Lab notebook

Name: Nelchi and Bethany
Date:5/03/18
Week#13

Objective: To observe the phenotype of *ex* RNAi injection.

Procedures:

- We observed the injected animals under bright field lighting
- Total larvae injected on 4/18/18 = 18

Results:

- We had six alive beetles
- Of the six, five had large wings that did not properly fold under their elytra
- All beetles had trouble righting when flipped on back

Date:4/26/18
Week#12

Objective: To observe the phenotype of *ex* RNAi injection.

Procedures:

- We observed the injected animals under bright field and GFP lighting.
- Total larvae injected on 4/18/18 = 18

Results:

- Number of animals still in larval stage = 1
- Number of animals dead in the pupal stage = 5 (some had blue injection buffer on them, died due to injection?)
- Number of pupae alive = 12
- Number of pupae that exhibited wing abnormalities = 6
- Number of pupae that looked normal (normalish) = 6

Documentation of the results -

Date:4/24/18
Week#12

Objective: To observe the morphology of our RNAi pupae

Methods:

- We observed our *T. castaneum* (n=18) under normal lighting and using GFP lighting.
- They were injected on 4/18/18 in the afternoon

Results:

- One was still in the larval stage
- Five pupae were dead (some had blue injection buffer on them, died due to injection?)
- 12 pupae were alive
- Of the alive pupae, 4 appeared to have wing abnormalities while the other 8 looked normal

Two examples of misshapen wings

Date:4/17/18
Week#11

Objective: To observe the morphology of larvae, pupae and adults of *Tribolium castaneum*.

Pupae

Adults

Date:4/12/18
Week#10

Objective: To remove single stranded RNA and DNA from the dsRNA we prepared on Tuesday and to purify the dsRNA.

Procedures:

- We first treated the dsRNA with a master mix containing nuclease free water, dsRNA, buffer, RNase and Dnase to remove ssRNA and DNA
- This was mixed by pipetting up and down ten times
- We then incubated this mixture for 30 min at 37 C
- We then purified the dsRNA by combining the incubated mixture with a master mix containing binding buffer, nuclease free water, and 100% ethanol
- The reaction was mixed by pipetting up and down
- It was then placed into a filter cartridge and centrifuged for 2 min at 13,000 rpm
- The filtrate was discarded
- We then added 500ul of wash buffer, centrifuged for 2 min at 13,000 rpm (repeated this step)
- We centrifuged again at 13,000 rpm for 1 minute to remove last traces of liquid from filter, the filter was then transferred to a clean collecting tube
- We then recovered the dsRNA by eluting in 50ul. After adding the elution solution it was incubated for 10 min in a 70 C heat block, then centrifuged at 13,000 rpm for 2 min.
- We repeated the same step with an additional 50 ul aliquot of elution solution in the same collecting tube.
- The 100 ul of elution containing dsRNA was then stored at -20C.

Date:4/10/18
Week#10

Objective: To purify template DNA for in vitro transcription.

Procedures:

- The PCR reaction mixture was combined into a new 1.5ml microcentrifuge tube from 8 PCR tubes (200ul in total).
- 1ml of Buffer PB was added to the tube. The tube was vortexed and spun down.
- 600ul of the solution was added to a new QIAquick column in the vacuum. The step was repeated one more time to get the DNA from the entire 1.2ml of the solution.
- 750ul of Buffer PE was added to wash in the vacuum for 30-60s.
- The column was centrifuged at 13,000rpm for 1 minute to remove residual wash buffer.

- The column was placed in a fresh 1.5ml microcentrifuge tube and 30ul of Buffer EB was added to the centre of the column to elute DNA. The column was let stand for 1 minute and centrifuged at 13,000rpm for 1 minute before checking the concentration using the nanodrop.
- The concentration of DNA was measured to be 141.0 ng/ul.

For in vitro transcription,

- Kevin made the master mix for the entire class with the following volumes for each reaction mixture of 20ul -

NTP mix - 8ul
10x Buffer - 2ul
T7 RNA polymerase - 2ul
Template DNA - 8ul
Total volume - 20ul

- We added 8ul of template DNA without diluting to make the total volume 20ul.

