, 0.4 kb inserts into a vector at one time. However, we recommend the assembly of five or fewer inserts into a vector in one reaction, in order to produce a clone with the correct insert. A strategy involving sequential assembly can be used if all of the fragments cannot be assembled in a single reaction.

Is this method applicable to the assembly of repetitive sequences?

Yes. However, one must ensure that each DNA fragment includes a unique overlap so that the sequences may anneal and are properly arranged. The repetitive sequence can also be internalized in the first stage of a two-stage assembly strategy. If having repetitive sequences at the ends of each fragment is unavoidable, the correct DNA assembly may be produced, albeit at lower efficiency than other, unintended assemblies.

What are the shortest overlaps that can be used with this assembly method?

Productive assembly has been achieved for DNA fragments with as little as a 12 bp overlap, however, it depends on the GC content of the overlap. We recommend using at least 15 bp overlaps, or more, for dsDNA assembly with a Tm ≥ 48°C (AT pair = 2°C and GC pair = 4°C). Increasing the length of overlap between fragments also reduces the amount of DNA needed for assembly.

What are the longest overlaps that can be used with this method?

Both the quantity of 5′ exonuclease in the NEBuilder HiFi DNA Assembly Master Mix and a 15 minute assembly reaction time have been optimized for the assembly of DNA molecules with ≤ 25 bp overlaps. If assembly reaction time is increased to 60 minutes, overlaps up to 40 bp may be used with the NEBuilder HiFi DNA Assembly Master Mix.

Can ≤ 200 bp dsDNA fragments be assembled by this method?

Yes. For optimal results, use these fragments in ≥ 5-fold excess.

Can ssDNA oligonucleotides be assembled with dsDNA fragments?

Yes. However, the optimal concentration of each oligonucleotide should be determined. As a starting point, we recommend using 45 nM of each oligonucleotide that is less than or equal to twelve 60-base oligonucleotides containing 30-base overlaps.

Can longer or shorter incubation times be used?

Yes. For assembling 2–3 fragments, 15 minute incubation times are sufficient. For assembling 4+ fragments, 60 minute incubation times are recommended. Reaction times less than 15 minutes are generally not recommended. Extended incubation times (up to 4 hours) have been shown to improve assembly efficiencies in some cases. Do not incubate the assembly reaction overnight.

Will the reaction work at other temperatures?

The reaction has been optimized at 50°C, but it has been shown to work at temperatures between 40°C and 50°C.

Is it necessary to purify PCR products?

Purification of PCR products is generally not necessary. You can use unpurified PCR products directly, as long as the total volume of PCR products in the reaction is 20% or less. If greater amounts of PCR products are used, a column cleanup kit is sufficient. It is advantageous to gel purify the target DNA fragment if the PCR product is contaminated by either non-specific amplification products, primer-dimers or large quantities of unused PCR primers.

Is it necessary to inactivate restriction enzymes after vector digestion?

Inactivation of restriction endonucleases is generally not necessary, but in some cases it might increase the transformation efficiency. If the insert and final assembled product also carry the restriction site that was used to linearize the vector it is necessary to heat inactivate the restriction enzyme or purify the cut vector if heat inactivation is not possible.

I would like to produce overlapping dsDNA fragments by PCR. Do I need to use PCR primers that have been purified by PAGE or HPLC?

No. Standard, desalted primers can be used.

I would like to assemble ssDNA oligonucleotides into dsDNA fragments. Do I need to use oligonucleotides that have been purified by PAGE or HPLC?

No. Standard, desalted primers may be used.

Can I use a 15-nt overlap that is entirely composed of His-tag repeats (i.e., CACCACCACCACCAC)?

No, you must flank the His-tag sequence on both sides with at least 2 nucleotides that are not part of the His-tag repeating sequence. Alternatively, intersperse CAC and CAT his codons to interrupt this repetitive sequence. You should avoid repeating sequences at the end of an overlap.

Can I PCR amplify the assembled product?

Yes. The assembled DNA molecule is covalently joined and can be PCR amplified. Additionally, if the final product is a closed circular DNA molecule, it can also be used as a template in rolling-circle amplification (RCA).

The control reaction is not resulting in any colonies. Why?

Our testing indicates that the choice of competent cells is critical. We recommend the use of high-efficiency chemically-competent cells such as NEB 5-alpha Competent *E. coli* (High Efficiency) (NEB #C2987).

What should I do if my assembly reaction yields no colonies, a small number of colonies, or clones with the incorrect insert size following transformation into E. coli?

- Assemble and transform the positive control provided with the NEBuilder HiFi DNA Assembly Master Mix/Cloning Kit (see page 11,12). Successful assembly of a positive control will demonstrate that the assembly mixture is functional and the transformation conditions are suitable.
- Analyze the reaction on an agarose gel. An efficient assembly reaction will show assembled products of the correct size and the disappearance of fragments.
- Check the primer design of the overlapping DNA fragments to ensure that there is sufficient overlap to facilitate assembly.
- Consider whether the cloned insert may be toxic to *E. coli* and a low-copy vector, such as a BAC, should be used.

How can I reduce the number of vector-only background colonies?

To significantly reduce the background of unwanted vector-only colonies, the vector should be a PCR product, rather than a restriction fragment. If background continues to be a problem, the PCR amplified vector can be treated with DpnI to remove the template carry-over, if applicable, and extracted from an agarose gel following electrophoresis.

What type of competent cells are suitable for transformation of DNA constructs created using NEBuilder HiFi DNA Assembly Master Mix?

The resulting DNA constructs are compatible with most *E. coli* competent cells. NEB recommends using NEB 5-alpha Competent *E. coli* (High Efficiency, NEB #C2987). If the assembled products are larger than 10 kb, NEB recommends using NEB 10-beta Competent *E. coli* (High Efficiency, NEB #C3019) or NEB 10-beta Electrocompetent *E. coli* (NEB #C3020). If the assembled genes contain repetitive sequences, NEB Stable Competent *E. coli* (NEB #C3040) should be used.

Can I use electroporation instead of chemical transformation?

Yes, electroporation can be used in place of chemical transformation.

Are there any differences between the requirements for 2–3 fragment assemblies versus 4+?

The major differences between the two are the length of overlapping sequences between the adjacent fragments and the incubation time of the assembly reaction. The 15 minute assembly reaction protocol is recommended for assembly of 2–3 fragments that are flanked by 15–25 nt overlaps. The 1 hour assembly protocol is recommended for the assembly of 4+ fragments, flanked by 20–40 nt overlaps. (see the chart on page 12).

Can I use PCR product amplified from Taq DNA polymerase?

Yes. The additional A base at the 3′ end of PCR product will be removed during DNA assembly if it becomes a mismatched residue once fragments anneal.

Troubleshooting

Positive Control Yields No Colonies Following Transformation into *E. coli*

- Be sure to perform a positive control assembly reaction with NEBuilder HiFi DNA Assembly Master Mix, as described on page 11.
- Use the competent cells provided with the cloning kit (NEB 5-alpha, Competent *E. coli*, NEB #C2987). The components of the NEBuilder HiFi DNA Assembly Master Mix may inhibit the functionality of competent cells from other companies.
- Perform the transformation procedure exactly as described on page 12.
- Competent cells may be thawed only once and cannot be repeatedly frozen and thawed without extreme loss in competency. Cells are best thawed on ice and DNA added as soon as the last bit of ice in the tube disappears.
- Do not vortex competent cells. Mix cells and DNA by gently pipetting up and down. Check cell competency by transforming 100 pg of pUC19 plasmid provided with the kit. Expect 1–3 x 10^9 colonies formed/µg DNA after overnight incubation on LB-ampicilin plates at 37°C.

