Argos* RNAi in *Tribolium castaneum
Experimental Study Notebook
Jessica Krandel & Hannah Abner

Date: 4/25 & 4/27
Week#: 13
Objective: (in one or two sentences)
Continue observing changes in beetles and recording changes via notes and pictures.

Procedures: (bullet point the procedures)
Observe beetles under the microscope and gfp scope. Record observations.

Note:
4/25
Some of the pupae have moved into the adult stage. We have 5 beetles alive in the adult stage. We have 1 beetle dead in the pupae stage.
The pupae appears to have lots of wing damage (most dead/decayed tissue is in the wing area as of today). Phenotype = lethal.
Many of the beetles have died. We did not observe any phenotypic irregularities in the eyes or wings of the surviving adult beetles (5 beetles). We put the remaining beetles (1 pupae, 5 adults) in ethanol for preservation and further analysis. Our study could benefit from sectional analysis to determine if there are irregularities in eye structure apart from the outward appearance of the ommatidia.

4/27
We looked at the adult beetles again (dead in Ethanol). No notable irregularities (compared to wild type).
For final report, talk about death seen in first week or two and discuss possibilities for those that survived to adulthood without any irregular morphology seen - maybe escaped the RNAi or small chance that the argos RNAi has effect other than morphological. Also, small chance that RNAi of argos enhanced the EGFR signals but not enough to have notable difference in phenotype. Other research has been done where EGFR was knocked out - resulted in smaller eyes (Tomoyasu, person to person communication) Since we did not see morphological changes when we knocked out argos, this suggests that enhancing the signal of EGFR does not have as great of an effect as knocking out EGFR considering other researchers have seen smaller eyes as result of doing so.

Conclusion:
Not much difference. Looks relatively normal. Questioning whether the RNAi worked on the adult beetles.

Final counts of mortality:
Injection concentrations: 0.5ug/uL and 1ug/uL
Number injected:

- 0.5 ug/uL - 15 organisms total injected
    - 6 pupae
    - 9 larvae

- 1.0 ug/uL - 10 organisms total injected
    - 0 pupae
    - 10 larvae

Survival:

- **(**4/18)
    - 0.5 ug/uL
        - 1 pupae dead, 5 pupae alive
        - 6 larvae dead, 3 larvae alive
    - 1.0 ug/uL
        - 0 pupae
        - larvae dead
- (4/20)
    - 0.5 ug/uL
        - 0 pupae dead
        - 6 larvae dead, 3 larvae alive
    - 1ug/uL
        - Still 0 pupae present
        - 6 larvae dead, 3 larvae alive, 1 larvae in pre-pupae stage
- (4/25)
    - 0.5 ug/ul
        - 1 pupae alive
        - 5 adults alive
    - 1.0 ug/ul
        - 0 pupae
        - All larvae (10) dead
- (4/27)
    - 0.5 ug/ul
        - 0 pupae alive
        - 5 adults alive
    - 1.0 ug/ul
        - 0 larvae alive
        - 0 pupae
        - 0 adults

Next step:
Write final report and present final results.

Date: 4/18 & 4/20
Week#: 12
Objective: (in one or two sentences)
Observe differences seen in injected beetles.

Procedures: (bullet point the procedures)
Check the beetles under the microscope and note changes

Note:
The beetles are mostly in the larvae stage this week so we will need to continue checking likely into next week.

Our beetles were injected with two concentrations:
1 microgram - 10 beetles
0.5 microgram - 15 beetles

We have 6 pupae this week (both Tues. and Thurs.). All 6 are from the 0.5 ug/ul injection. 2 of the 6 have abnormal wing structure and appear to be dying.
From the 0.5 ug/ul, as of Thursday there are 6 dead larvae and 3 alive larvae.
From the 1.0 ug/ul, as of Thursday there are 4 dead larvae, 2 on the last leg that look like they are about to die, 1 larvae that is pre-pupae stage, 3 larvae alive

On Tuesday we took a gfp scope photo of the ommatidia of one of the pupae. It looked slightly abnormal.

On Thursday, I noticed that 2 of the 6 pupae have one wing that is abnormal. I'm unsure if this is due to physical damage or due to the RNAi. It could be due to physical damage because the other 4 appear to have normal wing structure.
There is a gfp scope photo of ommatidia of a pupae from Thursday as well.

Conclusion:
There are abnormalities in the ommatidia and wing structure. Ommatidia seem to be spaced apart more than usual in multiple pupae and wings appear damaged in two of the pupae.

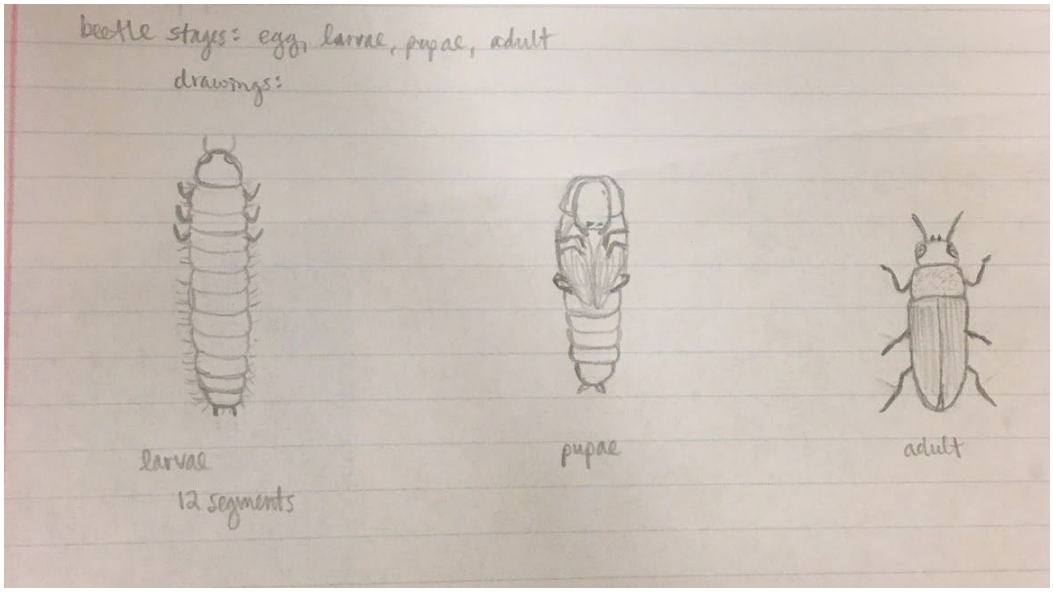
Next step:
Continue observing changes in the injected beetles.

Date: 4/11 & 4/13
Week#: 11
Objective: (in one or two sentences)
Injection (done by David and Yoshi)
Observe and take note of wild type Tribolium in 3 stages of development.

Procedures: (bullet point the procedures)
Use microscope to look at beetles at the larva, pupa and adult stages.

Note:
Used a microscope to observe the three stages of beetles and drew pictures of what we saw for all three stages.

Conclusion:
Some mortality observed. No apparent abnormalities observed, yet.



Next step:
Observe beetles that have been injected with dsRNA

Date: 4/4 & 4/6
Week#: 10
Objective: (in one or two sentences)
Do PCR purification (at beginning of lab) and then in vitro transcription. Then, do DNase treatment and dsRNA purification.

Procedures: (bullet point the procedures)
See last week for PCR purification procedure.
4/4
In Vitro Transcription

- Figure out how many ul of template is needed to get to 1-1.5 ug/ul concentration of template in solution
- To make in vitro transcription solution:
    - NTP mix 8 ul
    - 10x buffer 2 ul
    - Enzyme mix 2 ul
    - Template 8 ul (calculated to be highest conc up to 1.5)

    - Rnase free H2O 0 ul (add what's needed to get to 20 ul)
    - Total: 20 ul

4/6

RNase Reaction

- Put together the RNase reaction:
    - dsRNA 20 ul
    - Nucelase-free water 21 ul
    - 10x digestion buffer 5 ul
    - DNase I 2 ul
    - RNase 2 ul
- Incubate the reaction at 37 degrees C for 45 min