Date:4/05/18
Week#9

Objective: To synthesize a template for dsRNA using PCR

Procedures:

- Plasmid DNA was diluted from 430.4 ng/ul to 40 ul of 10ng/ul and the diluted DNA was used in the PCR.
- We prepared PCR to synthesize a DNA template for dsRNA synthesis using the following recipe which was then aliquoted into 8 PCR tubes:

Reaction

H_20	102 µl	
Green buffer	40 µl	
dNTP mix	16 µl	
Primer	30µl	
Plasmid DNA	10 µl	
Taq polymerase		2 µl
Total	200 µl	

Reaction Cycle

Program name:
Denaturation: 94 ℃ X2 min
↓
Denaturation: 94 ℃ X 30 sec
Annealing: 57 ℃ X 30 sec __ 35 cycles
Extension: 72 ℃ X 30 sec
↓

Extension: 72 ℃ X 5 min

↓

Hold: 4 ℃

Result:
PCR will be performed after class using this reaction.

Name: Nelchi and Bethany
Date:4/03/18
Week#9
Objective: To analyse the sequence of purified plasmid DNA and construct a map of the plasmid with the GOI insert.

Procedures:
Sequence of plasmid DNA was analysed and aligned with the predicted sequence to identify any differences between the two and to check the orientation of the insert in the plasmid.

Result:

Date:3/29/18
Week#8

Objective: To purify DNA from plasmids of cultured E. coli cells for sequencing.

Procedures:

- Last night (3/28/18), Kevin started cultures of our two chosen (2+3) colonies.
- Colony 2 was pelleted by adding 2ml of the culture to a 2ml tube and centrifuged for 3 min at 12,000 rpm. The resulting supernatant was removed, and an additional 2ml of culture was added and the process was repeated.
- 250 ul of buffer P1 was added and the pellet was resuspended in this buffer by vortexing

- 250 ul of buffer P2 was added to increase the pH, kill the bacteria, and destroy unwanted macromolecules. The solution was mixed by inverting the tube ~5 times.
- After exactly 1 min, 350 ul of buffer N3 was added to neutralize the solution, and the solution was mixed by inverting 4-6 times.
- The solution was then incubated on ice for 10 min.
- This solution was then centrifuged for 10 min at 13,000 rpm
- 750 ul was then applied to a QIAprep 2.0 spin column and vacuumed out
- The column was washed by adding .5 ml of Buffer PB and vacuuming it out
- The column was washed again by adding .75 ml buffer PE and vacuumed out
- The column was centrifuged for 1 min at 12,000 rpm to remove residual wash buffer
- The column was placed in a clean 1.5 ml microcentrifuge tube. To elute the DNA 50 ul of Buffer EB was added to the center of the spin column, it was allowed to sit for 1 min before being centrifuged for 1 min at 12,000 rpm.
- The column was then discarded, and the elution was saved for analysis
- We then measured the concentration of the DNA using the nanodrop.

Results: The concentration of our DNA was 430.4 ng/ul.
Conclusions: Next week we will sequence our DNA sample.

Name: Nelchi and Bethany
Date:3/27/18
Week#8

Objective: To run agarose gel of colony PCR product and choose the appropriate E.coli cells for further culturing and downstream applications.

Procedures:

- We made 80ml of 1.5% agarose gel (weighed 1.2g of Agarose and added it to 80ml of 1x gel green loaded TBE).
- Mixed it well and microwaved it at 30% power for 3 minutes and then again for 1 minute to get rid of any bubbles.
- We poured the gel into the mold and let it set.
- We added some water and removed the gel comb.
- The gel boat was placed in a gel box containing 0.5 x TBE.
- 2ul each of sample 1, 2, 3, and 4 were loaded into separate wells on the gel.
- 5ul of 50bp ladder was loaded.
- The gel was run for 25 minutes at 100 v.

Results:

The band size we were looking for was 451bp (351bp amplicon of primer set 2 + 50bp each of T7 and T3 sites in the plasmid).

Conclusions:
We selected colonies 2 and 3 to be cultured further and used for downstream applications.

