Lab in Cellular and Molecular Biology(BIO 464/564)

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Week 14

Final results:

Our hypothesis that RNAi of the TweedleD ortholog would cause a change in the axial ratio of Tribolium was not supported.

This could probably be because the TweedleD gene has 27 other paralogs which could have resulted in functional redundancy over the course of evolution. Also, the concentration of dsRNA injected could not have been sufficient enough to elicit an appropriate response in the beetles.

However, an alternate unexpected phenotype, where the beetles looked like they had "exploded" out of their cuticle was observed. The primary research paper about the TweedleD gene in Drosophila mentioned that this gene is instrumental in maintaining protein cross-linking for cuticle integrity. This could be related to the phenotype where the disintegration of the cuticle was observed in Tribolium after soaking in Ethanol for 48 hours.

Here it looks like a case of divergence of gene function though at one point of time it might have evolved from a common ancestor sharing the same function.

Fig 1: Normal axial ratio *Tribolium*

Fig 2: *Tribolium* exhibiting compromised cuticle integrity

Week 13

Objective: Look at Tribolium beetles. Count the living and the dead. Think about morphology.

Pupae:

Live:0 Dead:0

Beetles:

Live: 14 Dead:0

Of which:

Normal: 13

Kind of weird: 1 (it appears to have had a bad exit from the pupal case)

Week 12

Objective: Draw Tribolium larvae, pupae, and beetles. See injection demo for larva.

Count the living and the dead.

Larvae

Live: 1 dead: 0

Pupae

Live: 11 dead: 3

Beetles

Live: 0 dead:0

Comments: The larvae and pupae look disappointingly normal. Maybe the beetles will be tubby.

Count again on Thursday

Larvae

Live: 0 dead: 0

Pupae

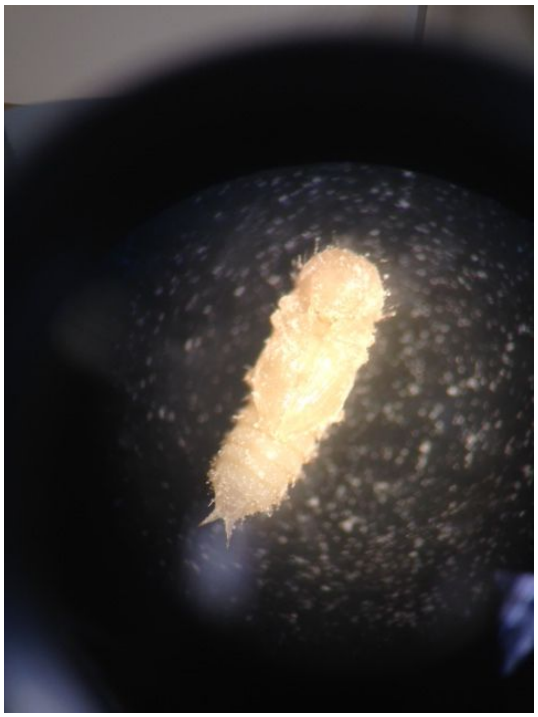
Live: 14 dead: 0

Beetles

Live: 0 dead: 0

Comments: Val and I are of the opinion that the pupae are kind of chubby. But not too chubby.

Say hello to our little friend!



Week 11

Objective: In-vitro transcription- Making RNA from our gene of interest

Quick recap: We have our gene of interest, flanked on both sides by T7 promoter

20µL solution
-8µL NTP Mix
-2µL 10x Buffer
-2µL enzyme mix
-8µL template (1 to 1.5µg of template present in the solution) (for a 1.176µg in our solution)
-µL RNAse free H2O to dilute, if necessary (not added in our case)

Objective: Purification of dsRNA. Using protocol: Nuclease Digestion to remove DNA and ssRNA.

Quick recap: we have our GOI in drRNA, ssRNA, and DNA form.