NEBuilder HiFi DNA Assembly Master Mix/DNA Assembly Cloning Kit Reaction Yields No Colonies Following Transformation into *E. coli*

- Assemble and transform the positive control provided with the NEBuilder HiFi DNA Assembly Master Mix/Cloning Kit. Successful assembly of a positive control will demonstrate that the NEBuilder HiFi DNA Assembly Master Mix is functional, and the transformation conditions are suitable.
- Check the primer design of the overlapping DNA fragments to ensure that there is sufficient and correct overlap to facilitate assembly.
- Avoid overlaps with highly palindromic sequences, as they may cause up to a 10-fold reduction in recombinant colonies. When assembling fragments into a multiple cloning site (MCS) of a cloning vector, it is strongly recommended that restriction endonuclease sites be located at the edges of the MCS to avoid overlap regions with highly-palindromic sequences. Plate higher amounts of transformation reaction when using restriction sites that are located in the middle of the MCS of the cloning vector.
- Repeat the NEBuilder HiFi DNA Assembly Master Mix/Cloning Kit reaction using higher concentrations of fragments and/or vector. Make sure that the total volume of PCR-amplified products does not exceed 20% of the high-fidelity DNA assembly reaction. If necessary, purify PCR fragments and/or PCR-amplified vector before the assembly reaction.
- Some DNA structures, including inverted and tandem repeats, are selected against by *E. coli*. Some recombinant proteins are not well tolerated by *E. coli* and can result in poor transformation.

- Test the success of the DNA assembly by performing PCR with primers that flank the assembled product.
- Consider whether the cloned insert may be toxic to *E. coli*, and whether a low-copy vector, such as a BAC, should be used.

NEBuilder HiFi DNA Assembly Master Mix Reaction Yields High Number of Clones with Incorrect Inserts

- Make sure that your PCR product is a single band of the correct size. If the PCR product is contaminated with non-specific bands, it is necessary to gel purify the PCR product to ensure cloning of the correct insert.
- Consider whether the cloned insert may be toxic to *E. coli* and whether a low-copy vector, such as a BAC, should be used.
- Consider using NEB Stable Competent *E. coli* (NEB #C3040) for inserts that contain repetitive sequences.

NEBuilder HiFi DNA Assembly Master Mix Reaction Yields High Number of Small Colonies

- Some recombinant proteins are not well tolerated by *E. coli* and can result in poor transformation efficiency or small colonies. Use a low-copy number vector (i.e., pACYC184) or a vector with tight control of protein expression. When assembling into the pUC19 vector, make sure that your gene is not in frame with *lacZ* alpha fragment.

NEBuilder HiFi DNA Assembly Master Mix Reaction Yields a High Number of Clones without the Insert

- PCR products may carry over large quantities of uncut plasmid template. To remove plasmid template, treat PCR products with DpnI restriction endonuclease before performing high-fidelity DNA assembly. Protocol for DpnI digestion can be found on page 11.
- Restriction enzyme-digested vector may carry over large quantities of uncut plasmid. Some restriction enzymes do not cut supercoiled plasmids to completion. The best way to reduce uncut vector background is to digest the vector with two different restriction endonucleases. If a single enzyme must be used, avoid restriction enzymes that leave four-base single-stranded overhangs rich in C/G (i.e., CCGG overhang). These overhangs may self-anneal to form the transformable form of the vector molecule. Also, increase units and/or incubation time and/or purify the linear vector from an agarose gel.

Appendix A

NEB 5-alpha Competent *E. coli* (High Efficiency, NEB #C2987)

Store at −80°C

Genotype: *fhuA2* Δ*(argF-lacZ)U169 phoA glnV44 f80Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*

CAUTION: This product contains DMSO, a hazardous material. Review the MSDS before handling.

Quality Control Assays

Transformation Efficiency:

100 pg of pUC19 plasmid DNA was used to transform NEB 5-alpha Competent *E. coli* (High Efficiency) following the protocol provided. 1–3 x 10^9 colonies formed/µg after an overnight incubation on LB-ampicillin plates at 37°C.

Untransformed cells were also tested for resistance to phage φ80, a standard test for resistance to phage T1 and sensitivity to ampicillin, chloramphenicol, kanamycin, nitrofurantoin, spectinomycin, streptomycin and tetracycline. The cells were shown to be suitable for blue/white screening by α-complementation of the β-galactosidase gene using pUC19.

Transformation Protocol Variables:

- *Thawing:* Cells are best thawed on ice and DNA added as soon as the last bit of ice in the tube disappears. Cells can also be thawed by hand, but warming above 0°C will dramatically decrease the transformation efficiency.
- *Incubation of DNA with Cells on Ice:* For maximum transformation efficiency, cells and DNA should be incubated together on ice for 30 minutes. Expect a 2-fold loss in transformation efficiency for every 10 minutes this step is shortened.
- *Heat Shock:* Both the temperature and the timing of the heat shock step are important and specific to the transformation volume and vessel. Using the transformation tube provided, 30 seconds at 42°C is optimal.
- *Outgrowth:* Outgrowth at 37°C for 1 hour is best for cell recovery and for expression of antibiotic resistance. Expect a 2-fold loss in transformation efficiency for every 15 minutes this step is shortened. SOC gives 2-fold higher transformation efficiency than LB medium; and incubation with shaking or rotating the tube gives 2-fold higher transformation efficiency than incubation without shaking.
- *Plating:* Selection plates can be used warm or cold, wet or dry, without significantly affecting the transformation efficiency. However, warm, dry plates are easier to spread and allow for the most rapid colony formation.

Antibiotics for Plasmid Selection

Antibiotics	Working Concentration
Ampicillin	100 µg/ml
Carbenicillin	100 µg/ml
Chloramphenicol	33 µg/ml
Kanamycin	30 µg/ml
Streptomycin	25 µg/ml
Tetracycline	15 µg/ml

Ordering Information

PRODUCT	NEB #	SIZE
NEBuilder HiFi DNA Assembly Cloning Kit	E5520S	10 rxns
NEBuilder HiFi DNA Assembly Master Mix	E2621S/L/X	10/50/250 rxns
COMPANION PRODUCTS		
Q5 High-Fidelity DNA Polymerase	M0491S/L	100/500 units
Q5 Hot Start High-Fidelity DNA Polymerase	M0493S/L	100/500 units
Q5 High-Fidelity 2X Master Mix	M0492S/L	100/500 rxns
Q5 Hot Start High-Fidelity 2X Master Mix	M0494S/L	100/500 rxns
NEB 5-alpha Competent *E. coli* (High Efficiency)	C2987I/H	6 x 0.2 ml/ 20 x 0.05 ml
NEB Stable Competent *E. coli* (High Efficiency)	C3040I/H	6 x 0.2 ml/ 20 x 0.05 ml
NEB 10-beta Competent *E. coli* (High Efficiency)	C3019I/H	6 x 0.2 ml/ 20 x 0.05 ml
NEB 10-beta Electrocompetent *E. coli*	C3020K	6 x 0.1 ml
SOC Outgrowth Medium	B9020S	4 x 25 ml

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NEW ENGLAND BioLabs Inc.

Version 1.2 12/15

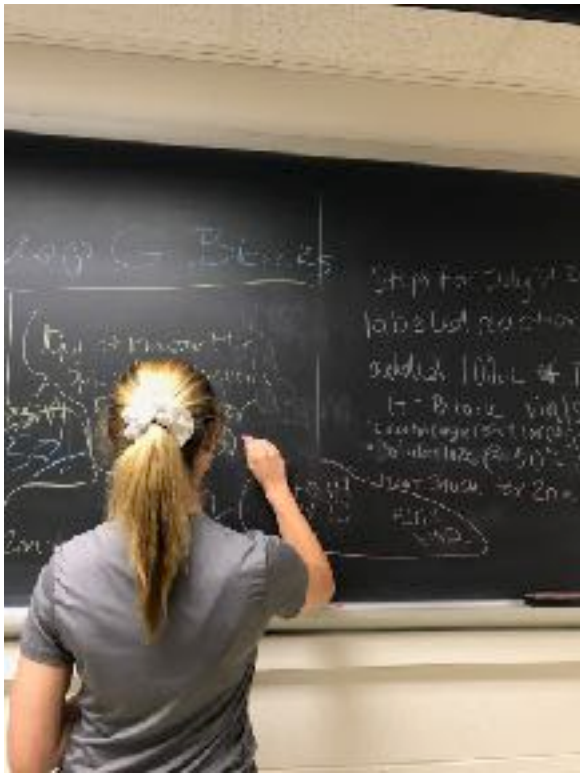
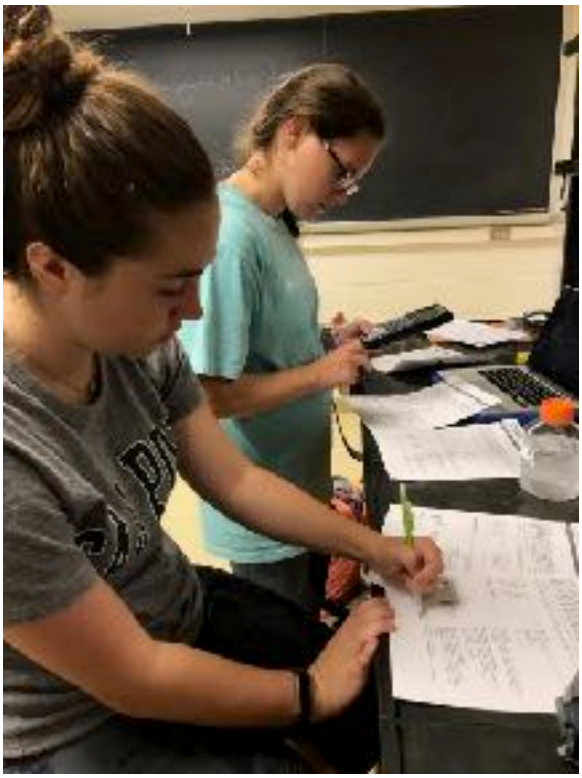


Figure 1: Maddie writes the start and end time on the board for incubation.

Figure 2: Kira works on calculations to know the concentration of bacteriocin to mix with the buffer.



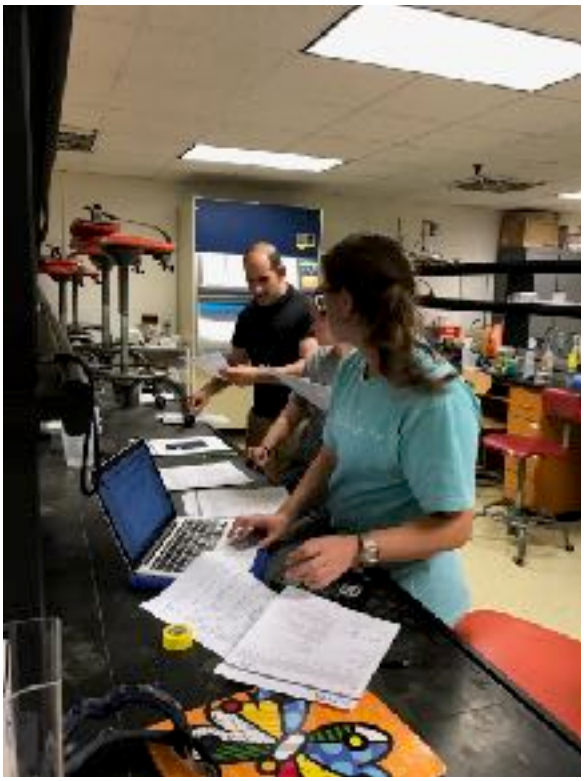


Figure 3 and 4: Team members work side by side to mathematically calculate concentrations while team leader Adam looks on.

August 2, 2017

Today team members met with the team advisors to discuss scheduling for next week. Next we inoculated colonies from Monday's transformations into flasks and tubes containing LB broth.

1. We added the appropriate amount of antibiotics into each flask and each tube. We added 1 uL of antibiotics in each container for every 1 mL of LB broth. The antibiotic solution was 34mg/mL solution of antibiotic and ethanol.
2. We performed the inoculation. We used bunsen burners and sterile plastic loops to transform colonies. We used a sterile loop to take 1 colony from each transformation plate, for each bacteriocin and CvaAB, and put the colony into the tube. Then we used a second loop to take 1 colony from each transformation plate and put the colony into the flask to let more cells grow.

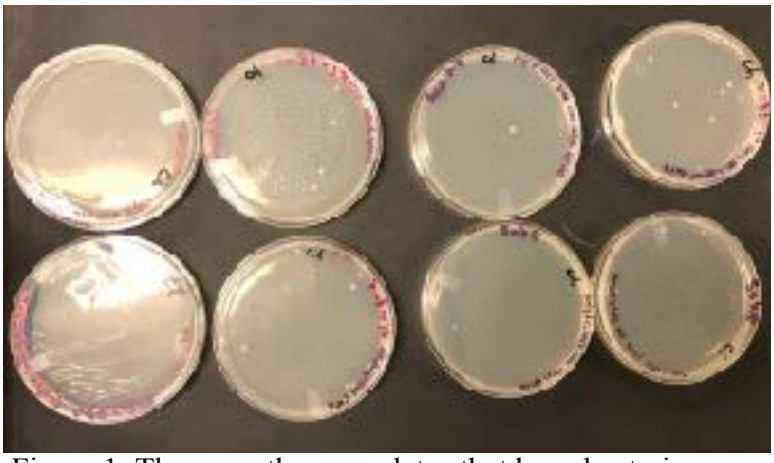


Figure 1: These are the agar plates that have bacteria cultures growing. Notice how none have a red or purple tint.

Figure 2: Team members work on organizing the agar plates that have our bacteria with our bacteria cultures growing on them.

Figure 3: Teammates Madlen, Yianni, and Kira look at the cultures on the plates and count out loud the numbers of cultures per plate which Adam wrote on the board (pictures in Figure 4).

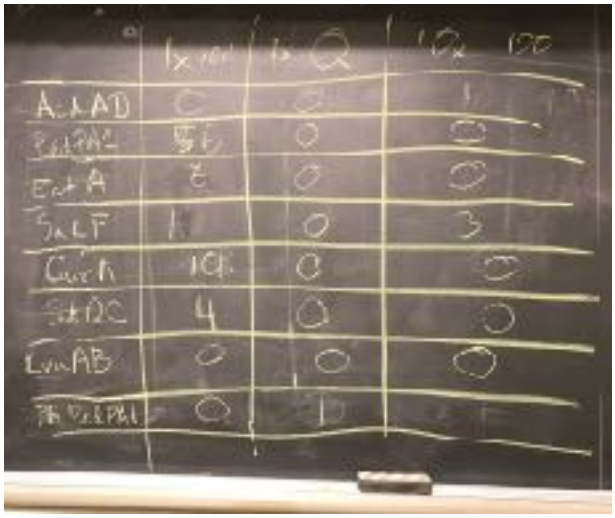


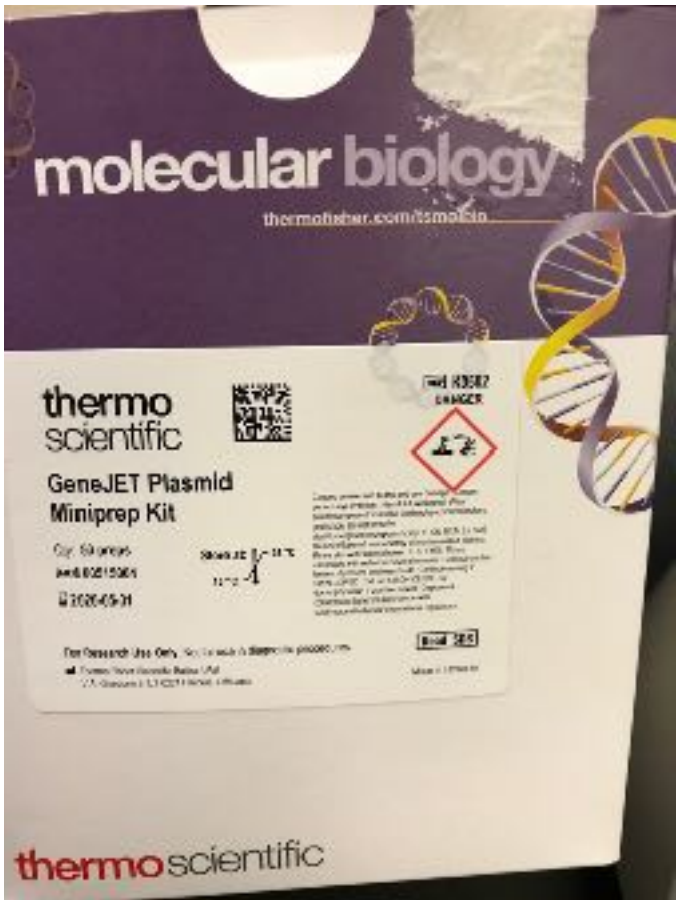
Figure 4: This is the tally of cultures per plate also shown typed on the next page (Figure 5).

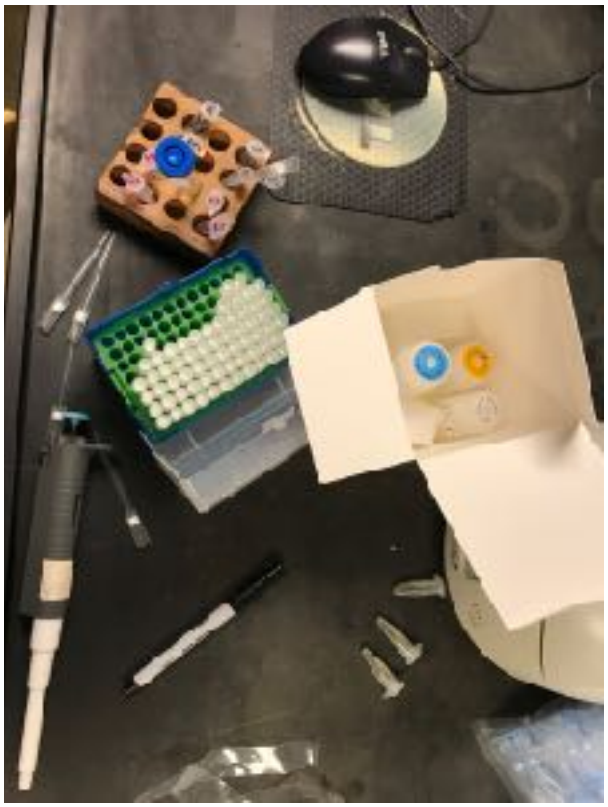
Colony Counts after First Transformation August 2, 2017			
Bacteriocin	Plate		
	1x 100	1x Q	10x 100
AcdAD	0	0	1
PedPA1	6	0	0
Ent A	6	0	0
Sak P	1	0	3
Cur A	10	0	0
Sak QC	4	0	0
CvaAB	0	0	0
PB Ped PA1	0	1	0

Figure 5: Colony Counts Data Table

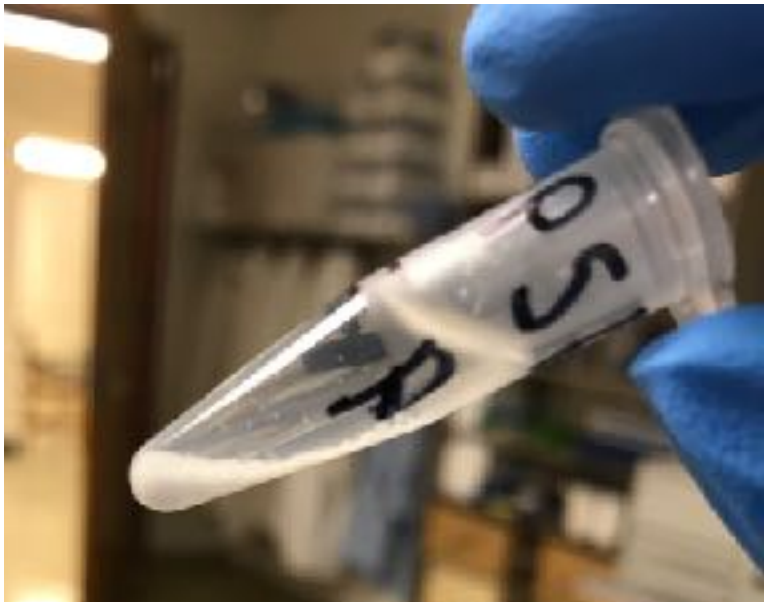
August 3, 2017

Today in the lab we broke up into two groups. Maddie and Sarah used the GeneJET Plasmid Miniprep Kit in order to miniprep four bacteria cultures. They used 2 pellets that had been centrifuged and combined them. Following the protocol for the kit buffer, lysis, and neutralization solution were added.


molecular biology
thermofisher.com/tsmolbio
thermo
scientific
GeneJET Plasmid
Miniprep Kit
thermo scientific



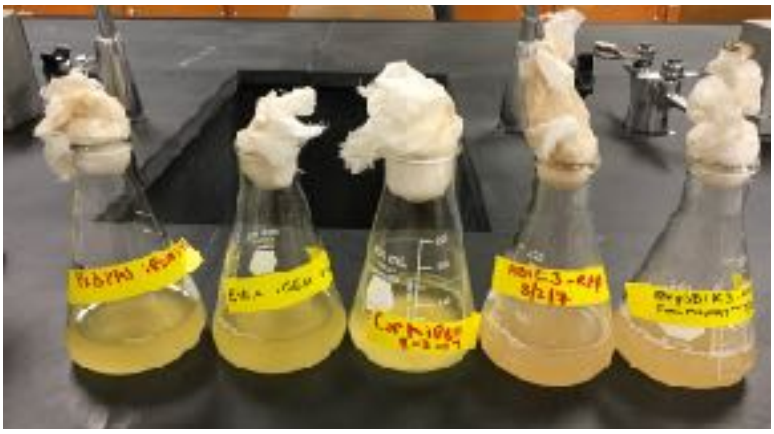

13:16, #002
4.3 ng/mL dsDNA
-0.018 Abs
0.080 Abs
1.90 ng/200
0.045 Abs
0.1808 Abs
08/03/2017
V1.32
CurA
4.2 ng/mL dsDNA
-0.112 Abs
2.08 ng/200
0.141 Abs
0.003 Abs
pSBIC3-RBS
4.0 ng/mL dsDNA
0.080 Abs
1.84 ng/200
0.014 Abs
0.023 Abs

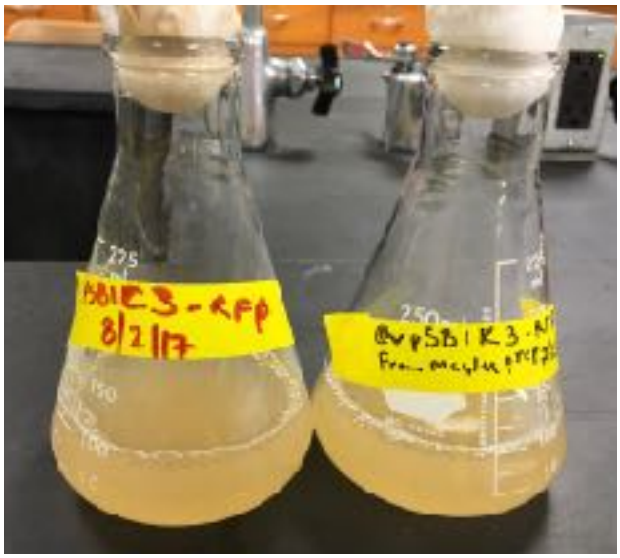


August 8, 2017

Today at 5:30 we put 100 μL of cells from the liquid bacteria culture, that had been incubating at room temperature in the lab for 5 days, into vials containing the antibiotic (5 μL) and 5mL of LB solution. The liquid cultures that we used to inoculate were for cultures of psB1K3, psb1C3, PedPA1, Ent A, and CurA. The cultures that we inoculated from agar plate cultures were SakQC and Sak P because of a labeling error on the flasks (we didn't feel confident in using bacteria from the liquid cultures because of this so instead we used bacteria from the original agar plates).

August 16, 2017





Today we met in Townsend Lobby on south campus to go over medal requirements and fill in our new team member Hannah on the Wiki since she will be our new website designer. Also in attendance of today's meeting was Ian, Adam, Seth, Yianni, Sarah, and Maddie. Sarah presented a short powerpoint to delegate tasks that need to be done in order to move forward. We planned on looking at a new lab space on the 18th.

August 18, 2017

Today Maddie, Sarah, and Yianni met with Mrs. Nemec to look at potential lab space in the Anatomy teaching department. However after further communication with EHS it was determined that the space is not fit for out bacterial strain. Further inquires of potential lab space are being looked into.

August 21, 2017

The RSO meeting went well, and we should be set for the first recognition day which is September 29th. At the meeting, Sarah and Yianni found out that Graduate students aren't really allowed to be in the executive board of RSO's but can participate as non-voting members. In figuring this out, we began talking about how we can better establish ourselves as an organized undergraduate club. So end result is that we want to meet sometime the first week of classes to have an "exec meeting" and start talking about how we want to run the club so it's successful and organized now and in later years. Yianni is going to start a separate doc which will start outlining how we will bring in students and how we can obtain a permanent research space and help from a professor.

August 27, 2017

August 27, 2017

Morris Library

2:00pm-4:20pm

Attendance:

Maddie

Sarah

Adam

Sam

Kira

Phil

Ian

Kelsey

Agenda:

-Main question: Do we have enough to present at the conference?

- Have we met all the medal requirements http://2017.igem.org/Judging/Medals

-Validated part?

-Sam suggests that we may not have a new part but instead we have red fluorescence

-Our gibson assembly pieces have been frozen for 4 weeks

-Money situation: Money for conference vs.

We are using the money we have intelligently

-October 27th is our end all dead line

-What do we need done: lab space and at least 12 hours in the lab over 3 nights

-Why are our colonies not the correct color?

-We need to leave time for error

-We need to present to doctor Wu (show our sustainability)

-show her what we have done so far

-present a budget

-we have committed under grads

-While we are looking for lab space Dr. Biddle has offered us storage space

-Adam talked to Dr. Biddle on Saturday about what we should cover in the presentation for Dr Wu

-We discussed team RSO positions and decided on the following for now:

President: Sarah

Vice President: Kira

Treasurer: Carissa

Historian: Maddie

Secretary: Maddie Temp fill in

Outreach Chair: Yianni

Website Manager: Hannah

PR Chair:

Education Coordinator: Ian

-We may want to add logistics manager in charge of supplies (especially perishable supplies)

Presentation for Dr. Wu discussion:

-we need to show our progress

-we have delegated positions for the RSO based on past experience of participating team members

-include the protocols and kits that we used

-how have we thus far documented out lab time

August 29, 2017

presentation

August 29, 2017

Biology Technology Institute

5:00pm-7:00pm

Attendance:

Maddie

Yanni

Kira

Sarah

Ian

Madlen

Jessica

Carissa

Sam (Grad Leader)

Adam (Grad)

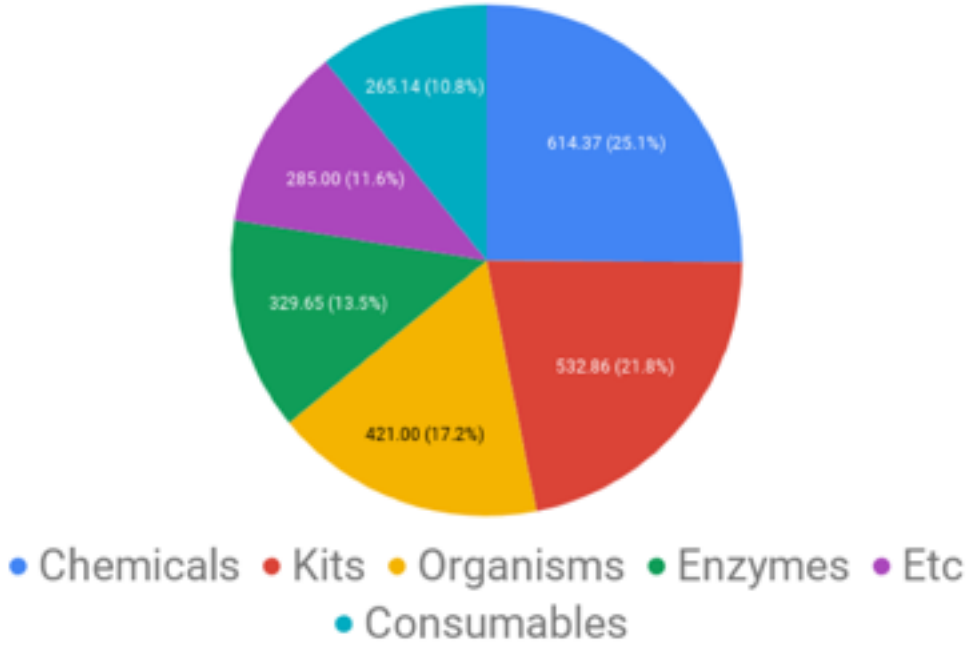
Karen Hoober
Dr. Biddle
Dr. Wu
Agenda/Notes

Project Overview

- Bacteriocins are toxins that bacteria produce to kill other bacteria
- We are engineering E. coli to overexpress bacteriocins
- Our bacteriocins have narrow killing ranges for targeted therapeutic value
- Bacteriocins are typically small peptides
- We chose our bacteriocins based on their narrow killing ranges

Budget

$10,000.00 : Initial Funds
-$4,500.00 : iGEM Registration
-$2,448.92 : Procard Expenses
$3,051.08 : Remaining Funds
$4,000 received from the Biology Department will be used for our project next year



Procedures So Far

- Gibson Assembly
- Transformation
- Inoculation
- Mini-prep

- Other procedures could not be performed due to the fire in McKinley Lab.

Background Knowledge We Have Gained

- Sterile technique
  - Bunsen burner practice
  - Bac-down and gloves
  - Sterile swabbing
- DNA Basics
  - Function of a plasmid
  - Restriction enzymes
  - Using Ugene
- Lab Safety
  - Sharps disposal
  - Waste disposal
  - PPE

iGEM Meetup at UVA

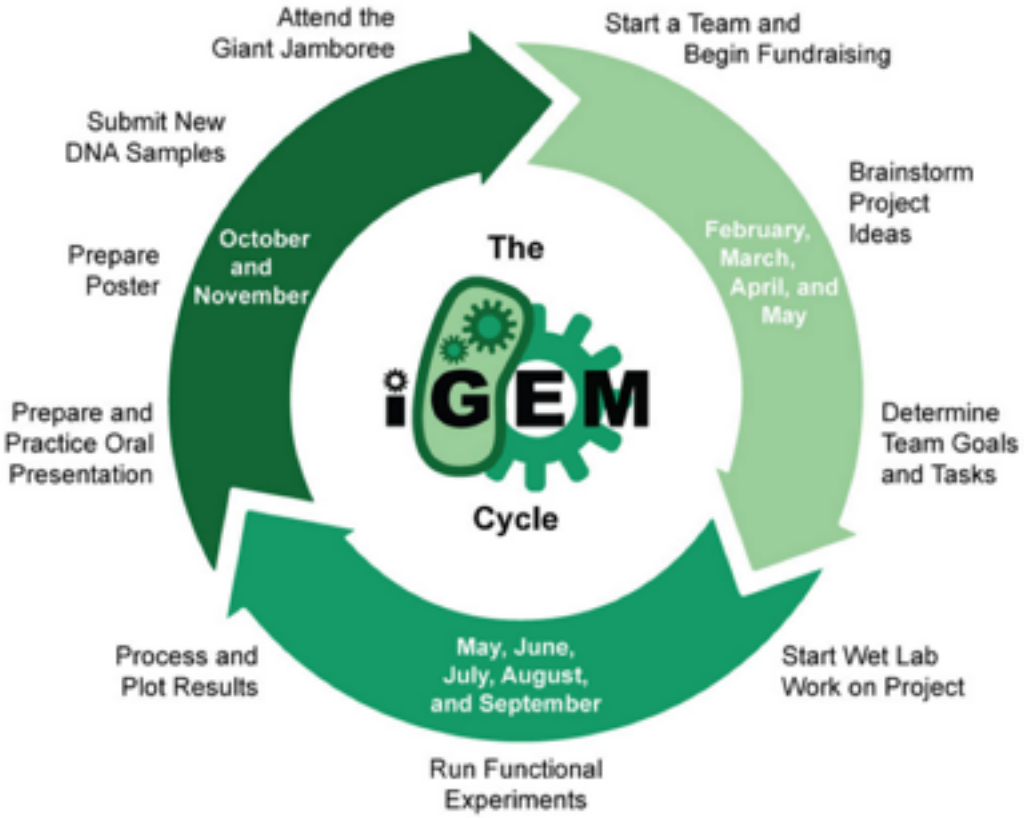
- We presented in front of 6 other teams
- Collaborated with other teams by answering questions and brainstorming ideas about how our project correlates to others
- Learned about Medal Requirements to qualify our team as a participating iGEM team
- Learned more about the opportunities and potential with iGEM
- Spoke with professionals about possibly bringing our projects into industry

Key iGEM Dates

- August 31, 2017 -- Conference Registration -- Pending
- September 1, 2017 -- Abstract Freeze -- Complete
- September 8, 2017 -- Roster Freeze -- Complete
- October 2, 2017 -- Safety Review -- Complete
- Deadline for DNA Submission is October 27th. -- Pending
- Judging Form Freezes October 27th. -- Pending
- Register constructs with iGEM by November 1st. -- Pending
- Wiki Freezes November 1st. -- Pending

Moving Forward

- We have started the process to become a recognized RSO and research team at the University of Delaware.
- 2017 Conference is in November
- Project will continue through the fall and spring in preparation for the 2018 competition



- This fall we will recruit and train new members for following years.

Establishing A Registered Student Organization

- President: Sarah Wenyon
    - Run meetings and handle day-to-day affairs
    - Delegate assignments and manage the team
- Vice President: Kira Dzedzy
    - Serve the role of President if the President is absent, help with delegation and communication
- Historian/Documentarian: Maddie Callahan

    - Maintain the official record of literature, protocols, experimental designs, and results

- Secretary: Kelsey Murray

    - Maintain laboratory calendar and meeting schedule, record minutes and events

- Treasurer: Carissa Walkosak

    - Responsible for all group expenditures and revenues

- Training Coordinator: Ian Pierce

    - Organize protocols and training sessions for new students

- Outreach & PR: Yianni Zerefos

    - Promote the team and our events

    - Organize outreach events and programs

Major Objectives Going Forward

Management

- Forming an RSO

- Student leadership roles

- Goal setting

- Scheduling / Calendar
- Organizing Supplies
- Recruitment

Networking

· Reaching out to our community (flyers, stickers, recruiting new members for next year)
· High school and camp presentation human outreach

Scientific

· Construct validation
· Double Transformation
· Functional Testing
· Product Purification

The Competition

<u>Bronze Medal Requirements</u>
Wiki:

- Document project progress and findings on webspace provided by iGEM
- Team member who can code HTML

Attribution:

- Distinguish between the work completed by undergraduate students, graduate students, and professors

Characterization/Contribution:

- Document new or validate existing DNA parts -- Chromoproteins

Silver Medal Requirements

Validated Part / Validated Contribution:

· Plan to redo mini prep and then run a gel electrophoresis to validate that our cells accepted our plasmid
· Plan to test whether the cells release bacteriocins

Human Practices:

· Urban Promise (week of September 27th)
· The Tatnall School (October 27th)

· We will be teaching younger students so that they have a stronger basic knowledge of STEM topics.

Collaboration:

· Brief conversation with Harvard, but we are currently in the process of contacting other teams

Possible Additional Costs for the Competition

· Conference Registration: $695 per person

- Transportation

- Hotel Costs

Lab Space Needed

- Enough space for 5-7 people is needed.

- Our meetings in the lab would be in the evenings or early morning

Maddie's Added notes:

-DBI outreach program reaches out to local high schools and could help sponsor an outreach trip for us

- At today's meeting we established that Dr. Amy Biddle professor in Animal and Food Sciences is our faculty advisor

- Career innovation Grant

-Dr. Wu mentioned an award that would not only help fund our project but also give us the recognition we need to be publicized in UDaily (she sent us an email about applying for this grant)

-NIBO initiative is a new building project that we can apply for space in for lab space for future years

-We need to coordinate a presentation directly to DBI

-We discussed if it was possible to hold our own iGem meet up at the University of Delaware'

-UD incubator student project HORN program (this is a far in the future goal)

September 5, 2017

September 5, 2017

Morris Library

5:00pm-7:00pm

Attendance:

Maddie

Kira

Sarah

Adam

Ian

Yianni

Kelsey

Emma

Phil

Agenda:

- Make a list of jobs and delegate positions

  list of metal requirements

  - wiki
  - collar with other team
  - outreach
    - politician
    - community
    - presentations

Scheduling meetings

Faculty outreach

Labspace/lab equipment

Recruitment

RSO

Sponsor outreach (thank you notes)

Budget

    conference budgets

Continuous training

New member training

CLC on the UD data base (UD developer)

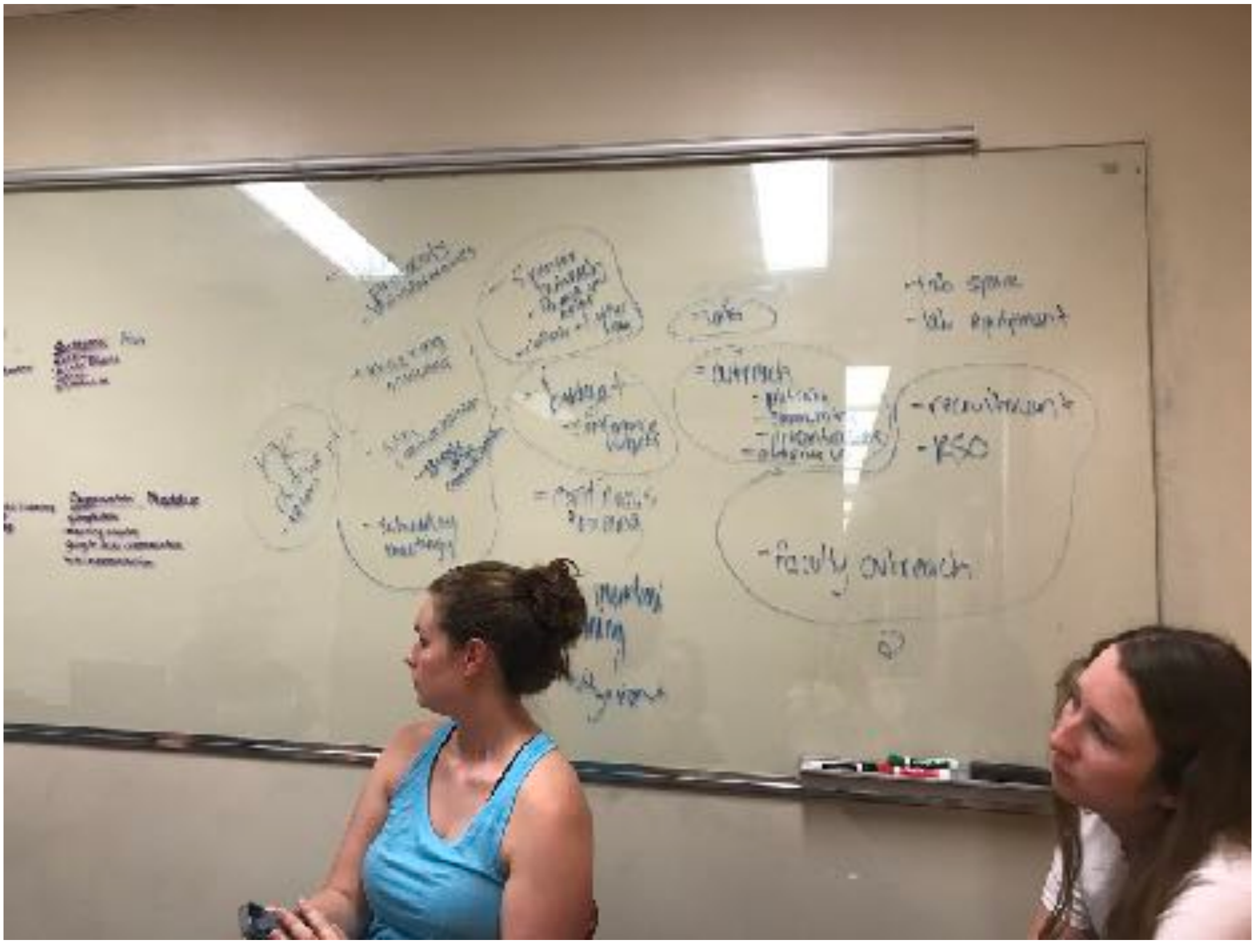
Lab protocols

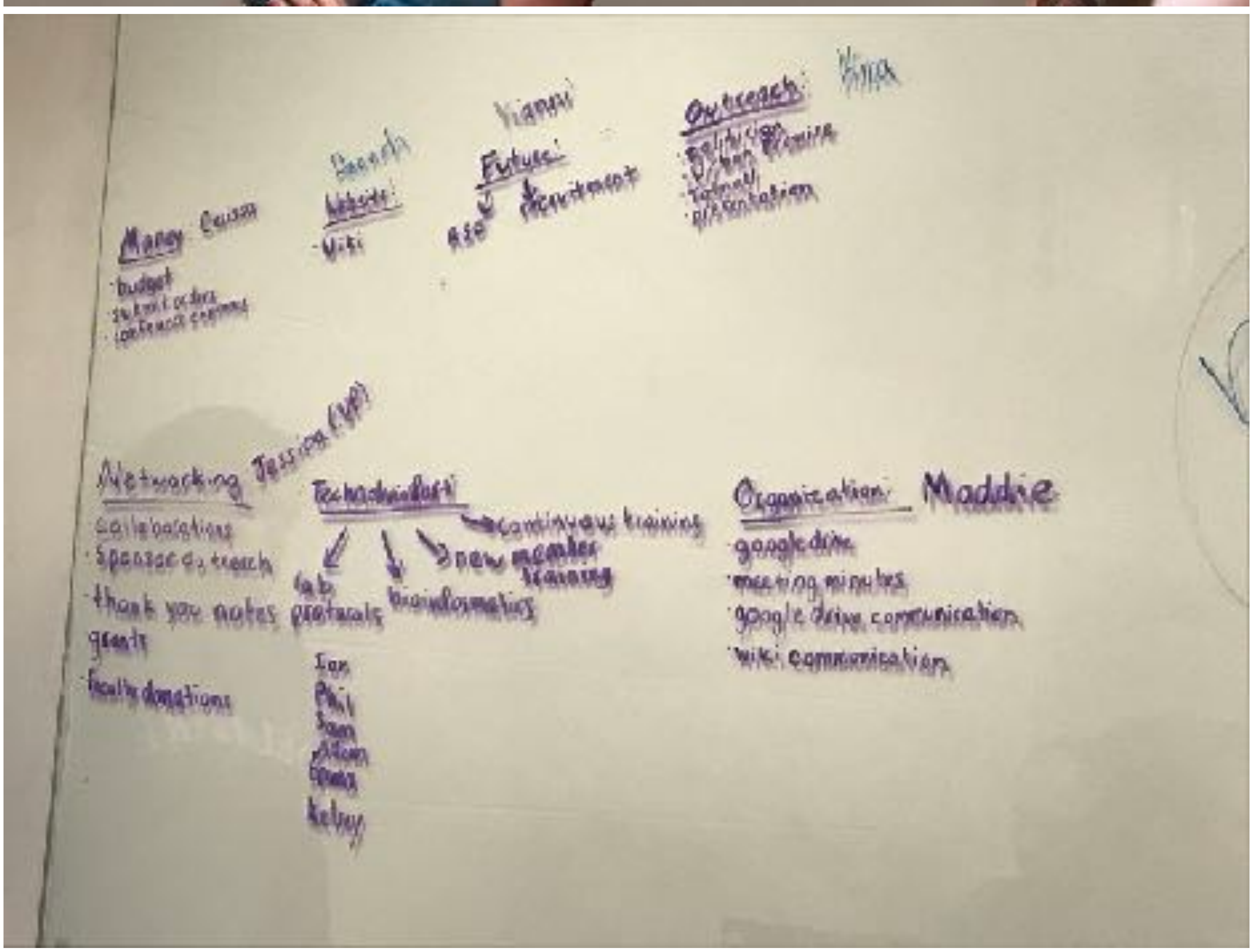
    bioinformatics

Meeting minutes

Review of mins

-MAKE A GOOGLE DRIVE FOR COMMUNICATION


- no space
- lab equipment
= outreach
- recruitment
- RSO
- faculty outreach


Money
· budget
Website:
· Wiki
Future:
RSO
recruitment
Outreach:
· presentation
Networking
· collaborations
· thank you notes
grants
· faculty donations
continuous training
new member training
lab protocols
bioinformatics
Ian
Phil
Sam
Kelsey
Organization
Maddie
· google drive
· meeting minutes
· google drive communication
· wiki communication

September 7, 2017

September 7, 2017

Morris Library

5:00pm-7:00pm

Attendance:

Maddie

Sarah

Sam

Yianni

Ian

Phil

Adam (5:45)

Agenda

What we want to do before we get into our new lab

- get the indicator strain and measure how much bacteria it takes
    - dosage amount (toxicity curve) minimal inhibitory concentration
    - MIC literature—titrate and see what the smallest amount to inhibit growth of bacteria
        - what makes the smallest amount difference
        - can we get liquid stage growth? important for culturing
- Viability of indicator strain?
    - concern with transformed and assembled cells
    - arrived from ATTC

- Ultimate goal: measure bacteria's effect we need a control
- Big challenge— getting method to work
- Lactobacilicius coniferous
- how narrow spectrum bacteriocins vs a broad spectrum antibiotic
- Saliva and testing the micro biome
- we need data to answer the over all question

1. ordering bacteriocin and getting minimum
2. successfully establish

- moving items from Mckinly to DBI
- goal of commercial bacteriocin is to work with our protein product
- engineer cells while working

compote cells from NEB DNA for IDT and Gibson assembly to get info into plasmids

we want to test miniprep and gel

- we need to repeat gibson (another transformation)
- measure DNA: we used biophotometer
    - Q-bit DR. Biddle's dna measuring machine

- Question for Adam: did we save the bacteriocin tubes from gibson assembly day?
- Google doc under gibson assembly
- during gibson assembly we used 5microliters of the bacteriocins
  - the master plates we made may not be viable (no red)
  - can we get DNA from them?

what we need to do?

look at constructs

Over all lab schedule once we get a lab space

- Inoculation
  - over night
- mini prep/ midi? (6hours)
- restriction
  - if we have plasmid… in the mean time gibson assembly of CVAB construct that works with all the bacteriocins that we have designed (about 8hours worth of work)
- 2x transformation—2hours
  - 48 hours over night
- Inoculation

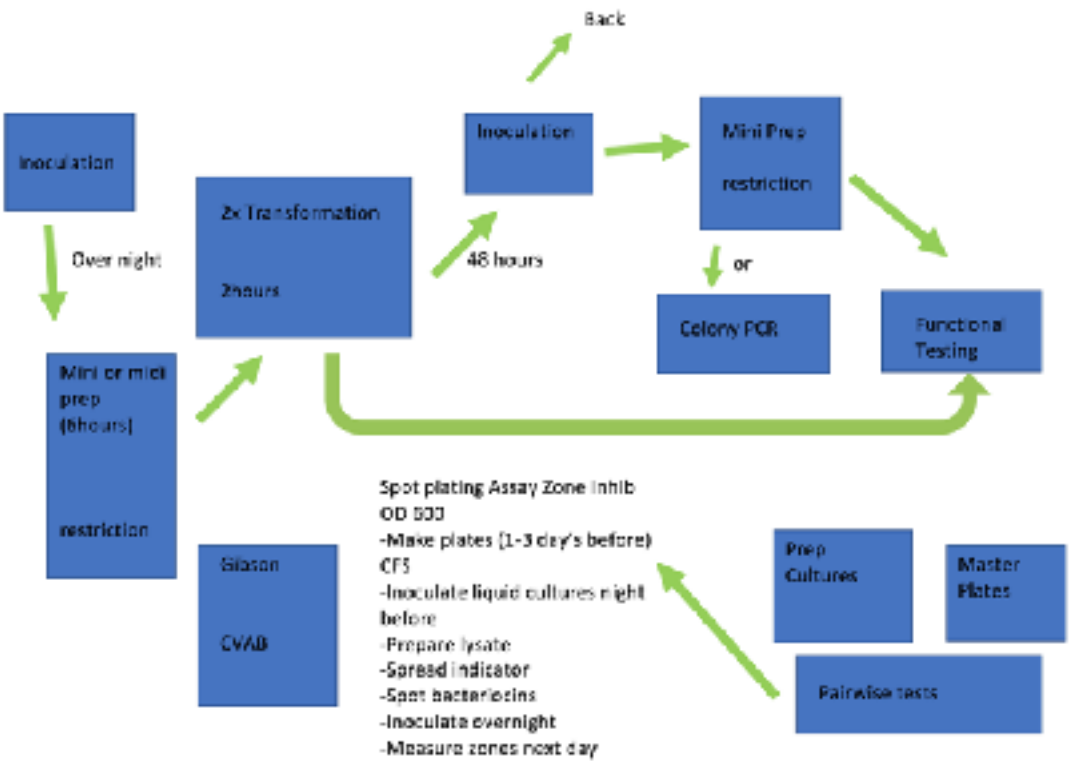
- miniprep/restriction
    - or color PCR
- functional testing (molecular side vs. functional side will be starting ASAP)
- prep cultures
- master plates
- pair wise tests
    - spot plating assay zone inhibit OD 600
    - make plates (1-3 days before CFS)
    - inoculate liquid cultures night before
    - prepare lysate
    - spread indicator
    - spot bacteriocins

if we only have an indicator strain what we are measuring is a titration curve

reach out to Dr. Biddle

BSL1

This is a digital rendition of the explanation that Adam made of our project during the meeting.



September 11, 2017

September 11, 2017

Morris Library

5:00pm-7:00pm

Attendance:

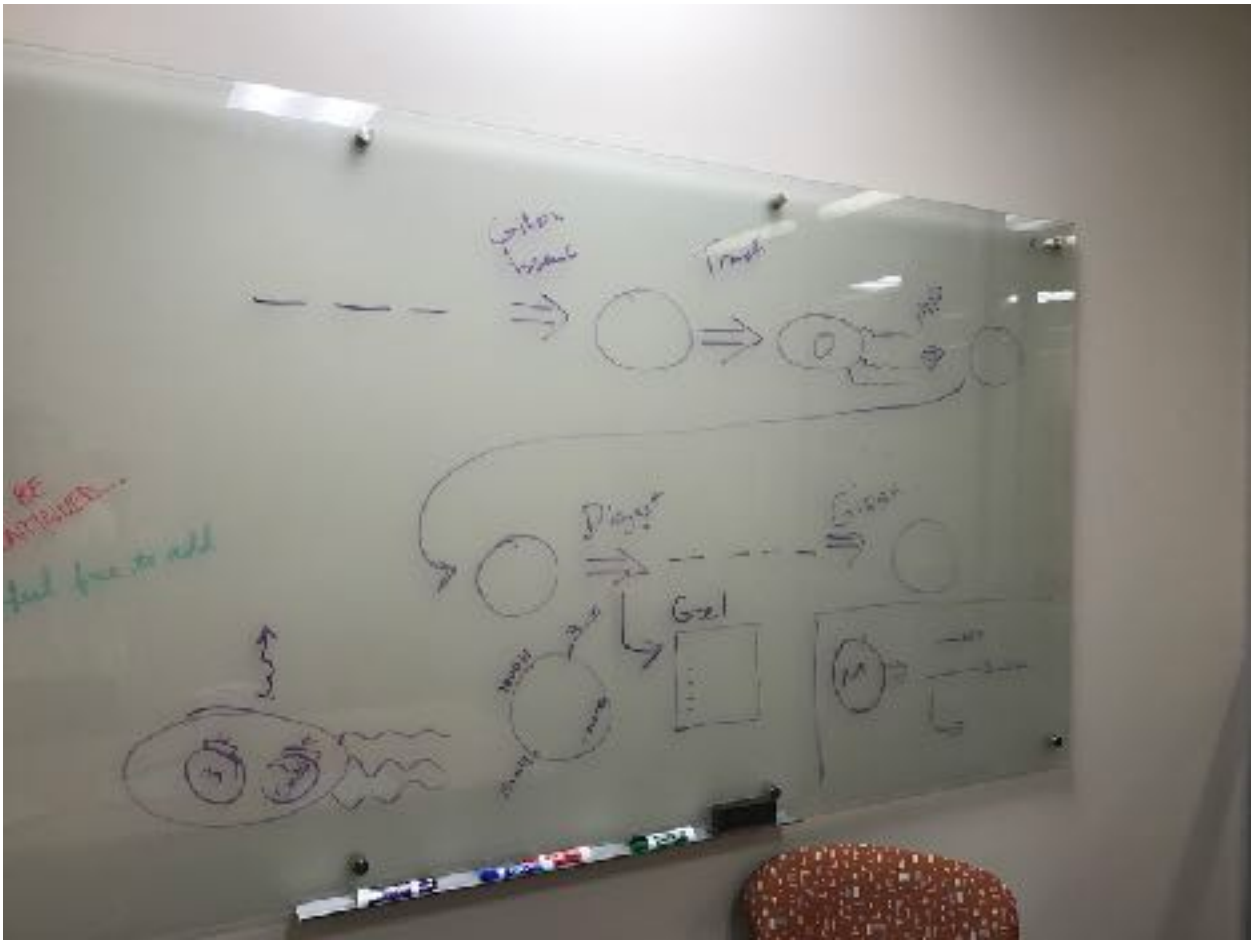
Maddie

Sarah

Adam

Agenda

Together Maddie, Adam, and Sarah discussed the research component of the iGem project. The idea for the meeting was so that Maddie and Sarah could further familiarize themselves and better understand the protocols and processes of the science portion of this year's iGem team.


Digest
Gel