Date:3/15/18
Week#7

Objective: To perform colony PCR on transformed *E. coli.*

Procedures:

- We first isolated colonies of our transformed *E. coli* into 15 ul of LB+amp (n=4).
- The remaining colonies on the plate were then saved and put in the fridge.

- The isolated colonies were cultured for 1 h at 37 C. Colonies were tapped gently every 15 min to mix.
- 4 PCR were then ran using the colonies and the following recipe and reaction cycle for each reaction.

Reaction

H_20	8.15 µl
Green buffer	4 µl
dNTP mix	8µl
T3 Primer (10µM stock)	2 µl
T7 Primer (10µM stock)	2 µl
Colony	2 µl
Taq polymerase	0.25µl
Total	20µl

Reaction Cycle

Program name:
Denaturation: 95 ℃ X 5 min
↓
Denaturation: 94 ℃ X 30 sec
Annealing: 55 ℃ X 30 sec __ 30 cycles
Extension: 72 ℃ X 30 sec
↓
Extension: 72 ℃ X 2 min
↓
Hold: 4 ℃

Next step:
Run the gel for colony PCR

Date:3/13/18
Week#7

Objective:
To clone the DNA (TOPO cloning), transform *E.coli* cells and plate the transformed cells.

Procedures:

- The frozen DNA was thawed, vortexed and spun for a few seconds.
- A TOPO cloning reaction mixture was made in a 1.5ml microcentrifuge tube with 4ul of purified DNA + 1ul of salt solution + 1ul of Topo vector.
- The mixture was kept at room temperature (RT) for 30 minutes.
- In the meantime, *E.coli* cells were thawed on ice for 10 minutes.
- After 30 minutes at RT, 3ul of the reaction mixture was added gently to the thawed *E.coli* cells and the pipette tip was swirled gently to mix well.
- The cells with the mixture was kept on ice for 5 minutes.
- The cells were then heat shocked at 42℃ for 30 seconds.
- The tube was kept back on ice for 5 minutes.
- 250ul of SOC was pipetted gently to the tube containing the cells and the DNA mixture.
- The tube was then kept at 37℃ for 45 minutes to 1 hour.
- 120ul of the solution was then pipetted out and plated onto fresh LB media and kept at 30℃ overnight.

Conclusions:
The cells were successfully plated onto fresh LB media.

Next step:
Transformed colonies will be selected and colony PCR will be performed.

Date:3/8/18
Week#6
Objective:
To isolate DNA using gel electrophoresis and ‘freeze and squeeze’ method.
Procedures:

- Gel electrophoresis was run before class to ensure that the PCR worked.
- In class, two groups made two 1.2 % gels (as described before).
- We ran gel electrophoresis of 5ul of 50bp ladder and 20 ul of PCR amplicon for 25 min at 100 V.
- We then cut out the piece of the gel with our chosen band (F2/R2) using an extractor tube.
- The DNA was then isolated using a freeze and squeeze tube that was put in the freezer for ~ 5 min then centrifuged at 13,000 g for 3 min at room temperature.
- The DNA was then preserved by **……**

Figure 1: Gel electrophoresis of PCR amplicons, from left to right, TC012545F1/R1, TC012545F2/R2, ladder, other groups F1/R1, and F2/R2.

Conclusions:
Our PCR was successful.

Next Step:
The isolated DNA will be put into plasmids next week.

Date:3/6/18
Week#6

Objective:
To learn how to set up PCR.

Procedures:

Date. 3/6/18
Aim: Set up PCR

Gene name: PREDICTED: protein expanded, TC012545

Pair 1:
Forward Primer: Tm=60.11℃: sequence 5'--GTTCTACGTGGACAGCCCTC
Reverse Primer: Tm=60.18℃: sequence 5'--CCTCCGTTATGAACCGGGAC

Pair 2:
Forward Primer: Tm=59.83℃: sequence 5'--ATTACGTGACCCTTCCGTCG
Reverse Primer: Tm=59.89℃: sequence 5'--GAGGAACGGAACTTGGGGTT

- 50 µl of Master mix with the following volumes of each (except the primers), was made on ice.