Create RNase digestion reaction mix
20µL drRNA
21µL Nuclease-free water
5µL 10X Digestion Buffer
2µL DNAse 1
2µL RNase

Mix everything in a tube. Incubate in incubator for 45 minutes.

To purify mixture from the degraded DNA and ssRNA, make binding mixture.

50µL dsRNA, fresh from incubator
50µL 10X Binding Buffer
150µL Nuclease-free water
250µL 100% Ethanol

Mix solution gently, then pipette entire solution onto filter in Filter Cartridge. Centrifuge at max speed for 2 minutes, discard flowthrough.

On top of filter cartridge, add 500µL Wash Solution (provided) and centrifuge at max speed for 2 more minutes. Repeat this step one more time with remaining 500µL Wash Solution. Centrifuge for 15 more seconds to remove remaining wash solution.

Apply 60µL hot elution solution to the filter in the Filter Cartridge.
Centrifuge for 2 minutes at maximum speed.

Go to nanodrop. Our results: 13.7ng/μL.
Give mix to Dr. Tomoyasu for refinement and concentration. Pray.

Comments
Our concentration of purified ssRNA was low, but it appears to have been the most-present solute in the solution, as the curve provided by the nanodrop had one distinct peak.

Week 10

Make 100μL of solution
46μL H2O
20μL 5x Buffer
8μL dNTP
15μL PCR4-TOPO
1.07μL plasmid mixed with 19μL H2O

TOPO cloning concept:

Thursday-

Objective: Check if PCR worked and then purify the PCR product to get highest concentration

PCR verification
Load 2 μl of the PCR product into the well (left side, well 2) to check if the PCR has worked.
Load the sample with 100bp ladder.
Estimate the size of the fragment thats running (it has promoters attached to it around 20 bps in size)

PCR purification
Add 5x buffer volume - 490 μl to PCR reaction
Follow steps on Qiatube sheet

PCR template DNA:
Date: March 30,2017
Concentration: 147 ng/μL
Length: 283 bp

Week 9- Spring Break

Week 8

Objective: use Miniprep to extract and harvest plasmid

Procedure: Miniprep

Spin down E. coli culture until it is a pellet- add 2 ml to tube, spin down, discard supernatant, add 2 ml more and then spin again. Place tube hinge side out (12krm, 2 minutes)
Remove the LB
Add P1* buffer, 250µL. Vortex well to resuspend cells.
Gently add P2 buffer (extremely alkaline!) for precisely 3 minutes, not shaking or vortexing.
Invert tube gently to kill the cells.
Add N3, 350µL, adding it slowly.
Place tubes on ice for 5 minutes
Spin down on centrifuge: 12krpm for 10 minutes.

Procedure: Plasmid extraction

Place centrifuged liquid into column tube: add 750µL supernatant to column tube.
Insert column tube into hydraulic vacuum pump
Hydraulically pump liquid down. Keep blue (upper) part of column tube.
Add 750µL PE to the still-pumping column tube
Vacuum down until dry.
Put column into a clean 1.5mL tube
Add 30µL of EB buffer**
Let sit 1 minute and then spin 12rpm for 1 minute
Throw away blue upper tube, keep lower 1.5 mL tube. It holds the plasmid.

Go down to CBFG. Use nanodrop to check ng/µL.

Send E. coli to company so they can purify plasmid.

Aim: Just remove plasmid alone (exclude membrane structures and genomic DNA)

Notes

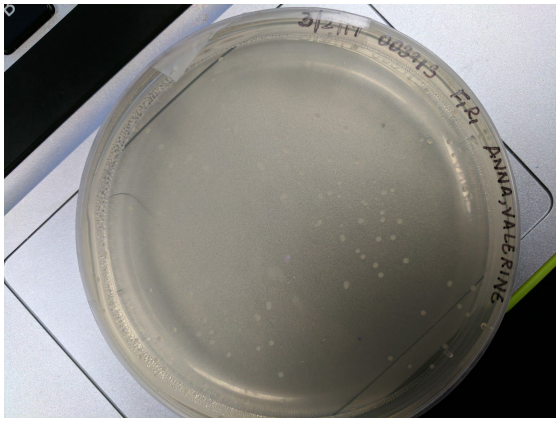
*During first resuspension of pellet in Miniprep procedure, we added PB buffer instead of P1. This is problematic.
**Add the EB directly onto the pallet, not down the sides of the tube.

Week 7

Objective: Perform colony PCR. Make sure that the E. coli colonies are with a plasmid and GOI.

Procedures: Use T3, T7, do colony PCR.

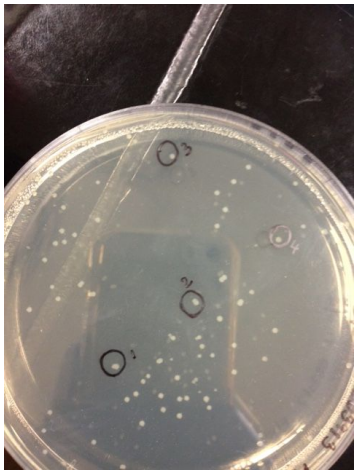
Notes



Bacterial colonies. E. coli and plasmid with GOI, some satellite colonies without GOI.

Protocol

Add 100µL LB to 1.5mL tube
15µl of LB-Ampr in each tiny tube (4 tiny tubes)
Use pipette tip to pick colonies
Pick 4 colonies
Put into 4 tiny tubes, swirling pipette in the tube.
Incubate at 37˚C for 1 hour.



Colonies taken.

Procedure for colony PCR

I) Required liquids

Liquid	µL	x5 (µL)
Water	8.2	41
5x Green Buffer	4	20
dNTP mix	1.6	8
Primer 1 F	2	10
Primer 2 R	2	10
Taq	0.2	1
Culture	2	2 per tube, each tube has own culture

Total volume 20µL (100µL Master Mix)

Mix everything in a 1.5µL tube except for the culture. Make 5 times the volume-- Master Mix.
Label 4 more tiny tubes- culture 1, 2, 3, 4.
To each tube, add 20µL mix and 2µL culture.

Perform PCR.

March 9:

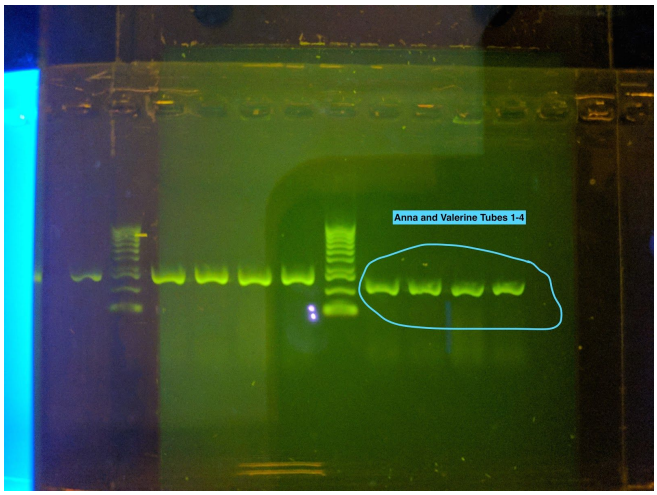
Objective: Run gel of the colony PCR, see if our band is in our predicted size

Procedure:

Run 2µl of our PCR reaction, tubes 1-4.
We are in tray B, slots 11-14
1.5% agarose gel
75ml TBE
Agar: 1.125 g

Notes:

Snapshot of our gel after running below.



Things to know:

1. What are the primers you used?- F1R1
2. What is the length of your PCR amplified fragment?

- Prediction: 350 bp
- Actual: 300 bp (estimated from gel)

Conclusion: Our gels were all approximately showing 300bp. The cleanest looking gel is from tube 1, but all appear to be more or less the same size.

Next step: Start *E. coli* culture.

Week 6

Objective:
To create a stock solution and working PCR solution of our forward and reverse TC# sequece.
To replicate our DNA segment through PCR.
Clone gene into E.coli
To watch Dr. Tomoyasu draw a cute Tribolium (haha jk)

Procedures:

- Run a gel
- Purify DNA fragment
- Clone DNA fragment into plasmid
- Transform E-coli with plasmid

Notes

I) Preparing primers

- Calculate the amount of water required to convert the dry sample of R1, R2, F1, F2 DNA into 100μM stock solution (volume of water is different for each sample).
- Dilute 10μL of each stock solution in 90μL water to make working solution.

Length of our primers- Set 1:243bp; Set 2:378bp

II) Components of PCR

Taq DNA polymerase
Set of primers: Forward and Reverse
dNTP mix
10x buffer (MgCl2)
Template DNA: GoI, **cDNA(better template because already spliced)**, genomic DNA
Make primer to 100μM
Make 100μL of 10 μM

Ideal size of the amplicon- 200-300 bp

III) Protocol

- Make 1.5 % gel
- Run the gel, using 5µL 50bp ladder
- Our samples were in slot 1 and 2, samples ordered 1(1) 2(2), gel on the right side (half further away from anode/cathode)
- Cut out DNA from gel with plastic cutter

Purify gel

Purifying the DNA: After running, choose the longer fragment. We chose our shorter fragment (F1R1) because the other had non specific binding. **Amplicon length- 243 bp**

We used a plastic cutter to cut the DNA from the gel and popped it into centrifuge tubes with column (Freeze N Squeeze). Froze the tube to solidify the gel and centrifuged for 3 mins at 14000 rpm. After spinning, the gel stays on the column, which is disposed and the DNA has percolated to the tube below.

Clone the DNA fragment into plasmid

Make double stranded RNA against our gene of interest in Tribolium and block the function of that particular gene and check its loss of function phenotype.

-Take the length of these DNA sequence that is longer (sequence between a set of primers).

-Linear DNA is highly unstable(transient). So we could make it circular? But in order to amplify the sequence, it has to be preserved. Therefore, we make the DNA as a plasmid

a) Make a plasmid
b) Put it into E.coli
c) Culture the E.coli
d) Called mini prep plasmid
e) Use this as a template
f) Use PCR to make DNA fragment with T7 promoter on both ends

- ❏ Putting linear DNA into plasmid makes it circular
- ❏ Digest plasmid and DNA with same restriction enzyme so that same ends are formed
- ❏ Use ligase to attach the two ends
- ❏ But Taq polymerase adds polyA tail at the end
- ❏ Ligation needs energy for the process
- ❏ So instead, we use topoisomerases
- ❏ Topoisomerases bind to TTT at the end of the plasmid and attach our PCR product to the plasmid
- ❏ Plasmid should have a resistance marker for selection

Cloning the DNA fragment into the plasmid:

- PCR product - 4µl
- Salt - 1µl
- Topo plasmid (plasmid+enzyme)- 1µl
- Incubate at room temperature for 15 min

- 3µl reaction mixture to E.coli
- 42 degrees for 30 sec (heat shock)
- 4 degrees for 5 mins(on ice)
- Add SOC 250 µl
- Plate and 40 min in shaker at 37 degrees

Transform E.coli into plasmid

Our kit has lacZ along with antibiotic resistance and marker called ccdB(kills the E.coli that doesn't take up our Gene of Interest). So only colonies that form have our gene of interest.
The plasmid is constructed and the E.coli is made to take it up by electroporation and change in medium salt concentration and slight heat shock
The E.coli is made to recover in Super Optimal Culture Medium before plating

Lab notebook - Week 5

10/10
Very good note taking! So, what is the length of your DNA? --Yoshi
Length of DNA- 350bp

Name:Valerine Rajathi & Anna Osatuke
Date: Feb 21, Feb 23
Week#: 5

Objective:
1)To learn how to accurately and precisely pipette solutions
2) To learn how to make an agarose gel for electrophoresis and separate different strands of DNA based on size

Procedures:

February 21-

a) Sterile techniques in fitting pipette tips
b) Using the right pipette for the right amount of liquid in order for it to be accurate and precise

February 23-

a) Making agarose gel mold: 1.2% w/v; adding 1.27g agarose to 100mL TBE buffer.
b) Using TBE buffer (with Gel Green visualising Dye)
c) Placing the gel in the buffer and an electric current is passed through the buffer
d) DNA is loaded in wells on the negative side of the electrode
e) After gel electrophoresis, the DNA sample A is compared to other DNA Samples to figure out the relative size of DNA sample A.

Note:

Agarose gel percentages for Dna fragment lengths:

- 1kb-10kb 0.7%
- 300bp-1kb 1%
- 50bb-300bp 1.5%

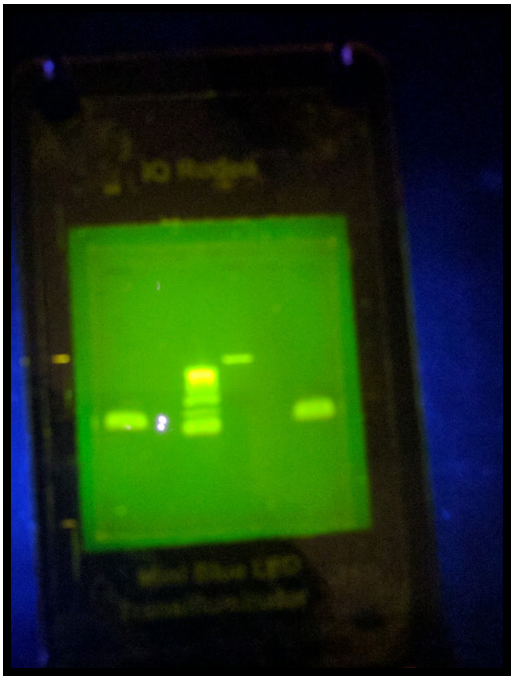
For bigger sizes of DNA, lower concentration of agarose will be beneficial

- Notes on preparing DNA samples for agarose gel electrophoresis
- Pre-prepared DNA samples are in vials, but no loading dye inside of DNA.
- DNA must be mixed with 1x loading dye before putting into solution. We have 5x dye, so must dilute it until it is 1x. For 4 microL DNA, add 1microL 5x loading dye.
- Since we will use 8microL DNA, we will mix 2microL loading dye. Using a 20microL pipette, combine DNA and then loading dye in a vial.
- Mix in vortex.

Notes for running the gels:

- Fill to the mark in two-lobed gel container with TBE not mixed with dye. The goal is to fill it up enough to submerge the gels.
- Put plastic lid on container, without having the gels inside but with the TBE, and run the electricity through. Watch for bubbles! Bubbles indicate activity, no bubbles is bad.
- (Connect cathode to cathode and anode to anode, and link to the electric control box.) Run at 100V.
- Squeeze water from squeeze bottle over solid agarose gels, and pull out slot inserter gently. After inserting the gels, watch the dyed slots for a few minutes to ensure that the gel is running in the correct direction.

- Electrophoresis gel results using Gel Green dye. Slots filled in order, from cathode/anode side to no cathode/anode side: 1(A) 2 (empty) 3 (ladder) 4 (C) 5 (empty) 6 (A). Run the gel, and watch for 1-2 minutes to see whether the direction is correct (towards the cathode.)
- Run gels in Gel green free TBE



Conclusion: Sample A is the largest of the DNA samples, as it moved the least of the way down the agarose gel medium.

Next step: Apply these methods to our TwdID gene sequence.