Purification of dsRNA

- Put together the dsRNA binding mix:
    - dsRNA 50 ul
    - 10x binding buffer 50 ul
    - Nuclease-free water 150 ul
    - Ethanol 250 ul
- Put all 500 ul of the binding mix into the filter and centrifuge - 13000 for 2 min
    - Then dump the flow through
- Wash the filter with 500 ul of wash solution, twice
    - Centrifuge after each wash
- Centrifuge again to remove any leftover liquid
- Apply 60 ul of heated elution solution to the filter
- Centrifuge again for 2 minutes
- Take 1 ul and mix with 19 ul of H2O
- Nano drop the dilution
- Elute again with 100 ul of heated elution solution and centrifuge

Note:

4/4

4/6

-use pre-heated elution buffer
-don't repeat and use 60ul elution buffer
-we actually did a 1:4 dilution

Conclusion:

4/4

The re-do of PCR mixture was successful. The PCR purification gave a product with a concentration of 133.4 ng/ul (good!)

4/6

Next step:
Injection of dsRNA

Date: 3/28 and 3/30
Week#: 9
Objective: (in one or two sentences)
Do mini prep
Analyze sequence and set up PCR. Run gel on Thursday.
Run gel for PCR product to confirm that PCR worked. If it worked, proceed to purification.

Procedures: (bullet point the procedures)

- Sanger sequencing done by company.
- Analyze the sequence (use form).

PCR

- ddH2O 40ul
- 5x buffer 20ul
- dNTP 8ul
- PCR4-Topo 15ul
- Plasmid 10ul
- Taq 1ul
- Need to dilute plasmid: 136.55 ng/ul → 10 ng/ul (20 ul)
    - Need 1.46 ul of plasmid to make 20 ul of 10 ng/ul solution
    - Double the solution since our micropipettes do not go down to 0.37 ul so make solution with 1.46 ul plasmid and 18.54 ul water.

Running the Gel

- Make a 1.5% gel: 40 ml + agarose
- Combine 4 tubes of 25 ul each from PCR to one tube of 100 ul total
- Vortex and spin down the 100 ul of PCR product
- Run 2 ul on the gel, use other 98 ul for PCR purification

PCR Purification

- Add PB buffer to the PCR reaction product (5:1)
- Put the mixture in a column (with filter)
- Centrifuge for 30-60 seconds or apply vacuum
- Wash with PE once and centrifuge 30-60s
- Centrifuge again for 1 min to remove wash buffer
- Place column in clean 1.5 ml in tube
- Add 30ul EB to membrane
    - (NOT 50ul like protocol says)

Note:
Sanger Sequencing of DNA
Read the gel from top to bottom to get the DNA sequence (Top- 3')

Use termination of the PCR reaction and a gel to read the sequence.
Our analysis of the DNA sequence shows no mutations in the plasmid sequence.
We analyzed it using the form provided (see filled out form in folder)
Do PCR with the T7; then, we will be able to make the RNA
*Mix non-enzyme components, mix, spin down, then add enzyme
Our PCR product is in the last slot (5) on gel 1
*PCR product = dsDNA, GOI with T7 tails

Conclusion:
Our PCR product did not show up on the gel so, we need to repeat the plasmid dilution and re-make the PCR mixture.

Next step:
PCR purification

Date: 3/14 & 3/16
Week#: 8
Objective: (in one or two sentences)
Do mini prep

Procedures: (bullet point the procedures)

- Mini prep - harvest the plasmids after killing the *E.coli*
    - Use centrifuge at high speed (pellet the *E.coli*)
    - Remove the culture medium (LB)
    - Add 250 uL P1 (PB)
    - Add 250 uL P2 buffer (high in alkaline, destroys membrane)
    - 3 minutes
    - Add N3 350 ul (add slowly)
    - On ice - 5 minutes
    - Spin down/centrifuge 12k rpm for 10 minutes
    - Add 750 ul supernatant to column
    - Vacuum
    - Add 500 ul PE
    - Vacuum again
    - Column to a clean 1.5 uL tube
    - Add 30uL of EB
    - Let sit 1 min and spin 12k rpm for 1 min
- Send plasmid to company for sequencing

Note:
E.coli - has genome, lipids, proteins

Measure time of P2 buffer precisely because it can destruct other parts of *E.coli* if left too long (we only want to disrupt the membrane). Don't shake either!
Use mini prep kit (has instructions)
Pipette 2 mL into small tube, spin down (12k rpm for 2 minutes), add 2 mL more, spin down
Accidentally added PE instead of PB to *E.coli*
**Re-do: pipette the 1 mL of culture left and spin down
Discard the culture soln. (keep the pellet)
Then, add the buffers (P1, P2, 3 min then N3)
Turn container upside down a few times to mix the N3 and then put on ice for 5 min
In this solution, we want the supernatant (not the pellets)
**Actually, P1 is different than PB… we should have added P1 but everyone added PB
When adding EB make sure you add it to the middle of the column where the white product is
After mini prep supposed to have a bell curve on from the NanoDrop printout

Conclusion:

Next step: After spring break, annotate the sequence and use as template to make dsRNA

Name: Jess and Hannah
Date: 3/7 & 3/9
Week#: 7
Objective: (in one or two sentences)
Pick up the colonies of *E.coli* with plasmids from plates and run PCR. Then, use product to run a gel.

Procedures: (bullet point the procedures)

- Colony PCR
    - Pick a colony and culture for about an hour
    - Set up PCR

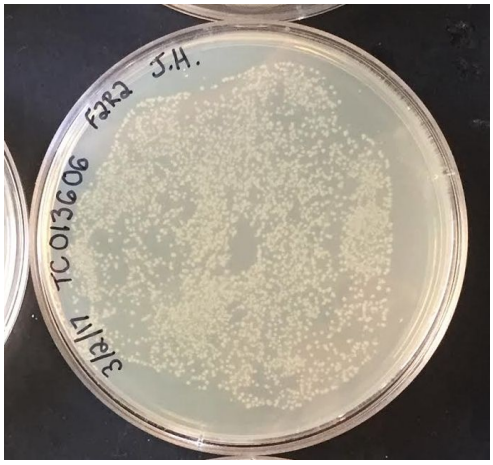
1. Add 15ul of LB+ampr to each of the four tubes
    a. 100ul LB into 1.5 ml tube
2. Use pipettes to touch colony and dip into the tubes, then immediately close (do in each of the four tubes)
3. Incubate in 37 degrees celsius for an hour
4. Run gel
    a. Gel: 1.5%, 75 mL TBE with 1.125 g agarose
        i. 75mL x .015 = 1.125g
    b. Run 2 uL of each colony (1, 2, 3, 4)
5. Examine gel under the light and find sizes of samples using 100 bp ladder.
    a. Compare these sizes to the predicted size (predicted is based on primers used)

“Today’s Recipe” - Colony PCR

H2O		8.2 uL	(41 uL total)
5x Green Buffer		4 uL	(20 uL)
dNTP mix		1.6 uL	(8 uL)
Primer 1 (T3); forward	2 uL	(10 uL)	
Primer 2 (T7); reverse	2 uL	(10 uL)	
Taq		0.2 uL	(1 uL)
Culture (in place of DNA)		2 uL	(8 uL)
Total		20 uL	(100 uL)

First, mix everything **except the culture**
Then split into four tubes each with 18ul, then add 2ul of culture to each tube
**always start with the biggest volume and pipette smaller into the bigger, always pipette in and out a few times, then vortex/spin down

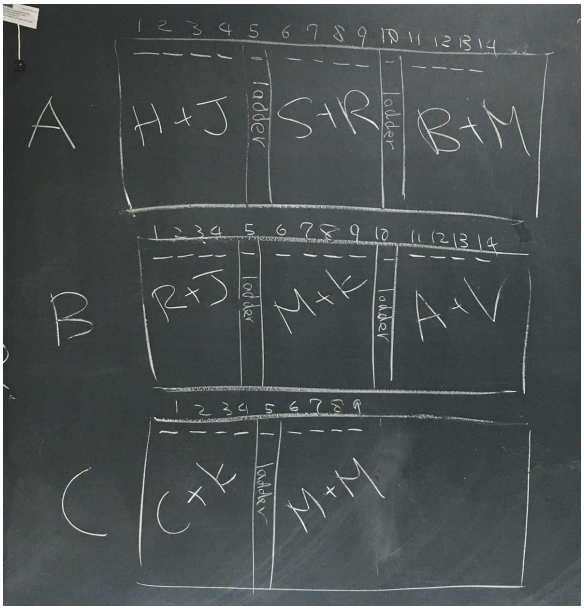
Note:
3/7



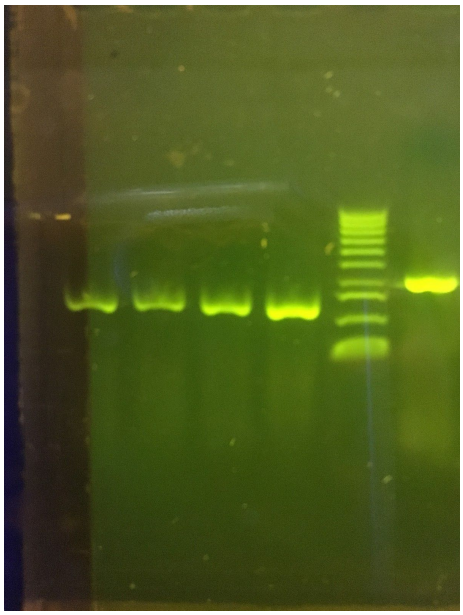
E.coli colonies on plate with ampicillin.

3/9
Look at gel to see if it has the band of predicted size based on our primers/gene of interest. We need to make sure that we picked the right colonies (colonies with the plasmid that has our gene). It’s difficult to be super accurate when predicting length of our PCR amplified region so keep this in mind. Predictions will be relative. Size should “match” actual size if we picked the right colonies.

1. What are the primers you used?
    a. F2, R2
2. What is the length of your PCR amplified fragment?
    a. Prediction: about 306 bp
    b. Actual: about 290 bp (overall)
    (estimated from gel)



Our samples were placed in gel A (box “H + J”)



Our samples are the four bands to the left of the 100 bp ladder. The bands look very similar in size although there is a little variation. The variation is likely caused by differences in concentration of the DNA samples. Sample 4 is farthest off of predicted size.

Conclusion: The DNA colonies we chose all appear to be similar to our expected size. However, because there is some variation we should use the band closest to our predicted 306 bp to use for the next step.

Next step: *E.coli* culture (choose one of the cultures to harvest) and mini prep to get the plasmid from the *E.coli*.

Date: Feb. 28th & March 2nd
Week #: 6
Objective: (in one or two sentences)
Perform PCR with primers specific to selected gene (*argos*). Then, clone the PCR product into plasmid and transform *E. coli* with the plasmid.

Procedures: (bullet point the procedures)
<u>Feb. 28th</u>

- Materials for PCR solution:
    - Taq DNA pol
    - Primer fwd/rev
    - dNTP mix
    - Buffer
    - MgCl2- can add
    - Template
        - GOI
        - **cDNA** (more concentrated than genomic)
        - Genomic DNA
    - Water
- Add H2O to primers to make 100 uM stock solutions (vortex to mix)
    - F1: 304 uL
    - F2: 243 uL
    - R1: 379 uL
    - R2: 313 uL
- Use stock solutions to make 100 uL of 10 uM primer solutions in new containers
    - Put 90 uL of water with 10 uL of the stock solution
- Set aside primer solutions
- Begin making PCR solution
    - Mix H20, 5x buffer, dNTP and cDNA in new container
    - Vortex and spin down
    - Add enzyme (Taq polymerase) and vortex quickly, spin down
- Split the PCR solution into two parts and then add the primers into the two separate tubes (one tube has R1F1 and the other R2F2)
- Vortex again briefly
- Spin down and put on ice

<u>March 2nd</u>

- Make gels (150 ml) 1.5% gel
    a. Use 2.25 g of agarose with 150 mL TBE (with stain)
- Run gels (20ul)
- Cut out DNA from gel

- Purify DNA
- Clone the DNA fragment into plasmid
- Transform *E.coli* with the plasmid

Date.__2/28/17__
Aim. Amplify Argos from Tribolium

Gene name.__Argos__
Primer1, Tm=__℃__: __5′__
__3′__
Primer2, Tm=__℃__: __5′__
__3′__

Reaction

H_20	µl	µl	
5 X PCR buffer	20µl	µl	
*$MgCl_2$ (25mM stock)	6µl (1.5µM)	µl (___µM)	
dNTP mix (2.5mM stock)	8µl (0.2µM)	µl	
Primer1 (10µM stock)	µl (___µM)	µl (µM)	
Primer2 (10µM stock)	µl (___µM)	µl (µM)	
Sample DNA ( )	µl	µl	
Taq polymerase (5U/ µl)	0.5µl (2.5U /100µl)	µl	
Total	100µl	µl	

*No $MgCl_2$ if using TAKARA Ex Taq or Promega GoTaq

Reaction Cycle **Result**

Program name: Electrophoresis:____%agarose, X TAE/ TBE

Denaturation: 95 ℃ X 5 min Loaded sample _____µl / lean

↓

Denaturation: 94 ℃ X 30 sec

Annealing: __*2__ ℃ X 30 sec ___ cycles

Extension: 72 ℃ X __*3__ m / s

↓

Extension: 72 ℃ X 5 min

↓

Hold: 4 ℃

*2: Primer Tm + 1-2℃

*3: 500bp/30sec, 1,000bp/1min

Note:
(describe what you actually did, along with all data you obtained (gel pictures, DNA quantification results, etc)

Feb 28th
What you need for primers:

1. Taq DNA pol
2. Primer fwd/rev
3. dNTP mix
4. Buffer
5. MgCl2- can add
6. Template
    a. GOI
    b. cDNA
    c. Genomic DNA
7. Water

Desired solution with primers: 100 uL of 10 uM

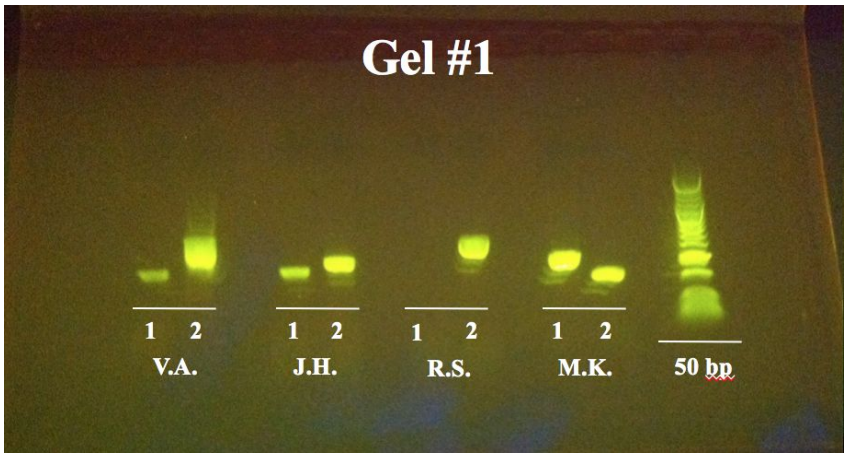
March 2nd
Choose the longer fragment to use today (F1R1 or F2R2)
The longer one is the F2R2

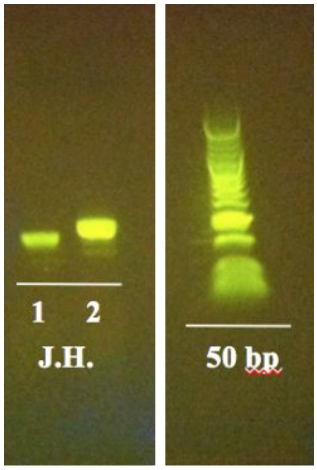
1. Make gels (150 ml) 1.5% gel
2. Run gels (20ul)
    a. Used 20ul of the green F1R1 and 20 uL of green F2R2 and put into gel in separate slots
    b. Viewed under light
3. Cut out DNA from gel (freeze & squeeze)
    c. Cut out the longer one (2) from the gel
    d. Freeze for 5 minutes
    e. Then 'squeeze' using centrifuge
        i. DNA and some liquid flows through to the bottom (gel purification) and agarose gets left behind
4. Purify DNA (freeze & squeeze did this for us)
5. Clone the DNA fragment into plasmid
    f. Mix PCR product (4ul)
    g. Salt (1ul)
    h. Topo plasmid (1ul)
    - Let this reaction sit at room temperature for 15 minutes
6. Transform *E.coli* with the plasmid

- Put 3ul of the reaction into the *E.coli* sample
- Heat shock the *E.coli* with the reaction solution: 42 degrees celsius for 30 seconds
- Let recover: 4 degrees celsius (on ice) for 5 minutes
- Recovery: Add 250ul of SOC
- Put in shaker at 37 degrees celsius for 30 min shake
- Plate 120 ul (on a plate treated with ampicillin)
    - Use plate spreader
    - incubate

Conclusion:

Feb 28th:

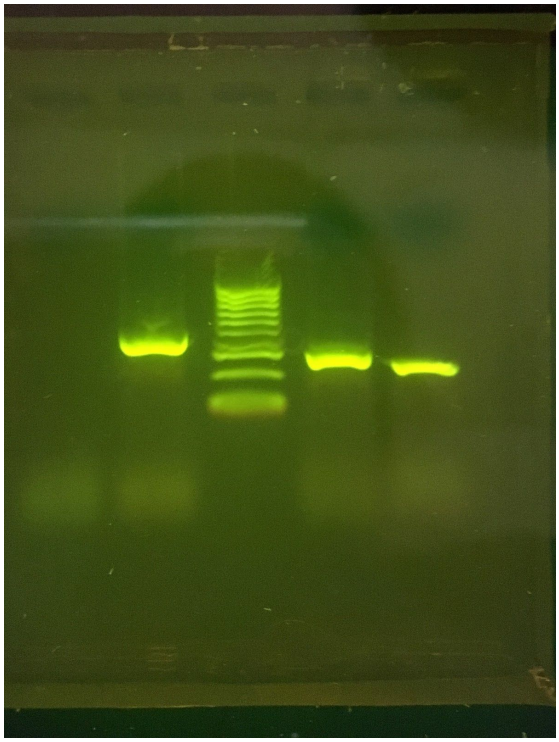




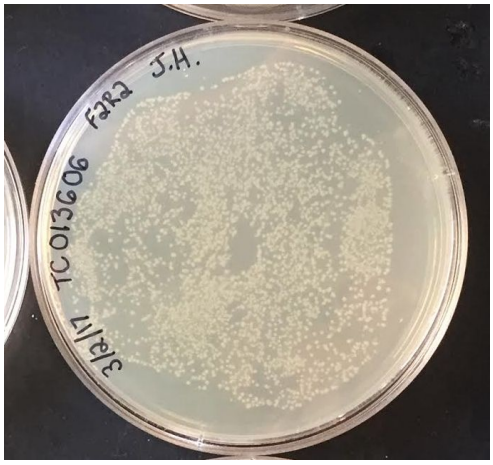
Our DNA is “J.H.” (the second from left)

Based on the 50 bp ladder, our DNA is 150 bp and 200 bp

March 2nd:



Our DNA is the first band to the right of the ladder, about 150 bp.



(plate results obtained March 7th)

Next step: Pick up colonies from plate and do PCR. Run a gel with product.

10/10
Very good note taking! Let's paste the gel picture and annotate the band size. --Yoshi

Date: Feb. 22nd
Week #: 5

Objective: (in one or two sentences)
Make and run a TBE agarose gel of 1.2% to find size of unknown DNA.

Procedures: (bullet point the procedures)

- Make gel
    - Mix purified agarose with TBE solution (with Gel Green stain)
    - Make 100 mL total so put 1.2 g agarose with 100 mL of TBE for 1.2% gel
    - Microwave the gel at 30% strength for about 2 minutes
    - Take the gel out with gloves and shake it. Microwave again until boiling
    - Take gel out and check for bubbles or uneven density
        - If uneven, microwave again
    - Pour the warm gel into mold and use a pipette tip to pop or move bubbles
        - Want the bubbles on the side of gel so they don't interfere with movement of DNA through the gel
- Electrophoresis
    - Fill the electrophoresis box with enough TBE to be able to submerge the gel
    - Be sure to place the gel in the box with the slots at the black electrode (negative) end since DNA moves towards the positive end
    - Loading the dye/DNA
        - Must dilute the 5x dye to 1x
        - Use 8 uL of DNA so mix this with 2 uL of the 5x dye (will give correct dilution)
        - Load this solution into the designated slot
    - Load 5 uL of the 100 bp ladder into a slot
    - Run the gel at 100 volts
- Find unknown strand bp size
    - Compare the unknown DNA to the ladder to get the size

Note:
(describe what you actually did, along with all data you obtained (gel pictures, DNA quantification results, etc)

Electrophoresis

Gel is made of TBE and agarose, typically used with 1% w/v (1 g/100ml TBE)
Negative at the top of the gel and positive at the bottom

Larger strands have harder time running down the gel and shorter strands have an easier time so will travel farther

Can use known kb strands to find an unknown kb strand
With condensed strands, hard to tell
Better resolution in longer DNA
Easier to tell with lower concentration of gel
Adjust the concentration with the size of DNA you expect to see
1 kb - 10 kb - try .7%

300bp-1kb 1%
50bp-300bp 1.5%
Use 1.2 grams of agarose, put in cylinder and microwave (watch carefully) for around 3 minutes
Power level 30% for 2 MINUTESSSSSSSS :)
Then take out the gel and shake
Put back in microwave

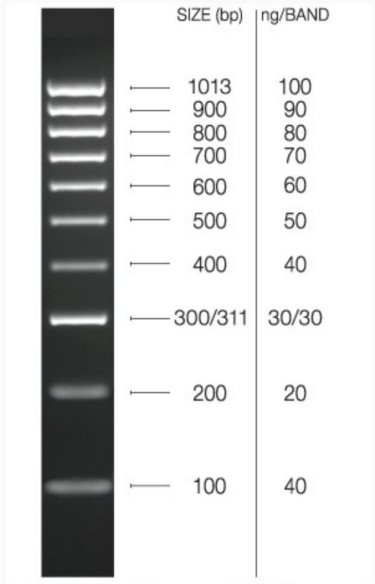
After gel has set, fill electrophoresis box with enough TBE solution (no stain) that gel can be submerged in it.
Set to 100 volts
Before using the gel… take out the plastic comb, rinse the gel, wipe off the mold (before any leftover gel dries in it)
The DNA will go towards the red electrode (positive) so, put the gel in with the slots (dark purple line) at the black electrode end

Loading dye:
Mix the DNA with loading dye, mixing will cause DNA to become dense
5x dye - we need to dilute it by 5 (1x is working solution)
Today want 8microliter DNA

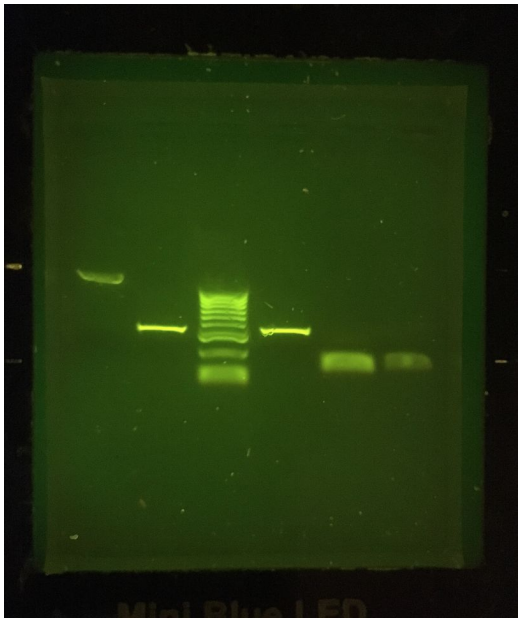
1. Mix with _2__microliters 5x loading dye
   5 microliters of 100bp ladder

We have unknown DNA A

Conclusion:
Based on the gel and the ladder, our DNA is about 200 bp.



5µl HyperLadder 100bp run on a 2% TBE/agarose gel and visualized using ethidium bromide



Our band is the farthest band to the right. It is about 200 bp.

Next step:
PCR