Second Week Observations: Analyzing the Injected *Tribolium castaneum* Specimen

Name: Brooklynne Dilley and Maddy Moe
Date: April 25th and April 27th, 2017
Week #: 13

Objective:
This is the second week after the Tribolium beetle larvae were injected with our plasmid, so this week we are continuing to make observations.

Procedures:
- Make observations about the samples of Tribolium that were injected with our gene.

Notes:
Yoshi made two different concentrations of our plasmid to inject into the Tribolium larvae: 0.5 ug/uL and 1.0 ug/uL, so we have two different samples from which to make observations.
We counted the number of specimen in each sample, and continued to make observations about the morphology of the specimen.

We made the following observations.

0.5 ug/uL	1.0 ug/uL
<u>Tuesday, April 25</u> 4 survived to adulthood, they probably won't survive more than a couple days Wings are shriveled in most, one has normal-ish characteristics. We believe this normal-looking specimen escaped the RNAi treatment since he is displaying no signs of abnormal phenotype. The other specimen are affected in similar ways: ● Tibia in leg should be 5x the length of the last four segments but it's about the same length ● All segments in legs are shortened and thicker around than normal ● Last four leg segments are fused together ● Dents in T1 segment of the body ● Holes of ventral side near the base of	<u>Tuesday, April 25</u> No new observations, they had not developed into adulthood.

the legs (associated with the wings, similar tissue) ● Antenna fused and the segments are smaller, we count four small segments + two large segments (should be 6 and 3) ● Wings are shriveled up and about half normal size ● No discoloration ● Wings are not organized, they cannot fold back in place ● Both elytra and hindwing are shriveled and affected	
<u>Thursday, April 27</u> ● Took pictures of elytra and wings next to wild type Tribolium. ● Elytra and hindwings were decreased in length	<u>Thursday, April 27</u> No new observations, they had not developed into adulthood.

Conclusion:
SUCCESS, the gene was definitely successful and there are several noticeable changes in the phenotypes of these specimen, throughout their entire bodies. Originally we just expected changes in the wings and legs but it appears that every appendage was affected, and even sections that we didn't imagine would be affiliated with wing/leg development are being affected.

Next Step:
Continue making observations as the samples grow and reach adulthood. We hope to dissect some of the specimen and mount the wings so we can make more observations about the differences in phenotype.

First Week Observations: Analyzing the Injected *Tribolium castaneum* Specimen

Name: Brooklynne Dilley and Maddy Moe
Date: April 18th and April 20th, 2017
Week #: 13

Objective:
This is the first week after the Tribolium beetle larvae were injected with our plasmid, so this week we are just making preliminary observations.

Procedures:

- Obtain the samples of Tribolium that were injected with our gene,
- Count the specimen
- Make observations.

Notes:
Yoshi made two different concentrations of our plasmid to inject into the Tribolium larvae: 0.5 ug/uL and 1.0 ug/uL, so we have two different samples from which to make observations.
We counted the number of specimen in each sample, and made observations such as:

- Development and growth
- How do they compare to the control Tribolium?
- Body segmentation
- Wing size
- Leg size/segments
- Appearance of the eyes

We made the following observations.

0.5 ug/uL	1.0 ug/uL
<u>Tuesday, April 18</u> 15 injected 8 in larvae stage (one deceased), 7 in pupae stage 13 body segments in larvae (comparable to control) In pupae, the wings appeared to be shriveled and much smaller (Yoshi observed that they appeared to be about half the size they should be)	<u>Tuesday, April 18</u> 9 injected All in larvae stage, all were alive 13 body segments in larvae (comparable to control) All were moving and lively Looked just like the control larvae, no discernable differences

In pupae, the legs were a bit shorter and located more towards the lateral side, rather than medial (middle) The pupae appeared to be otherwise pretty normal, the only discernable differences from the control Tribolium was the wing shape/size and the leg placement/size, as predicted One pupae is beginning to advance to its adult stage, we observed brown coloration around both proximal and distal ends (as opposed to the normal milky-white coloration across the entire pupae body)	
<u>Thursday, April 20</u> 8 larvae, 7 pupae Still no adults Last week there were 5 body segments between the bottom of the wings and the bottom of the body, this week there are 4.5 or 4 segments, so the wings might be growing or even different sizes between the different pupae One pupae has strange brown growth on one wing ??? Leg shortened, appendages messed up, we don't think he will survive, wing messed up somehow 2 larvae definitely dead, 3 larvae maybe dead or dying One pupae still looks brown on both ends as on Tuesday, we can't find the wings or at least they're very shrivelled and small 6 larvae living, 3 looking bad (almost dead)	<u>Thursday, April 20</u> Nothing new to observe, still in larvae stage 9 larvae, 2 looking dead or almost dead (not moving at touch)

Conclusion:
It appears that our plasmid was injected and integrated into the Tribolium's bodies successfully, there are already some noticeable differences when comparing them to the control Tribolium. It's a bit concerning that one larvae appears to be dead, but we believe that the remaining specimen will survive to adulthood so we can make more observations about the effects of this gene.

Next Step:
Continue making observations as the samples grow and reach adulthood.

Control Specimen: Making Observations about *Tribolium castaneum*

Name: Brooklynne Dilley and Maddy Moe
Date: April 11th and April 13th, 2017
Week #: 12

Objective: This week we aim to make observations about normal *Tribolium castaneum* so we can later compare our injected specimen to the control. This will allow us to observe how our plasmid affects the Tribolium, and therefore observe the effects of knocking out our gene.

Procedures:
This week we observed normal Tribolium castaneum under a microscope and made observations about them. We took several pictures and made some anatomical drawings as well.

Note:
We were given several samples of *Tribolium castaneum* in various stages of life. We looked at them under a microscope and made several observations about their morphology, including:

- Size and shape of the wings, legs, eyes, etc.
- Number of segments in body, legs, antennae, etc.
- General size, shape, proportions
- Direction of wings, antennae, and other appendages

We also took several pictures for later reference to compare to the Tribolium samples that will contain our plasmid. This will allow us to determine the exact effects of knocking out our gene.

Some Tribolium specimen were submerged in an ethanol solution temporarily to knock them out (or kill them, if left long enough) so we could successfully take pictures without the specimen moving around on the slide. It was very difficult taking pictures of moving specimen, but submerging the adults in ethanol allowed us to obtain several images that will serve us well later for comparison.

Conclusion:
There are no conclusions to be reached from this week, we simply made observations about the control specimen of *Tribolium castaneum* so we could reference the data at a later time.

Next Step:
David will inject new Tribolium larvae with our plasmid, and we will make observations about their growth over time, as well as any effects our gene might have had in the wings, legs, or general body morphology.

dsRNA of Dachsous

Name: Brooklynne Dilley and Maddy Moe
Date: April 4th and April 6th, 2017
Week #: 11

Objective:
This week we complete the last step to make dsRNA, which will later be inserted into the larval stage *Tribolium castaneum*.

Procedures:
- In vitro transcription
- Concentrate dsRNA

Note:
In order to convert our DNA into dsRNA we used the following procedure:

1. For in vitro transcript we mixed
    a. NTP mix : 8ul
    b. 10x buffer: 2 ul
    c. Enzyme mix : 2 ul
    d. Template: 8 ul (1-1.5 ug)
    e. RNase H2O: 0 ul
    f. ----------
    20ul
2. To determine amount of template to add
    a. Calculated: 196.86 ng/uL (measured)=> 0.19686 ug/uL x 8 = 1.57488
3. Mix solution, spin briefly
4. Put entire 500 uL sample in filter cartridge
5. Centrifuge at 13 krpm for 2 minutes
6. Use 500 uL of wash solution then centrifuge again
7. Use remaining 500 uL of wash solution then centrifuge again
8. After second wash centrifuge one more time without adding any more wash before proceeding
9. Add 60 uL HOT elution solution to the filter then centrifuge immediately for 2 minutes at 13 krpm

This will result in dsRNA for dachsous, which will then be inserted into the larval *Tribolium castaneum*.

Conclusion:
We successfully made dachsous dsRNA.

Next Step:
Inject dsRNA into larva.

Analyzing the Sequence

Name: Brooklynne Dilley and Maddy Moe
Date: March 28th and March 30th, 2017
Week #: 10

Objective:
This week we worked to compare our known sequence (from the first few weeks, obtained using FlyBase and Primer Blast) to the sequence that was obtained over spring break, by a company that sequenced our samples.