Reaction

H_20	___µl	30.5 µl
5 X PCR buffer	20µl	10 µl
*$MgCl_2$ (25mM stock)	6µl (1.5µM)	_____µl (__µM)
dNTP mix (2.5mM stock)	8µl (0.2µM)	4 µl
Primer1 (10µM stock)	___µl (__µM)	2 µl (__µM)
Primer2 (10µM stock)	___µl (__µM)	2 µl (__µM)
Sample DNA ( )	___µl	1 µl
Taq polymerase (5U/ µl)	0.5µl (2.5U /100µl)	0.5 µl
Total	100µl	50 µl

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

- Taq polymerase was added last and before adding Taq polymerase, the mixture was vortexed and spun.
- After adding each solution to the reaction, it was pipetted up and down a couple of times to mix it well.
- After adding Taq polymerase, the mixture was vortexed (for about 2 seconds) and spun for a few seconds.
- 23ul of master mix was added to two PCR tubes, each.
- 1ul each of forward and reverse primers (F1 and R1 in one tube, F2 and R2 in the other) was added to the two PCR tubes to make up the total volume to 25ul in each tube.
- PCR was set with the following reaction cycle program for 35 cycles.

Reaction Cycle

Program name:
Denaturation: 95 ℃ X 5 min
↓
Denaturation: 95 ℃ X 30 sec
Annealing: _*2_ ℃ X 30 sec 35 cycles
Extension: 72 ℃ X 30 sec
↓
Extension: 72 ℃ X 5 min

Result

Electrophoresis:__%agarose,__X TAE/ TBE
Loaded sample ___µl / lean

↓
Hold: 4 ℃

*2: Primer Tm + 1-2℃
*3: 500bp/30sec, 1,000bp/1min

Objective: 1
To dilute primers to 100uM stock and make 50ul of 10uM working solution from the stock solution.

Procedures:

- We labelled the primers with TC#012545 and F1/R1 and F2/R2 for primer sets 1 and 2, respectively.
- We then added 274ul of double distilled(DD) water to primer F1 to make the concentration 100uM.
- We added 262ul of DD water to primer R1 to make the concentration 100uM.
- For F2, we added 238ul of DD water and 324ul of DD water to R2 to make the concentration 100uM each.
- We vortexed the primers for a few seconds, twice.
- We then spun the primers for a few seconds.

Our 100uM stock of primers F1, R1 and F2, R2 were then ready.

- To make 50ul of 10uM working solution each, we added 5ul of stock solution of each primer to 45ul of DD water to 4 new microcentrifuge tubes.
- We labelled each tube with the TC# and the concentration along with the primer name on green labels.
- We then vortexed and spun them.

Conclusion:
We diluted the primers to 100uM stock solution and made 10uM working solution of each primer.

Date:3/1/18
Week# 5

Objective:
To learn how to make gels and determine size of unknown DNA samples through gel electrophoresis.

Procedures:

Fig 1. 50 bp Bioline Ladder

- We first made 1.2% gels using agarose and 1x Green gel loaded TBE. The gels were mixed then microwaved for 3 min at 30%. They were then mixed well again. After mixing, they were microwaved again for 1 min at 30% to remove air bubbles. Gel solution was added to gel mold. Any bubbles were popped with a pipette tip. It was then allowed to settle before being removed. Gel was put in gel box and filled with 0.5 X TBE.
- The three unknown samples had concentrations of A: 82ng/ul, B: 118 ng/ul, C: 115 ng/ul.
- The samples were diluted to 10ng/ul
    - A= 1.22 ul DNA + 8.78 ul DD H20
    - B= .85 ul DNA + 9.15 ul DD H20
    - C=.87ul DNA + 9.13 DD H20
- 2.5 ul of 5x loading dye were added to each 10 ul sample (to dilute loading dye to 1x).

- We ran 10 ul of sample and 5 ul of ladder at 135 V for 25 min.

Fig 2: From left to right gel of 50 bp ladder, unknown sample A, B, and C.

Conclusion:
Based on the gel, the unknown samples A, B, and C are approximately 300, 500, and 1200 bp long, respectively.

Next step: