Lab Activity Log - SECA NZ - iGEM 2017

Key Members:

- Jess Chase
- Judith Glasson
- Sneha Chatterjee
- Andra Popa

Friday 120517

Constructed and ordered DAIRIP4 gene for gateway plant expression with the aid of Geoffrey Thomson. Added attB1 and attB2 sequences to each end of the gene for insertion into the pDONAR221 vector. Optimised 6 codons for synthesis.

Monday 150517

Initial development complete.

Finished generating the reverse transcript genes with the added his tag and pelB fragment overlaps for the gibson reaction. From these generated the PCR primers for gene amplification. As all genes have the same insertion location and overlap, the same primers can be used for all of them.
Also generated PCR primers for the pET20 vector which would eliminate the MCS.

Talked to Shaun. Lab does not have a stock of pET20b+ vectors, but does have pET22b+. Functionally different only in that pET22b+ has a lacI region. His tag and pelB localisation sequence is identical, so constructs are compatible.

Gave Shaun viewers access to gene and primer files for error checking. Awaiting response.

Will now check sequences and primers against IDT to see if any sequence sections must be codon adjusted for production.

Wednesday 170517

Finished adjusting the genes for sequencing. Had to change a few of the codons fro reduce GC content, and change the codons in the his tag due to inability to synthesis 6 repeat sequences in a row. Added on an additional overlap to the his tag end to act as the homologous region for the Gibson's reaction now that the his tag is not identical to the one included in the vector.

Cannot claim free DNA from IDT until registration is finalised.

Tuesday 300517

Jess, Alastair and Judith completed the Plant Containment course required for entry into the PC2 Plant Hall under instruction from Davide Zazzaro. Pant manual can be found under Antifreeze vines: Safety and Containment.

Notes taking during session:

When entering the Plant Hall, write your initials on the whiteboard in the anteroom, to enure that fellow users are aware of who is in the room at any point in time. When you leave, remove your initials. If you are the last to leave, switch off the lights and lock the door behind you. Return key to draw.
The white board serves as a notice board - anything needing to be brought to Davide attention should be written here.

The plant containment operator is Jim Metson.
Wendy is in control of the laminar flow room.
If you see any seeds on the floor, wipe them up, place in the autoclave bag and tell Davide!
Update the registers regularly - these being the sowing and seed registers.

Even wt plants are autoclaved. Once inside the PC2 lab, everything is treated as GMO. The wt are not HSNO coded, but still require a sowing number, and are disposed of in the same manor as GMO’s. Initials and sowing number the most important information on the plant identification stakes.

Stakes do not go into yellow bags as they can break through on the way to the kitchen. Go in there designated bin.

All liquid must pass through the 100 micrometer filter. Trigene expires 6 months after dilution to 1/100. Spray the tray, wait 5 minutes, then wash.

Report any insects.

In the level 3 plant hall, everything gets autoclaved. In the level 5 transgenic green houses when working with medicargo, the soil and rockwool does not need to be autoclaved due to the seeds being easily spotted. Level 5 water can also pass directly down the drain.

DAIRIP4 gene arrived - resuspended and place in the freezer for us by Geoffrey.

Thursday 010617

Meeting with Davide to hand in our test sheets for the Plant Containment course.
Meeting with Jo and Geoffrey on plans with gene, experiment and transformation.
Gene has been placed in a blue box in freeze 1.
Had second enter check signed off by Jo.

Planting! Ran though planting process with Andrew, planting two trays of wildtype Arabidopsis.

Seeds are bleached to sterilise them, them cold treated for 3 days to promoter germination.

Rockwool - the prefered planting media for Arabidosis.
Place down a cutting mat, and cut the rockwool block to the width of the black planting trays.

Mix up some hydroponic media: Tank A and Tank B contain nutrients to help plant growth.
Add 40 ml of both A and B to the large green watering can, then top up to 8 litres.
Soak the rockwool in this liquid, pushing down and then leaving to soak for a while.

Make holes in the bottom of the black trays. These will sit inside of the large metal trays, and watering will be carried out by filling the base of the metal tray with water.

Use hammer and punch to make holes in the trays. Repeat process with the black plastic to prevent algae growth. There is a template sheet for you to work off.

The Arabidopsis seed are super tiny!

Air bubbles in the rockwool will appear as pale patches on the surface - press on them to release the trapped air.

Our sowing number: Q523
Wt Columbia
01/06/17
JC and JG

Remove seeds from tube by swirling with a toothpick. Then using a second tooth pick, collect one seed at a time and place onto the surface of the rockwool. Place two seeds for each hole, encase one does not germinate. Later, seedlings will be thinned.

Make a germination tent - increases the humidity to promote fast germination. Contruct with clear plastic and kebab sticks.

When finished, remember to return the key!

Tuesday 060617

Checked on the seedlings. Water still present in the bottom of the tray, so did not add any extra. Will check again tomorrow and hopefully start the regular Monday, Wednesday, Friday watering schedule.
We have germination! Left the plastic tent in place, as it is covering Geoffrey’s plants also, and I am not sure when it will be removed.

Granted access and given instructions on how to access the Share Plant Folder.

Wednesday 070617

Checked on seedlings. Have at least one growing per space. Talked to Jo, discussed timing of cloning and exam time tables. Suggested it might be easier to plant a second batch of seeds next week, and then do all of our cloning once exams are over. Will assess timetables and evaluate.

Friday 090617
Judith had final entry checked off by Wendy. Watered seedlings.
On Monday will thin out the seedlings to one per hole.

Monday 120617
Thinned seedlings, sent pictures to Alastair.

Friday 160617
Watered seedlings, took pictures.

Wednesday 210617
Watered seedlings, took pictures, sent to Alastair.
Organised to do seed sterilisation on Friday for a Monday planting.
Registered for the free DNA from IDT

Friday 230617

Began seed sterilization at 10am with Andrew. Did enough seeds for three blocks, in case we have issues during transformation and require more plants. However only a couple of healthy plants are required for transformation, so the spares will be used as experimental controls.

We were only sterilising wild type columbia, so only one tube was required. All seed handling is done in the white seed tray in the growth room. Before you begin make sure that the tray is feed of seeds from the previous user. Clean off the paper and tray into the bin if seeds can be seen, being sure not to drop any on the floor. Wipe down the tray with ethanol and a tissue.

Label the tube wild type, add the date and the previous sowing number for entry into the sowing register. Gently tap out some seeds into the new tube from the stock, and check the tray once you are finished for dropped seeds. If you have dropped, seeds, clean and dispose as previously mentioned.

The next steps should be carried out in the flow cabinets. These are turned on by pressing the fan on button, and takes 3 minutes to warm up. The inside should be wiped down with Trigene during this time.

The seeds will be sterilised in 4% bleach, which is kept in the top cupboard closest to the door. As we only had a small amount of seed to sterilise, we only required 200 µL volume. If you were doing a large sowing, you might need a full 1 mL.

Sterilastion:

1. Soak seeds in water; ~200 µL will be sufficient for our volume of seeds.
2. Give the tube a flick and mix around, so the seeds to do not sit at the bottom of the tube. Soak for 10 minutes, flicking periodically.
3. Spin down the seeds to collect at the bottom, so we can easily remove the water. Spin at 1000 rpm, 60 seconds.
4. Remove water, and add 200 µL of bleach.
5. Wait for 5 minutes, flicking the tube occasionally.
6. Remove bleach, and then 5x water wash with spin downs. 1000 rpm, 60 seconds. In the water wash you can use more liquid, as you want to be sure that all the bleach is removed. We used 1mL. If there are too many bubbles forming to allow pipetting of the water out, just spin a little longer to pop them.
7. Remove the water after the last wash, and add a fresh 200µL.
8. Chill at 4 degrees C for at least 2 days. Wrap the tube in tin foil.

The waste water containing seeds should be soaking in full bleach to ensure death of the seeds.

Chat with Shaun:
Accommodation in Boston must be through a University approved site/place. The hotel booked out by iGEM is expensive, but they got around this last year by using Flipkey, which is run by Tripadvisor. Rent an apartment for a few days, much cheaper.

Getting exams shifted or funded for sitting overseas needs to be done, but we have a good argument as we are representing the university at an international competition.

Jason Busby is the Gibson reaction main user, and Shaun will introduce us and hopefully he'll help us out.

Monday 260617
Sowing 2: Q567, Columbia, 260617, JG/JC
Sowing was carried out in same way as previously recorded, just with 3 seeds per hole instead of 2 at Wendy's suggestion.
Previous sowing number for wt seeds had been P977-1
Planted out 3 trays.

Thursday 290617

Collected the primers.
Can be suspended in TE buffer (10 mM Tris, 0.1mM EDTA, pH 8.0) or
Nuclease free water.

Choose to go with Nuclease free water. Spun down the tubes to ensure no dna had been dislodged from the pellet and could be lost. Quick spin down can be achieved by pressing the quick spin button on the benchtop centrifuge.

Trimmer the flower heads from plants, watered.

Friday 300617

Had lab meeting at Jess' house. Discussed the current lab plans with Peter, the process for recruiting a second year helper, and wrote out a labplan with expected timings.

Mondy 030717

Removed the germination tent from the seedlings, watered plants and trimmer the flower heads.

Tuesday 040717

Checked in on the plants and cut the flower shoots.
Talked to Davide about the electrolyte leakage assay and containment surrounding removing leaves from the pant hall of conductivity testing.
Continued the laboratory timeline refinement. Read the Gateway protocol in preparation for beginning the cloning process.

Wednesday 050717

Watered plants and changed out tray to reduce algae growth. Talked to Jo about getting started on the cloning process. Jo talked to her postdoc, Mau, who has agreed to help us with the Gateway.

Started that afternoon the Gateway process.

We made up a 1% agarose gel, as the vector is many kb, and larger fragments needs a less concentrated gel in order to migrate effectively.
Weigh out 1g of agarose per 100mL of 1x TBE buffer (Green labeled container for gels) in a clean weigh boat, and add to the bottle before the buffer, as the agarose adds volume.
Gel is formed by microwaving for 3 minutes, mixing every 30 seconds until the agarose is dissolved and the solution clear. This gel can be remelted, so larger volumes can be made.

Following the microwaving, your solution will be boiling hot, so should be allowed to cool for a time before use. If the gel is too hot when poured into the mold, you can crack the cast.

Once the gel has cooled a little, add the Redsafe DNA dye. For 100mL of gel you require 5μL of dye. Swirl in the dye in gently to prevent the formation of bubbles. The Redsafe dye is photosensitive, and should not be kept outside of its tin longer than necessary.

When pouring the gel, fill the gel holder to ⅓. Make sure the barriers and well comb is in place before pouring. The gel will be set in approximately an 40 minutes. If you are not using the gel straight away you can pour some TBE buffer over it to prevent dehydration.