Procedures:
- Obtain the sequencing results from the company that sequenced our fragments over the break
- Use finchtv to analyze the sequence, looking for any discrepancies or errors
- Directly compare the results to our expected sequence, and also determine the directionality
- Perform another mini-prep to purify the PCR products
- Measure the concentration of DNA in the sample

Notes:
March 28, 2017
At this point in the lab, we know that our primer should be inserted into the plasmid, but we are not sure which direction the primer runs in the plasmid, so we need to determine this. To do this, we compare the sequences (one obtained through FlyBase/Primer Blast, one obtained through a separate company (name of the company not provided by instructor) over spring break) using this website to compare them. We also downloaded a program called finchtv to directly view the sequencing results that were sent back to us over spring break. From the sequencing results sent back to us, we noticed a few points of insertion in which a new base appeared in the middle of the sequence, but upon viewing the results using finchtv we determined that these points were an error of the program interpreting the peaks incorrectly. It appears that our sequence was successfully inserted, and in the forward direction. We know the direction because the forward primer came first in the sequence; if the reverse primer had come first then we would need to flip it back, but this step was not necessary.
Now that the fragment is sequenced, we want to make dsRNA but we need RNA Polymerase. For this, we need the promoter site, which isn't currently on our fragment. Our fragment has the T7 promoter sequence on both ends so we can use this DNA as a template to make transcription from both directions.
Now the plasmid must be diluted to a concentration of 10 ng/uL. Originally, our DNA sample had a concentration of 265.74 ng/uL when it was sent back from the sequencing company.
Next, we set up a solution using a mixture of:
- 46 uL ddH2O

- 20 uL 5x Buffer
- 8 uL dNTP
- 15 uL PCR4-TOPO
- 10 uL plasmid (10 ng/uL concentration)
- 1 uL Taq Polymerase

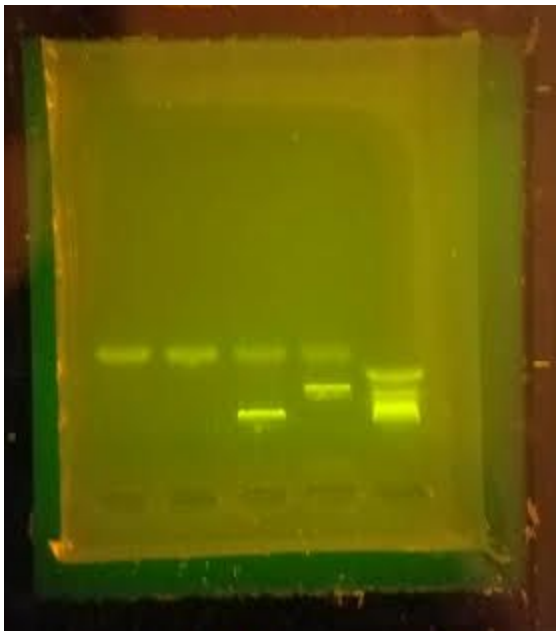
This mixture was used to run a PCR to ensure our fragment is the expected length (original fragment size + 20 + 20, because each primer is 20 bp long).

March 30, 2017

The size of the PCR products was estimated to be about 797 bp long (fragment size + 20 + 20). First the sample was mini-prepped using the following instructions:

1. We have 98 uL of PCR product to play with, so we use 490 uL of Buffer PB to the PCR reaction and mix (5:1 ratio)
2. Vacuum the tube
3. Add 750 uL Buffer PE while vacuuming
4. Centrifuge for one minute (after placing in clean 1.5 uL microcentrifuge tube)
5. Add 30 uL Buffer EB to the center of the white disc
6. Centrifuge for one minute (after placing in clean 1.5 uL microcentrifuge tube)
7. Measure the concentration of DNA

We measured a concentration of 196.86 ng/uL, which is favorable.
At this point, we performed another gel electrophoresis, and expected to see that our sample was about 800 bp long. Here are the results from the electrophoresis:



Our sample is the one in the middle, and you can see that it appears to be about 800 bp long, which was expected.

Conclusion:
It appears that our fragment was successfully inserted into the plasmid without error, so we can proceed with the experiment.

Next Step:
Make dsRNA for dachsous, convert this DNA to dsRNA.

Performing the first Miniprep

Name: Brooklynne Dilley and Maddy Moe
Date: March 14th, 2017
Week #: 8

Objective:
This week we will be mini-prepping our samples, preparing them for a PCR. After the PCR we will compare the results to our known data so we can ensure that our plasmid was taken up by the samples properly.

Procedures:
- Perform miniprep on our E. coli samples (see below for procedure)

Notes:
All we did this week was miniprep. This includes the following steps:
1. Pellet the E. coli samples by centrifuging the tubes at 12 krpm for 2 minutes
2. Remove LB, then add 250 uL of P1 Buffer, vortex well
3. Add xx P2 Buffer (high alkaline buffer) VERY gently, turn slowly to mix (this will destroy xx)
4. Wait 3 minutes
5. Add 350 uL N3 Buffer to neutralize the solution, the contents of the tube will now look like fluffy white fibers floating around (this is the DNA we want)
6. Put on ice for 5 minutes
7. Centrifuge at 12 krpm for 10 minutes (E. coli are now dead, hopefully the lipids and genome are destroyed as well while the plasmids are intact, the plasmids are in the solution)
8. Add 75 uL supernatant to blue column with white disc
9. Vacuum out any excess liquids
10. Add 500 uL PE Buffer (contains 95% ethanol)
11. Vacuum out any excess liquids
12. Column to clean 1.5 uL tube
13. Let sit for one minutes then centrifuge again at 12 krpm for one minute

Conclusion:
No conclusions can be drawn here, we are just prepping our samples for another PCR.

Next step:
Prepare our sample further for perform PCR, compare the results to our known data and sequence.

Culturing E. coli Samples with Plasmid Containing our DNA Fragment, and Performing a Colony PCR

Name: Brooklynne Dilley & Maddy Moe
Date: March 7th and March 9th, 2017
Week #: 7

Objective:
Take samples of four colonies and prepare the PCR solution, perform a PCR comparing the DNA in our colonies to the primers (F2 and R2) to ensure that our colonies were successfully integrated with our primer 2. Select colony based on the best PCR result.

Procedures: (bullet point the procedures)

- Add 100 uL of LB into a 1.5 mL tube
- Place 15 ul of LB into 4 separate tubes
- Take a medium sized pipet tip and scrap off a large colony from the agar plate
- Tap the tip of the pipet tip into one of the tubes containing 15uL of LB
- Tap tube to mix
- Do this again for the following three tubes using different colonies each time
- Culture at 37 degrees for 1 hour
- Setup Colony PCR (see notes)
- Choose the colony that is closest to our expected results

Notes:
Last week, we made plates of E. coli that were supposed to contain the plasmid with our primer (#2) integrated into it. They were cultured for a week, and this week we identified the remaining colonies as colonies containing the plasmid with our primer and the gene for antibiotic resistance (all other E. coli should have been killed off by the antibiotic).

We obtained 100 uL of LB (liquid agar solution for culturing E. coli) and split it into 4 tubes, each containing 15 uL. We then scraped off four colonies of E. coli from our plate, being careful to choose colonies that were isolated so we did not accidentally scrape off any E. coli that don't contain plasmid, but survived the antibiotic due to antibiotic resistant ring around E.coli with plasmids. We placed each colony into one of the four tubes (labeled C1, C2, C3, C4 with our initials) and cultured them for an hour at 37C with the LB + ampr solution. This allows for our cultures to multiply before we perform the PCR.

Meanwhile, we set up the PCR solution that contained the following ingredients:

- 41 uL of H2O
- 20 uL of the 5x GoTaq Buffer

- 8 uL of dNTP mix
- 10 uL of primer F2
- 10 uL of primer R2
    - Mix and spin down well
- 1 uL of Taq polymerase
    - Mix and spin again but not too long because could cause denaturing

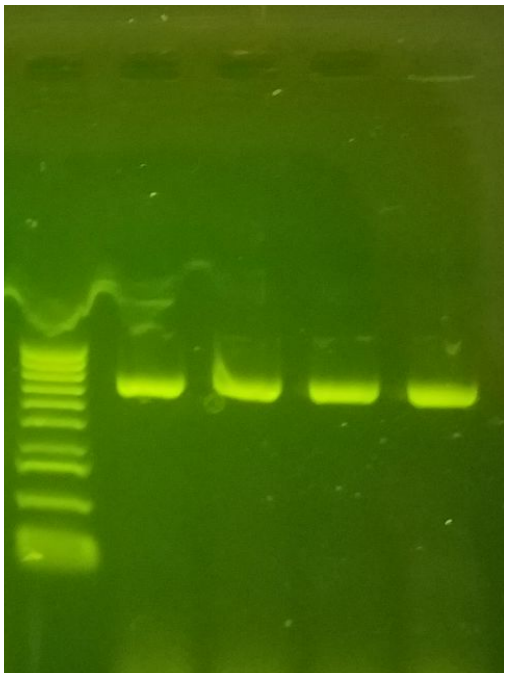
After combining these ingredients and culturing the colonies for an hour, we placed 18 uL of this solution into 4 tubes, then added 2 uL of the respective culture to each tube (i.e. 2 uL of C1 to the C1 PCR mix, etc.). In the end, we had 4 tubes containing 20 uL of colony PCR solution, each containing a different culture of E. coli that has the plasmid containing our primer.

A PCR was performed running each colony against a 100 bp ladder.

Conclusion:
Below is a picture of the colony PCR that was run. It's a little difficult to determine the size of the DNA in our colonies because it's a large sample, so it's closer to the + edge of the gel, where the ladder is more condensed. For clearer results a lower concentrated gel would need to be used. However, do to time and resources a 1.5% agar gel.

In the gel below, the wells are ordered as follows:

1. 100 bp ladder
2. Colony 1
3. Colony 2
4. Colony 3
5. Colony 4



After reviewing the results, we decided to go with colony 3, because it appeared to be the most centered and the gel is the clearest.

Next step:
After we choose the colony we want to use, next we will culture it so we can continue to use the strain of E. coli for the next steps.

Preparing the PCR Reaction

Name: Brooklynne Dilley and Maddy Moe
Date: February 28th and March 2nd, 2017
Week #: 6

Objective:
Prepare and perform PCR to localize and amplify desired gene. Perform TOPO TA cloning to selectively choose the plasmid with the DNA fragment of interest.

Procedures:
- Determine amount of water needed to make a 100 umol/1L solution with our primers
- Determine amount of the 100 umol/L solution needed to achieve a 100 uL of 10uM solution (ratio of 1:9 to achieve 100 uL). These are the primer solutions used in the PCR reaction
- Set PCR reaction (see notes)
- Place remaining primer solutions and buffer in freezer
- Provide Dr. Yoshi with the PCR reaction solutions (F1R1 & F2R2) for PCR to be performed
- "Freeze N Squeeze" the longest DNA fragment from the PCR
    - Isolate the section of the gel containing the DNA
    - Freeze
    - Centrifuge the sample to isolate the DNA
- Combine 4uL PCR product + 1 uL saltwater + 1 uL Topoisomerase plasmid
- Freeze for 15 mins
- Inject 3uL of the reaction into the *E. coli* culture
- Heat culture at 42 C for 30 sec
- Put culture on ice for 5 mins
- Add SOC 250 uL to the cells
- Heat at 37 C for 40 mins on a shake plate
- Plate 120 uL, scrape around the plate
- Incubate overnight

Notes:
<u>February 28, 2017</u>
To find out the volume of water to mix our primers with to achieve 100 uM of solution, we used this equation:
(1/100 umol)(amount given, nmol)(mol/10^9 nmol)(10^6 umol/mol)(1000 mL/L)

We calculated the following volumes for each sample:
F1: Given 26.5 nmol, use 265 uL water
R1: Given 34.7 nmol, use 347 uL water

F2: Given 27.0 nmol, use 270 uL water
R2: Given 26.8 nmol, use 268 uL water

After this we had to dilute the 100uM solutions to achieve 100 uL of 10 uM of each primer. To do this we used a ratio of 10:90 (10 uL of 10uM primer solution:90uL water). These primer solutions of concentration 10 uM were used for the remainder of the PCR setup.

For the remainder of this procedure, we made sure to keep the components on ice as we were mixing them to help foster the reaction.

To set up the PCR, we needed the following materials (list courtesy of Yoshi):

- 59.5 uL water
- 20 uL of the 5x GoTaq Buffer
- 8 uL dNTP
- 4 uL cDNA
- 0.5 uL Taq Polymerase

We combined all of these except the Taq Polymerase enzyme, then vortexed them well and spun them down. We then added the enzyme then briefly vortexed and spun down (careful not to denature the enzyme). This left us with 92 uL of solution that contains the enzyme but not the primer.

We then split the solution in half to achieve two identical solutions of 46 uL, because we will be performing two PCRs: one containing the F1/R1 primers, and one containing the F2/R2 primers.

To the first solution (labeled F1R1), we added 2 uL of each primer (F1 and R1) bringing the solution to 50 uL.
To the second solution (labeled F2R2), we added 2 uL of each primer (F2 and R2) bringing the solution to 50 uL.

We then transferred each solution (the full amount, 50 uL of each) to separate smaller capsules to perform the PCR. Our capsules are labeled MB1 and MB2.

The PCR will be performed later, today we just prepared the solutions.

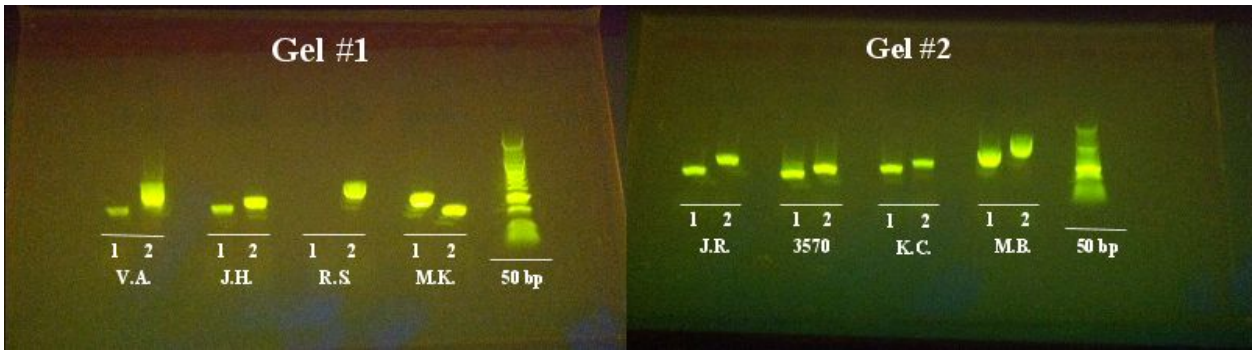
PCR program:
95C x 5 min
35 cycles:

- 95C x 30 s Denaturation
- 57C x 30 s Annealing
    - This depends on the melting temperatures of our primers
- 72C x 35 s Extension

72C x 5 min Addition extension step

Hold at 4C.

Yoshi performed a PCR on our samples during this time, the results are shown below.

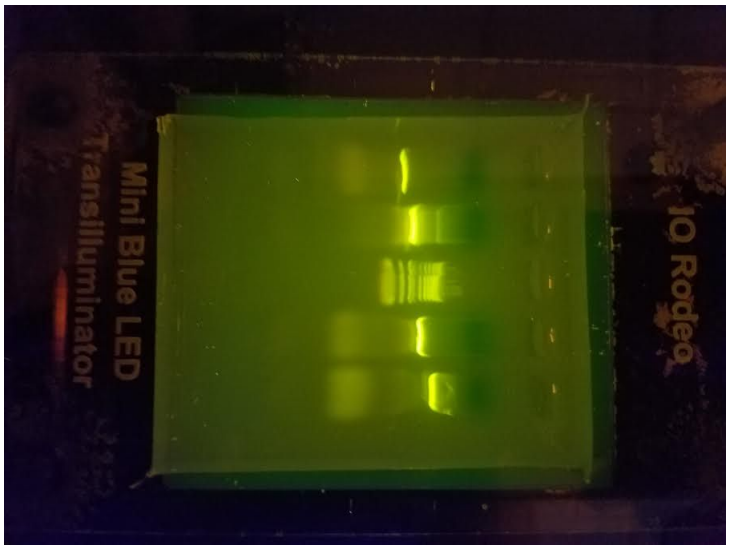


Our samples are labeled MB on the far right. You can see that both of our samples are viable, and they are distinctly different so it should be easy to choose a sample to use later.

March 2, 2017

The first thing that was done today was the preparation of the 1.5% agarose gel by another team. From here, we set up the PCRs in the wells by pouring the TBE into the box and placing the gel in the TBE solution.

We ran the following gel:



This is the order of the samples being run:

1. VA1

2. VA2
3. 50 bp ladder
4. MB1
5. MB2

From here we can see that one sample is distinctly longer than the other sample (one sample traveled further on the tray, indicating that it's a shorter length). We isolated the fragment that was the longest, **MB2,** (about 750 bp) by using the Freeze N Squeeze equipment: The first piece of equipment was designed to take out a portion of the gel that contains the DNA fragment. From here, we put the sample into the second piece of equipment, a specialized tube that contained a filter. We froze the tube for about 5 minutes.