On this gel we were to run three samples:
DaIRIP4 fragment, 744 bp long.
pDONOR221, 4762 bp
pB2GW7, 10882
The concentration of the destination vector was not listed on the tube, so to be safe we used 3μL. For the donor vector and gene we used 2μL.

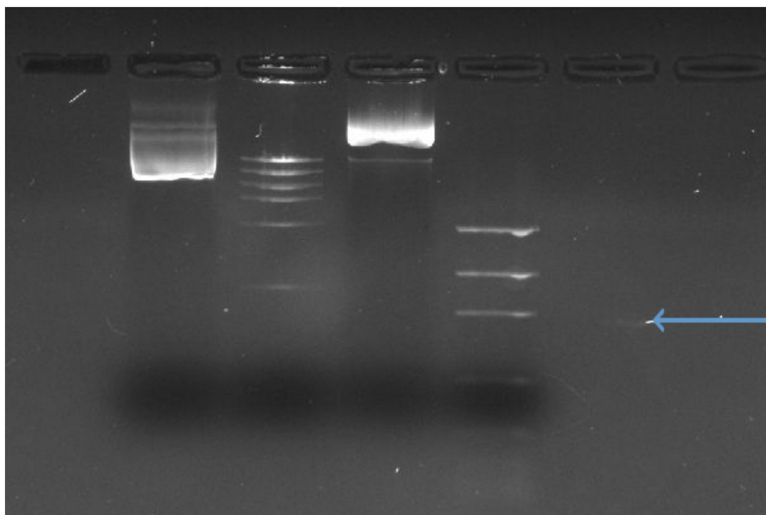
1μL of 5x GLB dye and 3μL of water were dropped onto a parafilm sheet, to which the sample volumes were added. When pipetting up the mixtures to load into the well is is recommended to have the pipette set to exactly the mixture volume or slightly below to avoid bubbles, which would drag some of your solution out of the wells.

The samples were loaded against both a high weight ladder for the vectors, and a low weight ladder for the gene. When loading the gene, we found the 5x dye was not dense enough to weigh it down, and much was lost from the well. We did not reload as the concentration of the gene was known, as was the length, and we were just running it for completeness.

Loading order:

pDONOR221	High Mass ladder	pB2GW7	Low Mass ladder	DaIRIP4

Run gel at 90V for 30 minutes.



The high mass ladder, loaded in the second well has bands corrisponding to 10 kb, 6kb, 4kb, 3kb, 2kb and 1kb in decending order. The pDONOR221, loaded in the second well sits just above the 4kb band. This corresponds well to its exected length, and as there is not evidence of small bands, we can be safe in the assumption that the vector has not degraded.

pB2GW7 can be seen to sit just above the the 10kb marker, which also corresponds well to its effected length. There is a second band, located at 10kb which is unexpected, but compared to the main band it is at an extremely low concentration.

The gene band, highlighted by the blue arrow, is very faint, which is expected condisering the loss of material during loading. However, is does fall just below the 800bp line on the low mass dna ladder, which proves the the fragment is likley the size we ordered.

Thursday 060717
When preforming the recombinations, the amount of DNA must be correctly balanced. The calculation shown below is used to convert femtomoles of DNA to nanograms of DNA.

$$ng = (fmol)(N)(\frac{660fg}{fmol})(\frac{1ng}{10^6 fg})$$

For our gene;
Ng = 50 x 744bp x (660) x (1/10^6)
= 24.552 ng

Gene suspended at 10 ng/μL.
V = 24.552 ng / 10ng/μL
= 2.45μL

Final solutions will be the following:

	Sample	Positive Control	Negative Control
	μL	μL	μL
Gene	2.445	1 (PEXP7)	1.225
Vector	1.0	0.5	0.5
Buffer	4.545	2.5	2.775
Clonase	2.0	1.0	-

The following is copied, with some editing, from the 2009 Gateway user manual.

1. Add the components, bar the clonase, to 1.5 ml microcentrifuge tubes at room temperature and mix.
2. Remove the LR Clonase™ II enzyme mix from -20° C and thaw on ice (~ 2 minutes).
3. Vortex the LR Clonase™ II enzyme mix briefly twice (2 seconds each time).
4. Add 2 μ l of LR Clonase™ II enzyme mix to the sample and positive control vials. Do not add LR Clonase™ II enzyme mix to the negative control vial. Mix well by vortexing briefly twice (2 seconds each time).
   Reminder: Return LR Clonase™ II enzyme mix to -20°C immediately after use.
5. Incubate reactions at 25° C for 1 hour.

Note: For most applications, 1 hour will yield a sufficient number of colonies for analysis. Depending on your needs, the length of the recombination reaction can be extended up to 18 hours. For large plasmids (≥ 10 kb), longer incubation times (i.e. overnight incubation) will yield more colonies and are recommended.

6. Add 1 μ l of the Proteinase K solution to each reaction. Incubate for 10 minutes at 37° C.
7. Proceed to transform a suitable E. coli host and select for expression clones.

Note: You may store the LR reaction at -20°C for up to 1 week before transformation, if Desired.

Our reaction was left in until 3pm, and began incubation at approximately 10:30-11:00.

Once we have formed our plasmids and transformed cells, plates will be required for growth and selections.

For 1L plating agar:
15 g Agar
10g Peptone
10g NaCl
5g Yeast extract

Mix the Peptone, NaCl and yeast extract with a lesser volume of diH20, say 700 mL until the powders are dissolved and the solution is clear. Then add the agar, with slight mixing and autoclave the solution.

As we needed two types of plates, the 1L solution was divided between two 500 ml bottles. The autoclaving process would cause the mixture to heat and melt, so the agar did not need to be fully integrated prior. We mixed up a LB broth solution to be autoclaved also.

While the agar was being autoclaved, we set up the laminar flow hoods.
Turn on the hood, and spray the work surface with Trigene. Allow to sit for 5 minutes, before wiping away. Follow with a ethanol wipe. When working in the laminar flow hoods, do not wear gloves. They are a fire hazard when using the bunsen burners. Remember to write your name in the roster when using the machine.
Clean hands with ethanol before beginning your work.

Set out all of your plates ready for pouring inside of the hood. You need to wait for the media to cool slightly before adding the antibiotics, and mix gently, but well. Avoid making bubbles, as this will effect the plate surface. Quickly pour the plates before the media begins to solidify.
Plates should be filled to about a 3rd of the dish, around where the edge of the lid sits when the plates are closed.

Allow the plates to cool with the lids off, to avoid the formation of condensation.

My (Judith's) plates had minimal condensation on use. Andra closed her plates a little earlier and has a more significant condensation, so at least 30-40 minutes of cooling time is recommended.

Store the plates back in their bags, upside down, in the fridge.

The donor vector concentration is 148ng
Destination is ~300ng we think
Buffer used was the TE buffer from the Gateway kit.

At around 3:00pm we added the proteinase K to the incubated solution, and incubated for 10 minutes. We then transformed the plasmids into the cells using electroporation.
For this, 2ul of plasmid was added to the tube of electrocompetant e.coli, which had been thawed on ice following removal from the -80°C freezer. Mix gently and pipette the contents into ice cold electroporation cuvettes. Pipette directly into the 1mm well, and avoid forming bubbles. Bubbles cause arcing, which will fry your cells. If there are bubbles, firmly tapping the cuvette on the bench can be effective.

Before placing the cuvettes in the electroporation machine, make sure to wipe down the sides of condensation. Set the voltage to 1.8V. Press both the charge buttons down simultaneously, and release when the bell tolls. Immediately add 500?uL of room temperature SOC solution to help the cells recover. (When dealing with argo bact, use cold SOC). Transfer the solution from the cuvettes to a tube, and incubate for 20 minutes to allow the antibiotic resistance genes to be transcribed.

Plated out the experimental, positive and negative controls. For controls? 100 uL onto amp plates.
For experimental, two plates are done, one with 100uL and one with 20uL to ensure isolated colonies are produced. Incubate plates at 37 overnight.

When cleaning the cuvettes, soak in 1% SDS for 20 minutes, before draining and leaving in drum ethanol overnight. Then drain, leave to dry and recap.

Friday 070717

Colony PCR time, to check whether the cells produced yesterday have the insert in their plasmid.

Will be using M13 forward and reverse primers for the donor vector.
Freezer Box Location:

5A F3 M13F
5A F4 M13R

The following volumes are for 1 15uL PCR run. All in uL.

Nuclease free water: 10.8
5x PHIRE buffer: 3
10uM dNTP: 0.3
10uM F primer: 0.3
10uM R primer: 0.3

Times by the number of samples, plus a water control and one extra for pipetting error.

Aliquot the master mix into the PCR tubes.

Grid up a master plate into 20, numbering each box. On one of the experimental plates with isolated colonies, mark out 20 colonies to test.

Working within the hood, take a sterile toothpick and touch a corner to a chosen colony. Touch the toothpick twice to the plate in the correct grid. Then take the pick and mix is quicky in the PCR tube to inoculate it. Do not mix for too long, as the wooden pick will begin to absorb the PCR mix. Repeat for all colonies, then seal and spin down the tubes.

Run a standard PHIRE PCR protocol on the PCR machine.
Placed the master plate into the incubator. Will need 24 hours, so will take out before leaving and will place back into the incubator on Monday.

The primers should produce a 1kb fragment if the insert is present. As this is a relatively small fragment, make a 1.5% agarose gel.

- Generated PCR products using gel electrophoresis

1. 5 µl blue dye (gel loading dye) added to fragments in all tubes using micropipette. Pipette tip changed each time dye added in new tube.
2. Vortexed each tube gently 2x using micropipette to mix fragments with dye, avoiding introduction of bubbles.
3. Loaded 15 µl of fragment-dye mixture from tubes into individual wells in gel for gel electrophoresis. Pipette tip changed each time fragment-dye mixture added into gel.
4. Wells 8 and 23 left empty and filled with low molecular-mass DNA ladder.
5. Second row of wells (well #16) was filled with fragments from tube 15.
6. Gel electrophoresis apparatus connected to power supply and set to 90 Volts for 35 minutes.
7. After allocated time, images taken of gel to show fragment migration:
    - Wells 2,6,9 and 22 (#22 is water control= no bacteria) showed no bands.
    - Only wells 10,12,13,15,16,18 and 19 generated 3 bands, with one of the 3 bands being bright.
    - Well 15 showed a brighter band with double the thickness. Possibility of double insert?
    - The rest of wells (1,3,4,5,7,10,13,15,18,19) each generated 2 faint bands.
    - Fragment size will be compared with the DNA ladder.

Monday 100717

Placed the master plate back into the incubator to increase colony size.
Set up the broths for plasmid purification.

For each colony to be amplified (we chose four, 9, 11, 12, 14) 4 mL of broth is required, with a final concentration of 50 µL/mL ampicillin.
We therefore made a master mix of 16 mL broth, to which we added 16µL of 50mg/mL stock ampicillin.

On removal of the plate from the incubator, we found that there had been bubbles formed between the agar and the base of the plate. They were not affecting our colonies, and we suspect it was a result of the agar being heated to quickly following direct removal from the fridge to be placed in the incubator.

Once the bacterial colonies on the master plate were sufficiently large, we scraped off one of the two colonies of each of the chosen type and inoculated the broth tubes by dropping the toothpick in. These we incubated with shaking at 37 degrees overnight.

We also investigated which restriction enzymes could be used for a restriction digest once the plasmids were purified. We found that SstI was a gene specific, single cutter, which when combined with HincII, a single plasmid cutter, would give us evidence of correct insertion should the correct fragment sizes be observed.

Tuesday 110717

Purified the donor plasmids.

The following protocol was used from the Zymo Research **ZR Plasmid Miniprep™-**Classic kit.

1. Centrifuge 0.5 - 5 ml[1,2] of bacterial culture in a clear 1.5 ml tube at full speed for 15 - 20 seconds in a microcentrifuge. Discard supernatant. Repeat until all 4 ml of broth has been pelleted.

    During this step I spilt ~1mL of sample 12 broth.

2. Add 200 µl of **P1 Buffer** (Red) to the tube and resuspend pellet completely (i.e., by vortexing or pipeting).
3. Add 200 µl of **P2 Buffer** (Green)[3] and mix by inverting the tube 2 - 4 times. Cells are completely lysed when the solution appears clear, purple, and viscous. Proceed to the next step within 1-2 minutes.
4. Add 400 µl of **P3 Buffer** (Yellow) and mix gently but thoroughly. Do not vortex. The sample will turn yellow when the neutralization is complete[4]. Allow the lysate to incubate at room temperature for 1-2 minutes.
5. Centrifuge sample(s) for 2 minutes.

6. Place a **Zymo-Spin™ IIN** column in a **Collection Tube** and transfer the supernatant from Step 5 into the **Zymo-Spin™ IIN** column. When pipetting the supernatant be careful not to disturb the green pellet to avoid transferring any cellular debris to the column.
7. Centrifuge the **Zymo-Spin™ IIN/Collection Tube** assembly for 30 seconds.
    Note that the collection tube assembly does not have a lid, so tubes must be sealed with parafilm.
8. Discard the flow-through in the **Collection Tube**, making sure the flow-through does not touch the bottom of the column. Return the **Zymo-Spin™ IIN** column to the **Collection Tube**[5].
9. Add 200 µl of **Endo-Wash Buffer** to the column and centrifuge for 30 seconds.
10. Add 400 µl of **Plasmid Wash Buffer**[6] to the column**.** Centrifuge for 1 minute.
11. Transfer the column into a clean 1.5 ml microcentrifuge tube and then add 30 µl (of **DNA Elution Buffer**[7] to the column. Centrifuge for 30 seconds to elute the plasmid DNA.

Once purified, the plasmid concentration was determined using the Nanodrop.

The lab in which the Nanodrop is located has strict health and safety restrictions, so be sure to always wear safety glasses. Gloves are needed, but are not to be worn when opening doors. Bring your own pipettes and tips, as these are not provided in the spec area.

When first using the nanodrop, ensure it is clean. Pipette 1µl of water onto the stage, close the lid to wash, and then wipe the stage and lid with the fine tissues.

Select Nucleic Acids from the menu. Place 1µl of the DNA elution buffer in which the plasmids were suspended onto the stage, close the lid, and press Blank.
Wipe down the stage and lid with a tissue, the pipette 1µl of the plasmid solution onto the stage. Close the lid and press Sample. Record the displayed concentration.
Wipe down the stage and repeat with the remaining samples.

Final concentrations are:

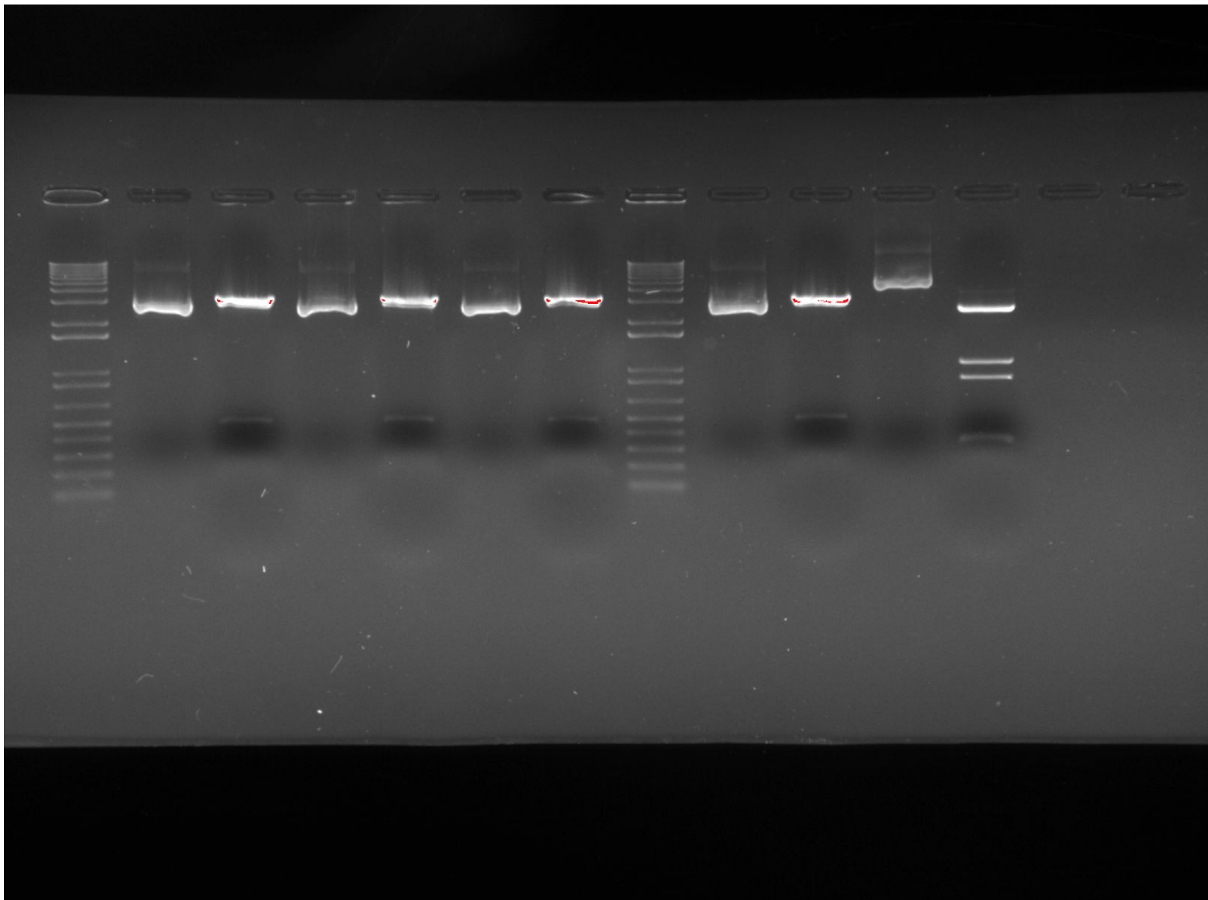
9	144.45 ng/µl
11	131.80 ng/µl
12	129.65 ng/µl
14	163.45 ng/µl

Began the restriction digest to test insert was correct.
Digested all 4 plasmids plus the donor vector as a control.
Made up the following master mix:

H_2O	9µl	45µl
10x Sst1 buffer	2µl	10µl
10 u/µl Sst1	1µl	5µl
10u/µl HincII	1µl	5µl

13µl of the above master mix was aliquoted out into 5 tubes, and then 7µl of the appropriate plasmid was added to each sample. This was incubated at 37 degrees C. Left to digest until ~3:00pm.

Made up a 1% agarose gel and ran both the undigested and digested fragments.
Order is as follows:
High mass DNA ladder, 9 uncut, 9 cut, 11 uncut, 11 cut, 12 uncut, 12 cut, high mass DNA ladder, 14 uncut, 14 cut, donor uncut, donor cut.



All of the digested samples showed the expected fragment, and none were cut multiple times like the digested donor plasmid, showing that the multiple cloning site had been removed.

Following confirmation of the insert, we proceeded to the LR Gateway recombination reaction.

Decided to proceed with colony 14 since it had the highest concentration. Required approximately 75ng of plasmid, so decided to add 0.5µl to a total of 81.7ng.

Made up the following three solution in pcr tubes:

	Sample	Positive Control	Negative Control
	µL	µL	µL

Gene	0.5	1.0 (pENTR-gus)	0.5
Vector	0.5	0.5	0.5
Buffer	7.0	2.5	2.0
Clonase	2.0	1.0	-

As last time, solutions added buffer, vector, gene, with clonase last. The LR clonase is stored in box 1B15. Left for 16 hours at 25oC from 4pm till 9:30am the following day.

Wednesay 120717

Added the proteinase K to the recombination samples. Incubated for 1 hour.
Transformed DH0B cells via electroporation. During the process the positive control sample arched, so no colonies expected to form. Added the SOC and incubated from 10:50 to 11:50 to allow antibiotic resistance expression.
During this time let the spectinomycin plates prewarm in the laminar flow hood.
Plate out as previous, with 100µL of controls, and both 100µL and 20µL of sample. At 12:15pm plates were placed in 37oC to incubate.

Began plant experiments.

Went down to the Warehouse a purchased two plastic tubes with lids large enough to fit the plant trays for $9.00.

To ensure the conditions between the experimental and control tub would be as similar as possible, I covered the inside of the control tub with tinfoil to block out the light. This is to mimic the lack of light experienced by the experimental plants during their time in the fridge.

I took 6 mature leaves from the plants to use for the electrolyte leakage assay. These were washed and dried to remove any transgenic pollen contamination. Following this they were placed into 15mL falcon tubes, and 14ml of deionised water was added.

To prep the plants I removed all of the flowers stalks and placed them into the containers. I did not water the plants beforehand to try and reduce excessive condensation/humidity in the room temperature control container. The samples for the electrolyte leakage assay were placed into the tubs beside the plants, and the containers sealed. The control was left in the growth room, and the experimental tub placed in the fridge. Incubation began at 2:30 pm.

Thursday 130717

Tray 1 = Control
Tray 2 = Experimental

The control tub had condensation, however this was to be expected.

The control plants had regrown new flower stalks overnight, whereas the experimental plants showed no such growth.

Plants were returned to growth trays and watered. Tubs were washed with water and trigene, then placed on a shelf for later use.

Friday 140717

Gene specific primers for next batch of colony PRC had still not arrived.
Spent morning updating lab log. Cleaned up lab space and organised samples.

Experimental plants has resumed growth of flower shoots. No apparent signs of distress. Suspect that the fridge is not cold enough to cause cellular damage, definitely need below freezing temperatures. If none of the equipment in the plant hall can go subzero, may be able to mimic the effects by packing ice around the containers.

Wrote the doodle poll to send out to potential second year lab assistants.

Monday 170717

Still no primers.
Moved on to protein prep work. Talked to Jason about next steps, and found we need to produce a new batch of pET22b + plasmid for recombination.

As there was no pet22b+ plasmid purified, we need to grow some more up from a frozen glycerol stock of bacteria. Bacteria do not like to be freeze/thawed, so we took a pipette tip and scraped some of the top layer of the frozen tube and plated those. Plate was a standard ampicillin plate.

Plant experiment - no sign of permanent injury to experimental plants group. Possibility that the cold exposure has increased the rate of flowering due to stress. Watered plants and trimmed flower shoots.

Received an email from IDT stating that our primers were missing in transport, and would likely be further delayed. Window for plant flowering is closing, discussed options with Geoffrey. He has primers which are not gene specific, but will sufficient to make a hailmary go ahead much less risky.

Tuesday 180717

If needing to do further seed sterilisation, wt columbia seeds are located in Box 7, J6/J7

Primers:

pBG2W7 R	Box 14J7
35SF	Box 5I10

Ran a colony PCR reaction with Geoffreys primers due to the delay with our own.

	15μL Run	x22 Run
Water	10.8	237.6
PHIRE Buffer	3	66
10mM dNTP	0.3	6.6
10mM 35SF	0.3	6.6
10mM pBG2W7R	0.3	6.6
PHIRE TAQ	0.3	6.6
DNA	Swab	6.6

Ran 20 colonies and made a spec master plate. Used Geoffrey's PRC settings which includes and extra boiling step to ensure complete cell lysis before the amplification begins.

For protein work, inoculated liquid culture with the pET22b bacterial stock that had grown on the plate. With will allow amplification for plasmid purification on Wednesday.

Wednesday 190717

Firstthing in the morning I took the PCR tubes out of the machine and placed in the fridge. Also placed master plate into fridge.

Peter completed the plasmid miniprep extraction. Plasmid stock still needs to be nano dropped.

Made up a 1.5% medium long gel. Watered the plants and cut flower shoots. No evidence of damage to experimental plants.

Ran the gel for the colony PCR products and the water control. X2 15 well gel.

Added 1μL of 10x Bluejuice to each PCR tube, mixed and spun down before loading. Added 4μL of ladder to wells 8 and 23. Ran for 35 mins at 90V.

Inserts are as expected for our gene being present, so chose to continue despite not having used gene specific primers. Colonies 8, 9, 10 and 12 chosen.

Grown up overnight in 4mL of LB with 50 μg/μL spectomycin.

Thursday 200717

Investigated restriction enzymes for use in digest. Found the in the destination vector Sst1 cuts once in the gene as per last time, but also once in the vector.

Carried out miniprep plasmid extraction. Took 1mL of the colony 8 solution, spun down, discarded supernatant and resuspended in glycerol to make a frozen stock.

Stock number 5141 Box 3G-2 D5

Nanodropped purified plasmids

#8	127.35 ng/µL
#9	138.00 ng/µL
#10	141.30 ng/µL
#12	133.80 ng/µL

Carried out the Sst1 digest on the plasmids to double check insert. Started digest at 12:47pm

H_2O	10µl	40µl
10x Sst1 buffer	2µl	8µl
10 u/µl Sst1	1µl	4µl
Plasmid	7µl	-

Friday 210717

Ran a small gel of the digest, which was removed from incubation at 9am.

2µL of sample and 1µL of dye for the uncut samples, 8µL of sample and 2µL of dye for the digested samples. 4µL of DNA ladder. Ran the gel for 40 minutes at 90V.

8uc, 8c, 9uc, 9c, Low Molecular weight ladder, High molecular weight ladder, 10uc, 10c, 12uc, 12c.

Plants - Cut the flowers from both trays. No argobacterium ready to go so needing to cut the bolters in order to promote further flowering.

Talked to Lulu about the agrobacterium transformation process.
Argo strain GB1310 in box 5316?

Needed new plates for agrobacterium expression containing Rifampicin, Gentamycin, and Spectomycin.

Made up 500ml of agar to be bench-topped autoclaved.
Weighed out 5g peptone, 5g NaCl, 2.5g yeast extract, mixed with 300mL diH2O. Topped up to 500mL, then added 7.5g agar.

Set up the bench top autoclave by adding 800mL of deionised water. Place the lid on with the arrows aligned, twist to tighten. Press go, and will run for approximately 20 minutes followed by a period of depressurizing.
Tip out the water once you are finished to prevent the growth of organisms in the autoclave. The sides are not insulated, so DO NOT TOUCH! Goes up to 121 degrees.

Stocks		Final
Gent	50ng/mL	50μg/mL
Spec	100ng/mL	100μg/mL
Rif	50ng/mL	50μL/mL

500mL of agar, so requires 500μL of antibiotics. Used 20x plates.

Monday 240717

Continued work with Lulu. It is good practice to wipe down your pipettes before use in the hood.

Transform the agrobacterium as you would transform E.coli (via electroporation), with the slight change being the addition of cold SOC instead of room temperature SOC after applying the current. We chose to use sample/plasmid strains 9 and 10 to go on with, and added 2μL of plasmid to each argobacterium tube for transformation.

Had repeated arcing in sample 9. Possibly there is too much salts in the plasmid.

Later at 4:00pm in afternoon- plating of antibiotic resistant plates

1. 2X agar plates of spec, gent, and rif., were taken out of fridge to warm up= 6 plates in total. Cells grow better on warmer plates rather than cool.
2. Label agar plates with name, date, room number, either sample 9 or 10, and either 100 μl, 10 μl, or 5 μl.
3. Samples 9 and 10 added to lab incubator (37 $\circ C$ for 5 min) to warm.
4. Laminar hood workspace sterilised
5. Glass spreader submerged in ethanol
6. For each sample (9 & 10), prepared and labelled 3 tubes to pipette 100 μl, 10 μl, 5 μl of the sample. 6 Tubes in total.
7. Label agar plates with name, date, room number, either sample 9 or 10, and either 100 μl, 10 μl, or 5 μl.
8. Take beakers containing samples 9 & 10 out of incubation
9. 90 μl of LB agar was added to the 10 μl and 5 μl tubes for both samples 9 & 10.
10. Add quantities of either sample into respectively labelled tubes (i.e 5 μl of sample into 5 μl-labelled tube). Done this for both samples 9 & 10. Mix by pipetting liquid up and down.
11. Glass spreader sterilised by taking out of ethanol submersion and flaming.

12. Pipette each sample-LB agar mixture from tubes onto agar plates containing different antibiotics.
13. Use sterilised glass spreader to spread mixture evenly on dish
14. Leave under fume hood with lid off to air-dry, before returning plates to incubation room for 5-6 days.

Thursday 270717

Miniprepped pET22b+ vector to increase our stock for future transformations. Protocol took two days. Nanodropping showed the final concentration of pET22b+ to be 137.2 ng/µL.

Friday 280717

Miniprep extraction of plasmids from argobacterium colonies grown overnight previously 1, 2, 3, and 4. When nanodropped, the final concentrations were:
Colony 1: 26.7ng/uL
Colony 2: 87.45 ng/uL
Colony 3: 99.800 ng/uL
Colony 4: 40.550 ng/uL
Prior to obtaining these results, we had decided that a concentration of roughly 50ng/uL would be suitable for continuation, but below that was unlikely to yield productive results. Therefore, we chose to continue transformations using only plasmids from colonies 2 and 3, the two which had higher concentrations than this.

Transformation into E.coli was done via electropoation, as before, using strain DH10β. One line was created for each of the two plasmids, and have been appropriately labelled. This was done to ensure the plasmids in our Agrobacterium transformant strains were still carrying the insert.

Monday 310717

Trimmed plants of flowers in preparation for transformation on Thursday.

Monday 310717 Later that day

Did colony PCR

1. 8 colonies of each plasmid done in total.
2. Colonies 3mL and 2mL (DH10β & LZ) chosen for 2 spectiomycin plates.
3. Prepared 80ulL of PCR stock solution in 16 PCR tubes (2x sets of 8, for colnies 3ml and 2ml).
4. On each spec. petri dish, drew grid lines to mark 16 areas (in a 4x4 pattern).
5. For plate 1 of sample DH10β (2mL):
    a. Used thin end of sterile toothpicks to do a bacterial swab of 1 individual colony from the plate.

    b. Toothpick then dabbed onto area 1 on spec. Plate (determined by grid lines).
    c. Toothpick then gently shaken in 1 of the PCR tubes with PCR solution already present to introduce some colony bacteria into the tube.
    d. Repeated for steps a) - c) for 8 more samples from the gene plate onto the spec plate.
6. For plate 2 of sample DH10β (3mL):
    a. Repeated steps 5. a) - d).
7. **Table 1**: A master mix of PCR volumes/ concentrations was made using Lulu's template of:

Substance/ Concentration	Volume (uL)	Temperature (degrees Celsius)	Duration (seconds)
5x buffer	2	94	600 (=10 min)
25um DNTP's	0.08	98	30
10um Forward	0.25	98	10
10um Reverse	0.25	55	10
Taq enzyme	0.2	72	10
H20	6.22	72	60
Bacterial dilution	1	15	Keep until use

Note: The Forward, Reverse, and Taq were run at 35 cycles.

8. **Table 2**: To make the PCR master mix, the volumes of each of the substances were increased 18x from original volumes to generate:

Substance/ Concentration	Volume (uL)
5x buffer	36
25um DNTP's	1.44
10um Forward	4.5
10um Reverse	4.5
Taq enzyme	3.6
H20	111.96
Bacterial dilution	18

9. The substances, along with their respective volumes, were all pipetted into a PCR tube, starting with the water first (to prevent contamination).

10. The master mix was then centrifuged for 30sec to ensure all contents were mixed thoroughly.
11. 8 new, empty, PCR tubes were dedicated to sample DH10β (2mL), and 7 new, empty, PCR tubes for sample DH10β (3mL), creating 17 PCR tubes. The final PCR tube (#18) was used as a control with just water added.
12. 9mL of the PCR master mix was added to each new tube for both samples.
13. 1mL of the old PCR tubes (where the toothpick containing the bacteria was swished inside) was added to each new PCR tubes for both samples, with respect to the sample (i.e old PCR tubes containing the 2mL bacteria was placed in the new PCR tubes designated for the 2mL samples, etc).
14. Centrifuge both sample sets of the PCR tubes for 30sec to mix contents. Make sure no bubbles were introduced. If there are bubbles, centrifuge again.
15. Put both PCR tube sets in the incubator, following the temperature and duration instructions from **table 1**, taking note of the cycle number.

Tuesday 010817

Prepared a medium-short 1% agarose gel for running the E.coli (containing the agrobacterium plasmids extracted and transformed on Friday) colony PCR. Used a 20 well comb. Into each PCR tube we added 1μL of 10x Bluejuice loading dye, mixed and spun down. The samples and a low molecular weight DNA ladder were loaded in the following order:

Ladder, Colony 2-1, Colony 2-2, Colony 2-3, Colony 2-4, Colony 2-5, Colony 2-6, Colony 2-7, Colony 2-8, Ladder, Colony 3-1, Colony 3-2, Colony 3-3, Colony 3-4, Colony 3-5, Colony 3-6, Colony 3-7, Water Control.

Insert Picture

When loading the gel, we found sample 3-2 had a much lower volume than the other samples.

Began the flower dip protocols, as provided by Lulu, shown below. See notes on today's activities after.

Arabidopsis transformation (Christine)

Solutions required:

YN (1L)
3 g Beef Extract
5 g Bacto Peptone
8 g NaCl
10 g Yeast Extract
Adjust pH to 7.3 with NaOH

IM (Infiltration media) (1L)
4.4 g of MS plus vitamins powder
50 g sucrose
Adjust pH to 5.8 with KOH
Just before use add 10 μl of BAP 10 μg/L (final conc)

Things to be aware of:

- Contained as much as possible the transformation area
- Gloves, plant material etc go in bug bags (incinerated)
- *Agrobacterium* culture, glassware are treated with hypochlorite

Growing plants for transformation

- Vernalize seeds as usual and spread out on a small block of rock wool in a grid like manner

Day 1

- Set up 10 ml culture in 50 ml flasks. Use YN supplemented with the appropriate antibiotic. For pART27 add 10 µl Spectinomycin (stock 100 mg/ml), 10 µl Gentamycin (stock 50 mg/ml), 10 µl Rifampicin (stock 50 mg/ml).
- Grow overnight at 28 °C with shaking.
- prepare 1L YN in a sterile 4L flask and autoclave
- prepare 500mL IM per 1L culture you want to grow and autoclave

Day 2

- In the afternoon (~2-3 pm) inoculate 1L of YN (+ antibiotics) with 2.5 ml of the overnight culture. Keep the remaining culture at 4°C.
- Optional: Perform DNA extraction to check presence of the right plasmid
- put the IM in the fridge

Day 3

- Check O.D when you arrive in the lab. Continue to grow the culture until the O.D reaches 0.8 at 600 nm.
- Centrifuge the culture at 4500 rpm, 4 °C, 20 min, switch on the pump in the greenhouse, it needs to run 30 min prior use. Follow strictly the instructions of “how to use the pump” that are on a sheet close to the pump.
- Resuspend the pellet (from 1L culture) in 500 mL of cold IM (may require the use of a 5 mL tip) Remember to add 5 µl of BAP (stock solution is 1µg/µL) in the IM medium

In the greenhouse:

- Cut already existing siliques off the plants that will be transformed
- Add 100 µl of Silwet-L77/500 mL to 500mL of *Agrobacterium* culture in IM and mix.
- Transfer *Agrobacterium* solution into two (or more…) small beakers and place in the vacuum chamber.
- Take the rock wool blocks and place upside down onto the beakers. Check that most of the inflorescences are in the *Agrobacterium* solution.
- Apply vacuum for 10min
- Remove the plants and lie the blocks onto paper towels to let the *Agrobacterium* solution drip off
- Return the plants to the cabinet and keep them there until they start dropping seeds

Began by making up 2L of YN.

Weighed out the ingredients as listed above x2 into a 2 litre beaker. The beef extract is very sticky, and should be weighed out directly into the beaker. The other powers can be weighed in a boat as usual.

Topped the beak up to ~1.5 litres with diH2O and mixed using a stirrer bar. Once all ingredients were dissolved, we adjusted the pH to 7.3 using the pH meter and NaOH.

When using the pH meter, the probe should remain in its storage solution when not in use. When ready, remove the probe and rise with diH2O into a waste beaker. Place the YN beaker onto the stirring plate, and set the stirrer bar to a medium speed. Lower the probe into the solution, ensuring the end of the probe is fully submerged.

Using a plastic pipette, slowly add drops of NaOH, pausing to allow the reading to stabilise, until the pH is at the desired value of 7.3. Once this is complete, remove the beak and rinse the probe down with diH2O, before returning it to the storage solution.

Once the pH of the YN is adjusted, pour the solution into a large measuring cylinder, and top up to 2 litres. Divide this solution between two 3L flasks, cover with tinfoil, and set aside for autoclaving.

Next mix up 1 L of infiltration media.

Weigh out the ingredients as above, place into a beaker and top up to ~800mL with diH2O. Mix until dissolved. The MS powder is located in the top right hand shelf of Jo’s fridge door.

Again adjust the pH, this time using KOH. When doing this we overshot to pH 6.4, so brought it back to 5.8 using HCl.

Once adjusted, pour into a measuring cylinder and top up to 1L. Split the solution between 2 1L bottles.

Took both the YN and IM to the kitchen for autoclaving.

Using some of Lulu’s YN, we made up 20mL of YN + Spec + Rif + Gent solution in the hood. Divided between two 50mL flasks. Inoculated flasks with strains 2 and 3 of agrobacterium from the master plate by scrapeing the colon off with a toothpick, and mixing around in the YN. Bunged and covered flasks with tinfoil and placed in the 28 degree C shaker for overnight incubation.

Collected solutions, placed YN on bench and IM into fridge for later use.

Wednesday 2/08/2017

Using antibiotic solution prepared by Lulu we prepared two large 1L flasks of YN+antibiotic

solution (Should Probably have final Conc of Antibiotics here). Due to lack of growth by strain 2 both flasks were inoculated with 2.5mL of previously grown strain 3, and left to incubate overnight in the 28 degree shaker.

03/08/17
At 9:00am the OD of the two flasks was:
1 - 1.082
2 - 0.940
Flask to is closest to the desired OD of 0.8, and so will be the solution we use.

Following the instructions list under Day 3 in the above protocol, we transformed 10 plants.
[Get specific pump use instructions from plant hall.]

Transforming plants is equivalent to a second sowing, so plants were applied a new sowing number of Q585.

We also made a -80 stock of our transformation strain, with the following details.
Locations: 3G-2, D7.
5143 Stock
Agrobacterium, GB3101, strain 3.
pBW2G7, DaIRIP4

07/08/17
Watered the plants. Was advised by Davide that they would require bagging tomorrow.
Talked to Wendy and will also bag some of the wild type plants to replenish the columbia stock we have been using. 4 plants will be plenty, be sure to pick healthy ones.

10/08/17
Sterilized some more P977-1 seeds with Andra.

14/08/17
Planted out 2 blocks of seeds, sowing number Q599.
Aquired remaining parts from last years distribution kit. Stored into freezer box and fridge 2.

15/08/17

Ran linear PCR reaction using pET22b+ vector and 2x Kapa HiFi HotStart readymix (KHH)

As the full concentrated stock solution of our pET vector was 137.2ng/ul (as prepared on Thursday 27-07-17), this generated to 0.036ul, after calculations, to be micro pipetted into each PCR tube. This is too small to be pipetted, so we diluted our vector 1:1000 to generate a concentration of 0.1372ng/ul and volume of 0.7288ul which we can use. From this, we set up the following mixtures in 2 PCR tubes:

Substance	Volume (ul)
KHH	25
Forward primer (stock at 10uM)	1.5
Reverse primer (stick at 10ul)	1.5
Vector	0.7288
Water	21

Then ran PCR program on machine, using:

- Initial denaturation- 5min at 95 degrees (celsius)
- 30 cycles of:
    1. Denaturation- 20sec at 98 degrees
    2. Annealing- 15sec at 55 degrees
    3. Extensions- 5min at 72 degrees
- Final extension- 5min at 72 degrees

After PCR process finished, took PCR tube-set out, cut them into individual tubes (as only 2 out of the whole set were filled with solution), and left in freezer to use tomorrow.

17/08/17
Ran a 1% gel of the pET22b+ linear PCR fragments for 35min and 90V. Found there to be multiple bands with a background smear. After discussing with Shaun we have found it likely that the annealing temperature was too low, and non specific binding has occurred. Will need to run further trials to determine the best temperature and achieve a single band.

18/08/17

Decided to use gradient PCR machine to test range of annealing temperatures (55-62 degrees celsius) to hopefully get better results.

Set up master mix for 12 new PCR reactions using the standard ingredients and volumes (PHIRE TAQ polymerase, 10 uM forward primer, 10 uM reverse primer, 10 uM dNTPs, water, 5x buffer, and DNA*).

*In this case, the DNA was our vector, of which we used 3 ul in our mastermix.

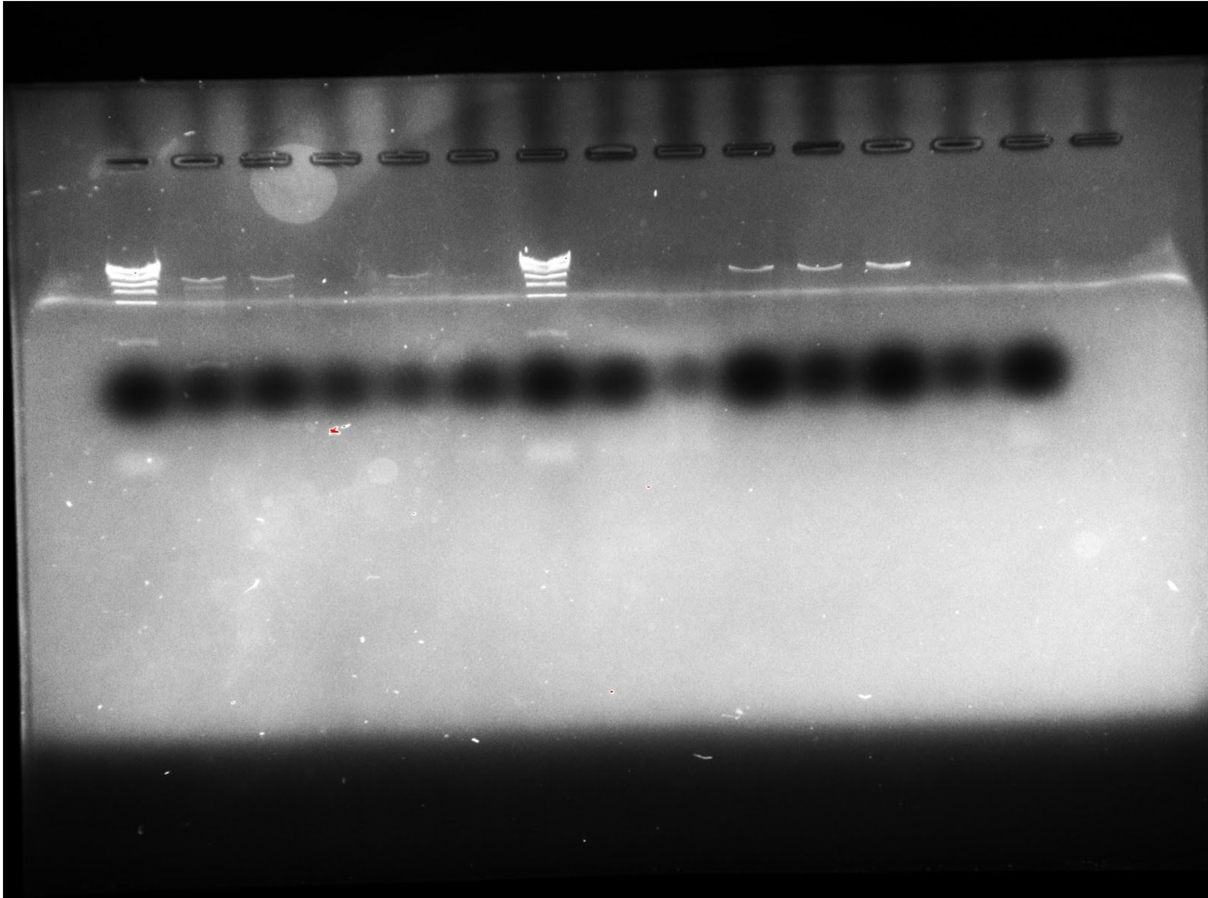
Centrifuge mastermix to ensure proper mixing.

15ul of mastermix solution was added to each of 12 PCR tubes.

The tubes were then centrifuged to ensure proper mixing, before being placed in the gradient PCR machine, with each tube receiving a different annealing temperature- ranging from tube 1 at 55 $\circ C$, to tube 12 at 62 $\circ C$.

Once PCR reaction finished, ran a 1% gel of the pET22b+ linear PCR fragments for 50min at 90V.

Made mistake: well 14= PCR tube #6. Everything else correct.



Both the ladders in wells 1 and 7 were not sufficiently dispersed. This could be due to the run-time being too short, as we had both a large fragment to work with and used a 1% gel mould. We can try with 3% gel. However, we did get 3 clear, single bands in wells 10, 11, 12, which correspond respectively to temperatures: 60.7 $\circ C$, 61.4 $\circ C$, 61.8 $\circ C$.

As we know that these temperatures yield successful results, we will use those temp. values in subsequent reactions.

21/08/17

Did nanodrop on PCR samples to check plasmid concentration.

Sample 9: 493.60ng/ul
Sample 10: 503.65ng/nl
Sample 11: 655.45ng/nl
Sample 12: 588.80ng/nl

Also repeated gel electrophoresis using samples 9-12 again to see if we could get evenly distributed bands. Used a 1kb+ DNA ladder at 5ul volume (as was diluted with water and already contained blue juice), a medium-short gel size on 1% agar, and a mini gel size containing 3% agar. Left both for 40min at 90V.

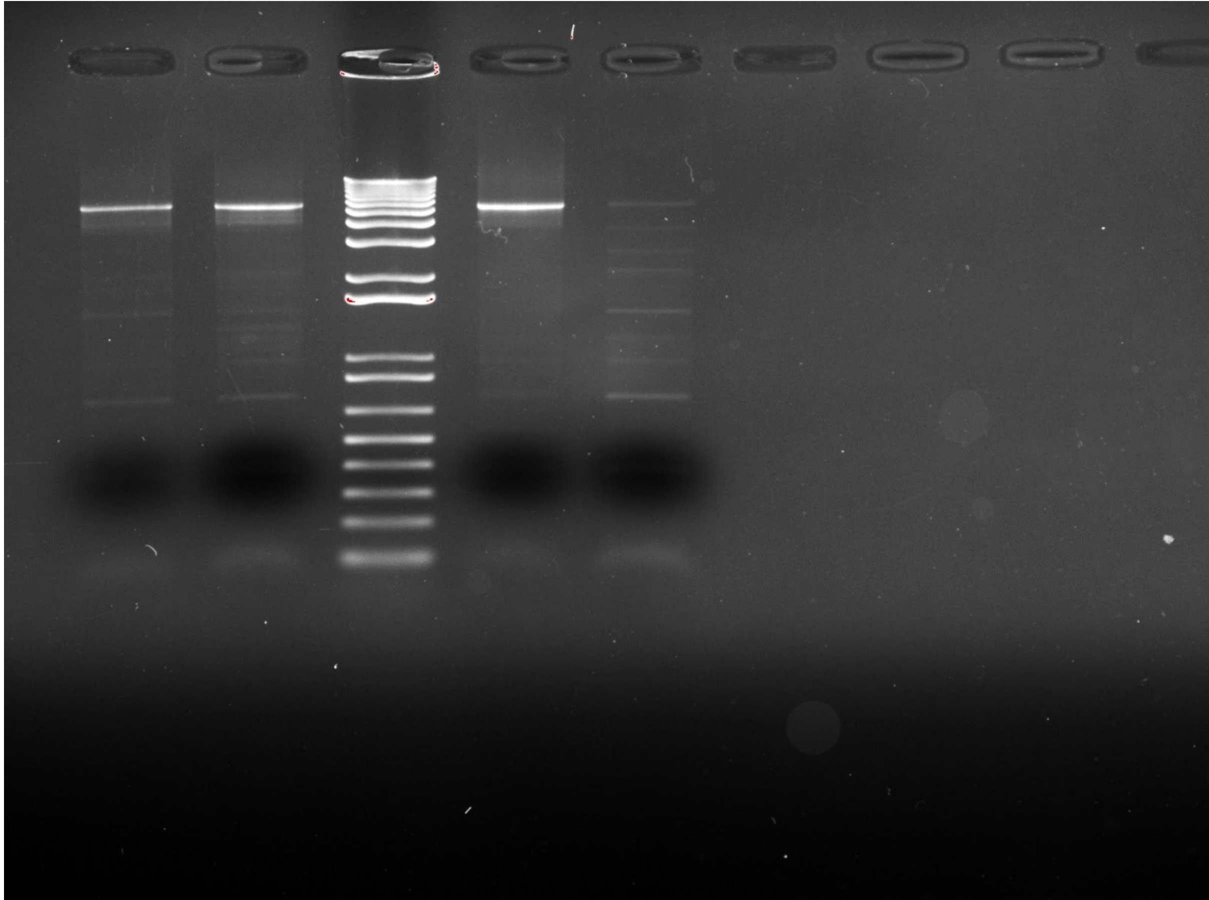


Image 1: Medium-short 1% agar gel with 1kb+ ladder after 40 minutes run-time

This ran very well, and we got bright, clear bands in wells 1, 2, and 4, indicating samples 9, 10, and 11 respectively. No band in well 5 (for sample 12). All 3 bands are identified to be around 5000bp according to the ladder in well 3.

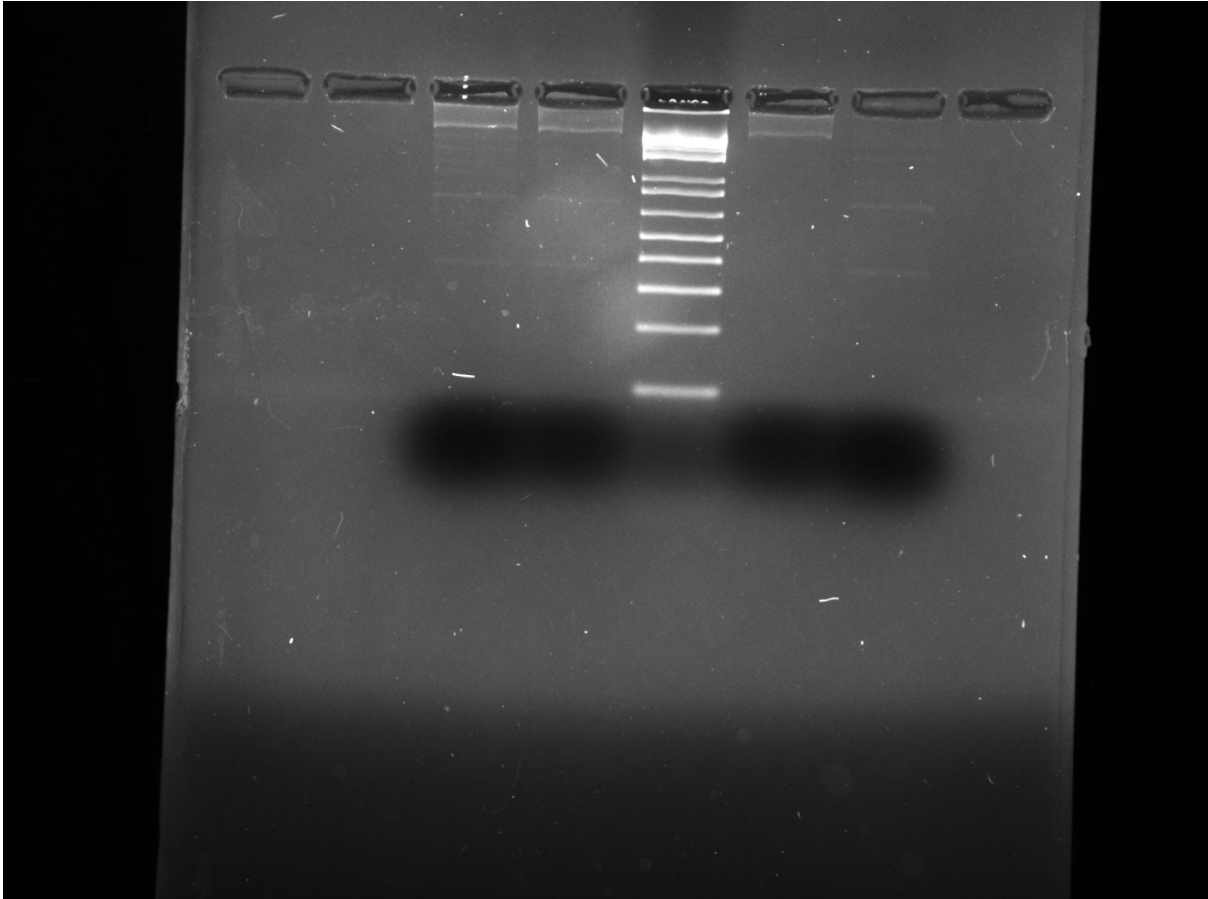


Image 2: Mini gel on 3% agar using 1kb+ ladder after 40 minutes run-time

The results were not as expected as the fragments in wells 3, 4, and 6 ran very slowly and were not spaced out clearly. No band observed for well 7. The 1kb+ ladder in well 5 could also have been more spread out. As a result, it was decided to let the gel run for another 40min.

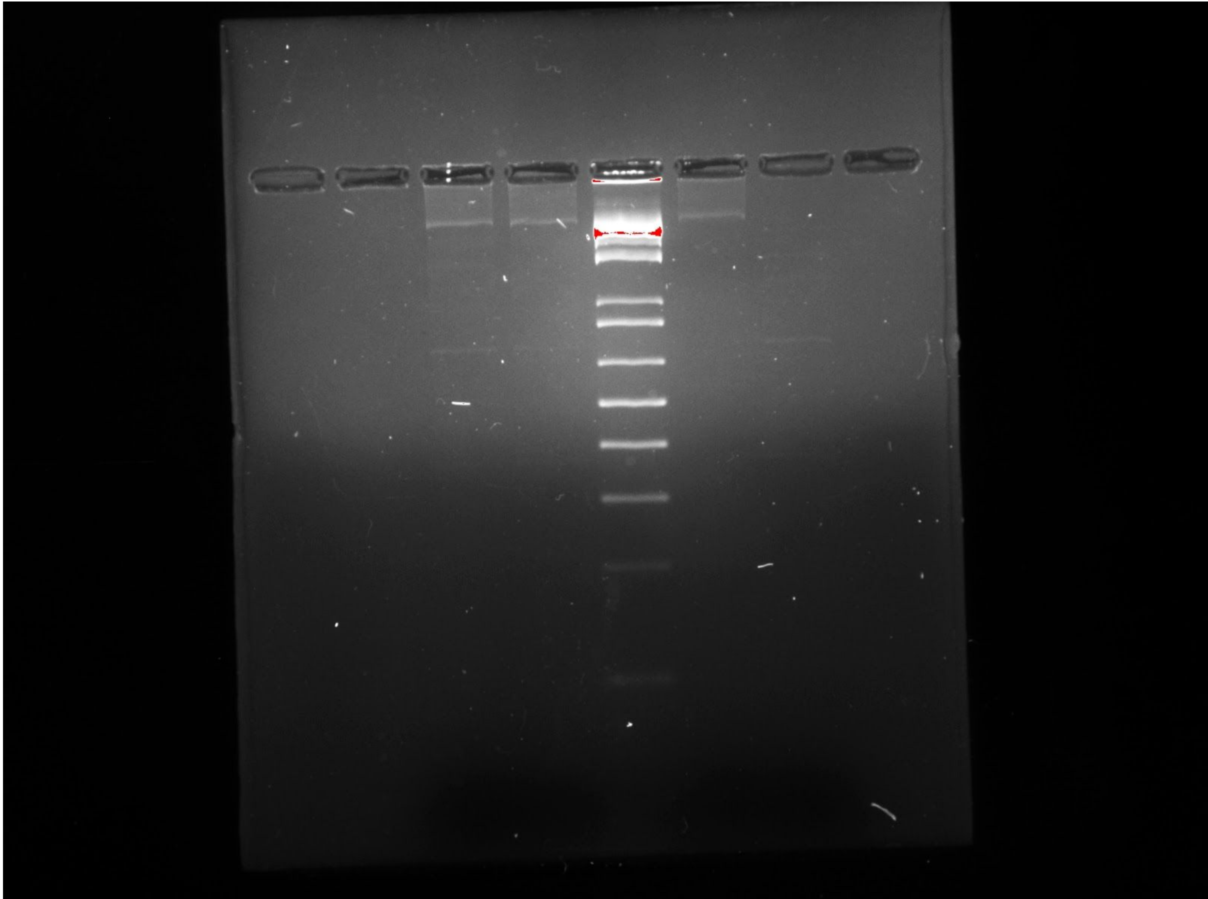


Image 3: Mini gel on 3% agar using 1kb+ ladder after 80 minutes run-time

The bands dispersed slightly better here, but the ladder was close to running off the gel. It is not clear which band on the ladder our fragment samples in wells 3,4 and 6 correspond to. As a result, this gel will not be of use to us, and results from the the medium-short gel on 1% agar will be considered instead.

Note: thicker gels, such as the 3% agar, should be used for smaller fragments only (around 2kb). This helps them run more slowly and disperse properly. As our fragment was around 5kb, a 0.5% agar gel would have been more appropriate, and would decrease the run-time to a more realistic timeframe.

23/08/17

Resuspended our genes using ultra pure water to make them around 10ng/ul concentration.

Made a mastermix PCR solution for both our vectors and genes, (insert quantities)

Added tubes to PCR machine and ran standard program, with the exception of changing our annealing temperature to $61.8 \circ C$, which we determined previously from gel electrophoresis as the optimal temperature.

25/08/17

Checked on plants and folded them down into bag. Stopped watering.

05/09/17 (red=comments. To be removed)

Hypothesis: Get clear bands for our PCR'd vector and genes using gel electrophoresis after varying the PCR annealing temperature and PCR mastermix volume.

Method: A standard PCR reaction was set up for both vector and DaIRIP4 as follows:

In PCR Mastermix	Vector mastermix volumes (ul)	Gene of choice mastermix volumes (ul)
5x Phire Buffer	10	10
10uM dNTP	1	1
10uM F Primer	1	1
10uM R Primer	1	1
Phire TAQ	1	1
pET22b+ Vector	2	NA
Gene of choice	NA	1
Water	34	35

Mastermixes were made in small PCR tubes for vector and gene using the above volumes (We used the really tiny ones, not the standard-sized PCR tubes).

Once both sets of mastermixes were centrifuged to ensure mixing, they were placed in a PCR machine (Selected the furthest one in the lab, next to the gradient PCR) with the following settings: (select user: Geoffrey, then edit)

1. Initial denaturation- 98.0 $\circ C$, 00:30 minutes
2. Denaturation- 98.0 $\circ C$, 00:15 minutes
3. Annealing- 60.8 $\circ C$, 00:20 minutes
4. Extension- 72.0 $\circ C$, 01:30 minutes
5. Final extension- 72.0 $\circ C$, 01:00 minute
6. Storing conditions- 15.0 $\circ C$, Indefinitely/ until use

Numbers 2-4 were run at 35x cycles.

Upon PCR completion, a 1% agarose gel and redsafe mixture was made in a mini mould and left to set for 30min.

On parafilm, 2ul of the vector, gene, and High Density ladder were each mixed with 2ul of BlueJuice.

All 3 were then transferred into wells 3, 4, and 5 (correlating Vector, DNA ladder, and Gene respectively) on the agarose gel, covered with buffer, and the gel electrophoresis set to 90V for 40min.

Results & Discussion: It was hoped that by increasing the volume of the vector in the PCR mastermix from 1ul to 2ul, and decreasing the PCR annealing temperature from 61.8 $\circ C$ to 60.8 $\circ C$, brighter and cleaner bands would show after gel electrophoresis. However, this was not the case for the vector.

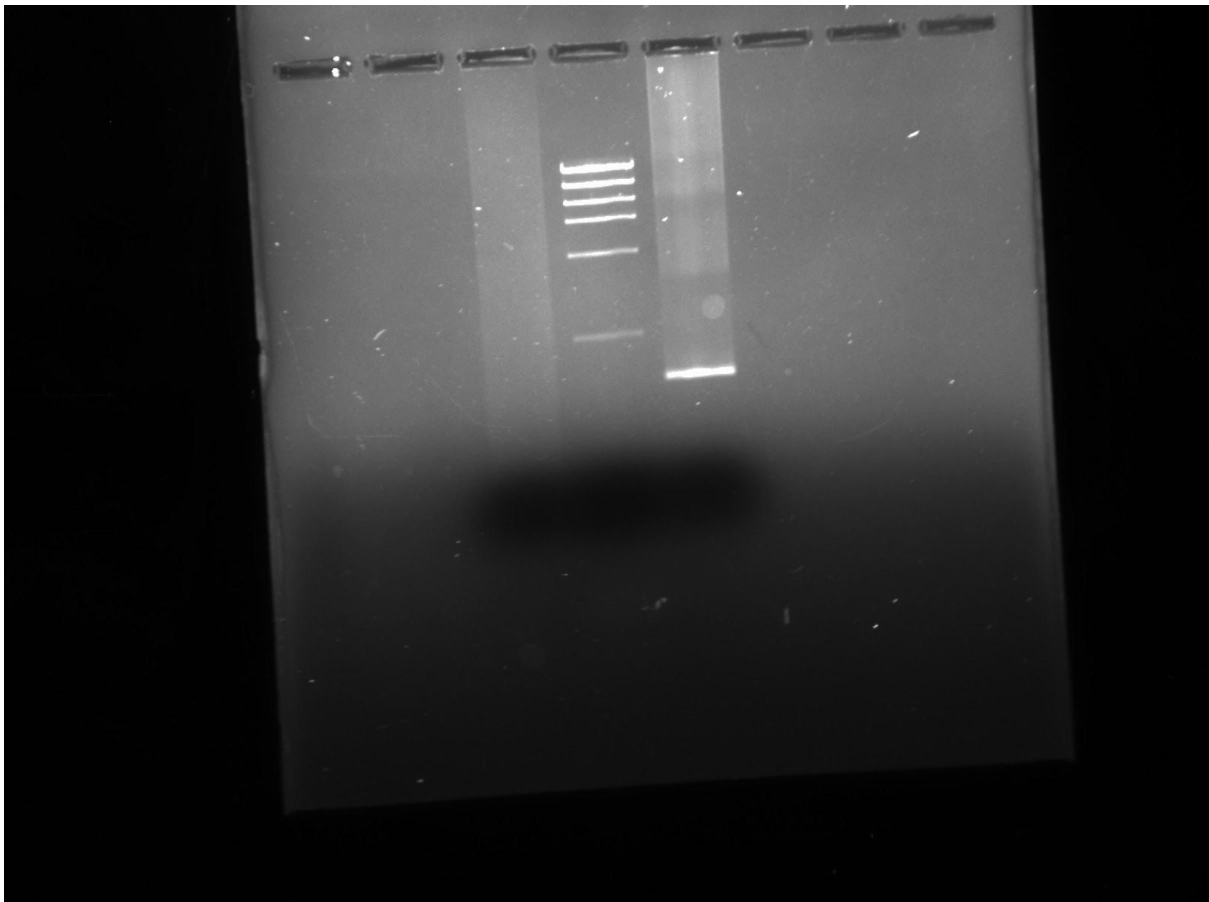


Image 1: The pET22b+ vector, High Density ladder, and gene were added in wells 3, 4, 5 respectively.

Well 3 containing the vector has no bands. Both the ladder and gene successfully produced bright, clear bands. It is uncertain what caused this vector result, however, it could be because its concentration was too high.