After freezing, we placed the tube in the centrifuge, which "squeezed" (separated) the DNA sample from the agarose gel. The gel is capable of freezing more quickly than the DNA, so the filter in the tube captured the frozen agarose gel, while the DNA was allowed to travel through the filter into the body of the tube.

We discarded the filter, then put the tube containing the DNA on ice.
After we isolated the DNA, we combined it with salt water and Topo plasmid:

- 4 uL PCR product
- 1 uL salt water
- 1 uL Topo plasmid

We combined these materials in a new tube, then capped it and froze it for 15 mins.
Place E.coli on ice and limit amount of time off ice because fragile. Place 3 uL of reaction in E.coli cells then heat shock it at 42 C for 30 seconds. Then, place it on ice for 5 minutes. After the 5 minutes add 250 uL of SOC to the reaction.

Place this in the incubator shaker for 40 minutes. Take 120 uL of reaction and place it on plate. Then plate the plate on rotator disc and use hockey stick to plate it. Lastly, turn upside down and incubate overnight. The only E. coli to survive will contain the plasmid containing our gene.

Conclusion:
This week,using PCR, we were able to amplify our desired gene. By amplifying the gene we were able to perform TOPO TA cloning to selectively choose the plasmid with our DNA fragment of interest (Dachsous.)

Next step:
The next steps are to mini prep the plasmid (use that as a template) and perform PCR to make DNA fragment with T7 on both ends.

The Gel Electrophoresis

Name: Brooklynne Dilley and Maddy Moe
Date: February 23, 2017
Week #: 5

Objective: Learn how to run a gel electrophoresis and analyze the results

Procedures:

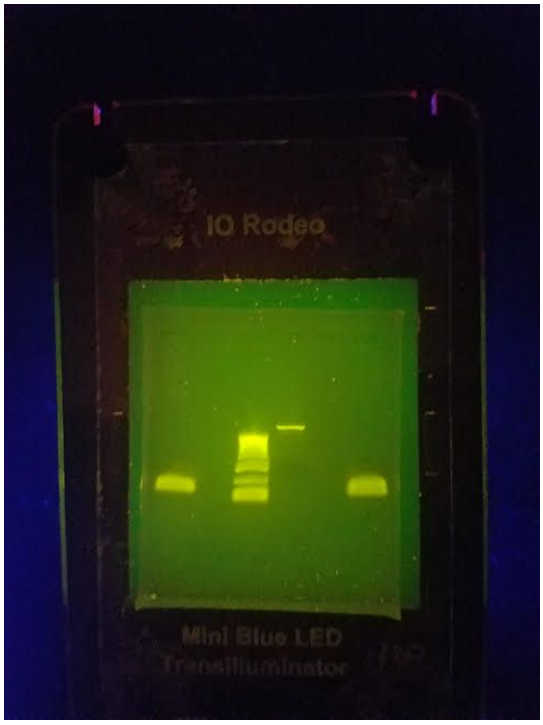
- Learn the components that go into the gel and how to calculate the gel concentration (%)
- Learn the procedures for running the gel
- Run a sample gel to understand how it works

Notes:

We mixed an agarose gel with 1.2% concentration using 1.2g agarose and 100 mL of water. It was microwaved on power level 30% for 2 minutes to help it solidify, and we added some time to achieve a boiling solution. Once it was boiling we took it out and shook it up a bit, then microwaved on the same setting until it boiled. This was repeated until it appeared to be fully mixed. It was poured into the wells, about ⅔ full. We waited until it solidified, then poured TBE without Gel Green into the electrophoresis box. The machine was turned to 100 V. Water was added to the box, being careful not to spill the water or the gel. A pipette was used to mix the DNA with the loading dye so that we can directly see the DNA. We combined 4 uL DNA with 1 uL dye to obtain a 5 uL sample. Next, DNA was added to the wells, being careful to add it to the right side of the gel. Because DNA is negatively charged, it was added to the negative side of well so it would travel to the positive side.

We used DNA sample C for this portion of the experiment. It was loaded next to a 100 bp ladder, and the results are shown below.

Our sample is the third sample, directly adjacent to the 100bp ladder.



Conclusion:
Today we learned how to run a gel electrophoresis, which will be used several times during this lab.

Next step:
We will begin the microbiology portion of this experiment, and work to designing our primers.