The next steps will be performing the PCR reaction and gel electrophoresis again, but varying the concentrations of the vector. This may produce more favourable results.

Hypothesis conclusion: partly correct; The gene produced favourable results. The vector did not.

Wednesday 060917

Aim: To investigate the effect of vector concentration on successful PCR amplification.
Hypothesis: After Andra's discussion with an Honours student about what might be going wrong, I would expect that the lower concentrations (e.g. using 0.5 or 1 uL of vector) might be more successful.

I decided to run the PCR of just the vector (no gene) using volumes of 0.5, 1, 2, and 3 uL of pet22b+ (PCR dilution). I also chose to include a negative control (0uL of vector) I created a 6x master mix as follows:

In PCR Mastermix	1x rxn(ul)	6x rxn (ul)
5x Phire Buffer	10	60
10uM dNTP	1	6
10uM primer pet22b+ outwards from His	1	6
10uM primer pet22b+ outwards from pelB	1	6
Phire TAQ	1	6
Water	33	198

PCR tubes were then created as follows
0 = 47uL of master mix + 3uL of PCR grade water.
0.5 = 47uL of master mix + 2.5uL of PCR grade water + 0.5uL of pet22b+ PCR dilution
1 = 47uL of master mix + 2uL of PCR grade water + 1uL of pet22b+ PCR dilution
2 = 47uL of master mix + 1uL of PCR grade water + 2uL of pet22b+ PCR dilution
3 = 47uL of master mix + 3uL of pet22b+ PCR dilution

These tubes were stored in the fridge for a time prior to PCR due to availability of the machine. Refrigeration was an attempt to prevent degradation during this wait period, although I should have thought to check availability before starting.

The tubes were centrifuged and then run on the same PCR cycle done previously:
Initial denaturation- 98.0 $\circ C$, 00:30 minutes
Denaturation- 98.0 $\circ C$, 00:15 minutes

Annealing- 60.8 $\circ C$, 00:20 minutes
Extension- 72.0 $\circ C$, 01:30 minutes
Final extension- 72.0 $\circ C$, 01:00 minute
Storing conditions- 15.0 $\circ C$, Indefinitely/ until use

This was done on the old PCR machine at the back of the lab.

Following this, tubes were removed from the machine and stored in the refrigerator until they could be run on a gel to check the result.

Ran a 1% gel of the PCR products from this morning.
Hypothesis: Hopefully we will see a single band corresponding to 5.4 KB, which would represent our linear pet22b amplified product.

Methods
Used up the last of the 1% gel stock to cast a mini gel with an 8 well comb.
While the gel set, I made up a new batch of 1% gel → 300mL using 3g of agarose.
Mixed 2μL each of the samples and a high mass DNA ladder with 2μL of 10x Bluejuice. The surface tension of the samples was low, forming much shorter, wider droplets than the ladder.

Once the gel was set, loaded as follows

Lane	1	2	3	4	5	6	7
Vector volume, μL		0	0.5	1	High Mass Ladder	2	3

Set gel to run for 40 minutes at 90V.
Placed PCR tubes into freezer box.

Conclusion: Ladder did not separate. 3μL well has given a clear band that appears to be at the expected length (for what little we can see by the ladder), however there is also a second much smaller band which is of some concern.

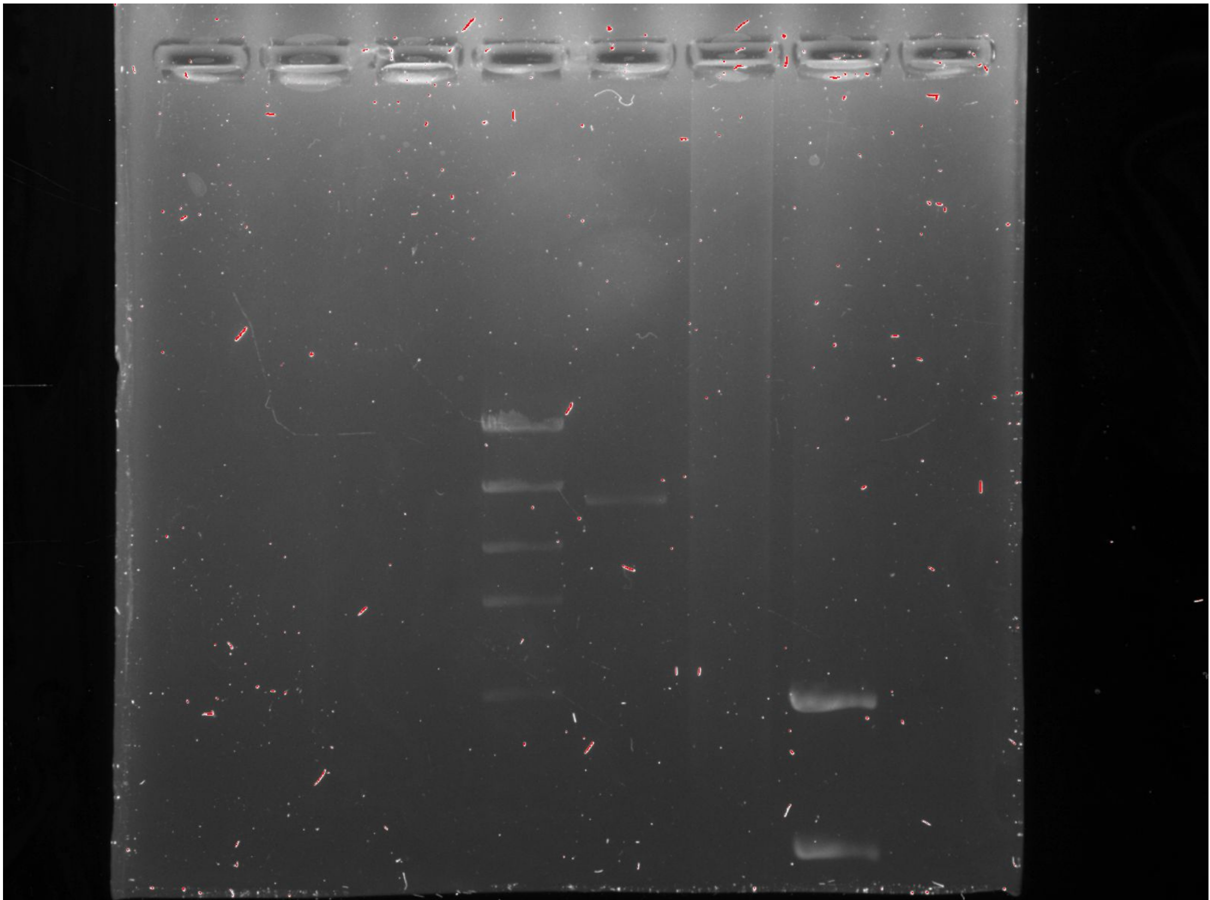
Friday 080917

Davide advised more frequent checking on the plant to prevent further mentions in the surveys. Equired with many parties for assistance with first seed harvesting. Wendy may be able to help us on Monday afternoon.

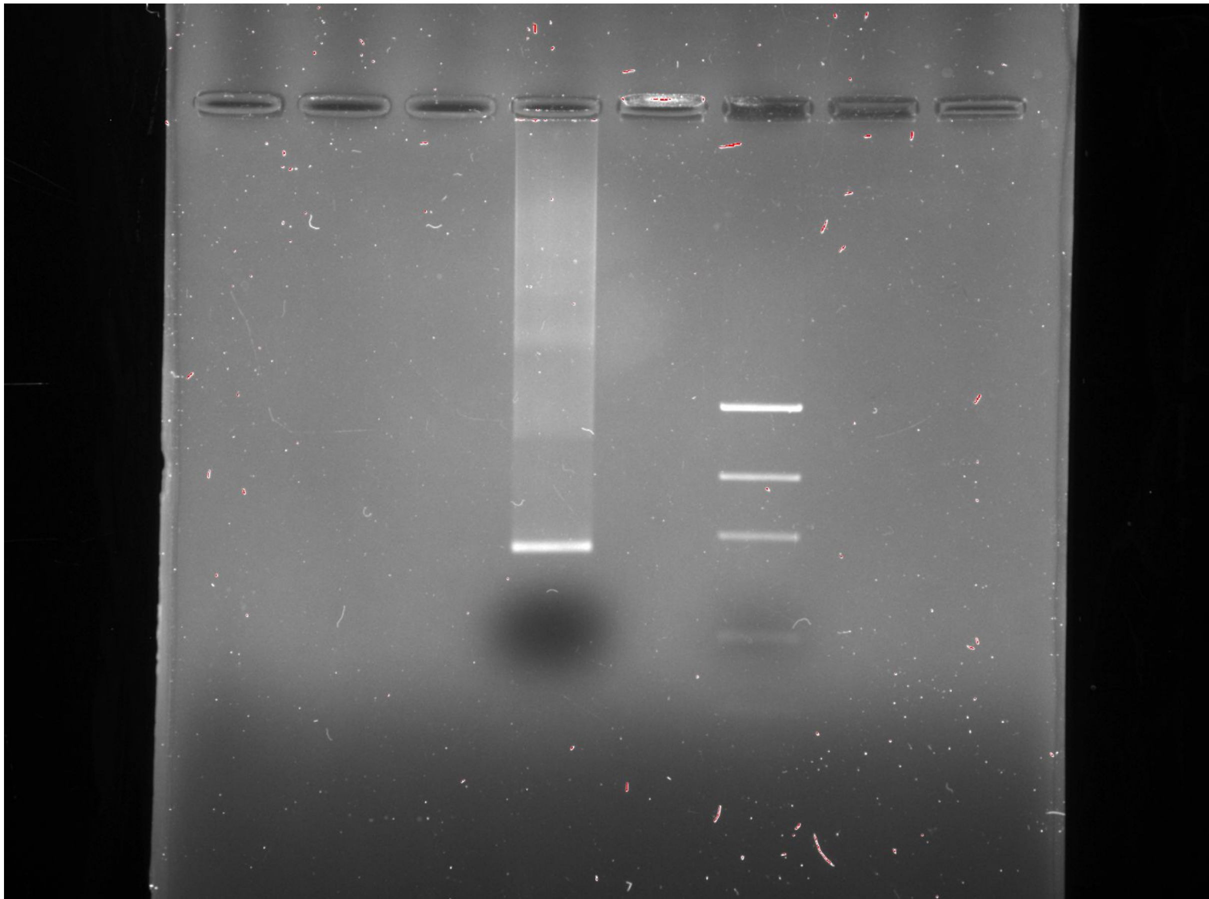
Prepared a 1% gel of the pet22b and DaIRIP PCR samples with a low and high mass dna ladder. Ran for 80 mins at 90 Vs.

The low mass ladder and gene frgament ran off the gel, which I had known would be a possibility, but we did get good seperation of the high mass ladder. The vector pcr product sits just below the 6KB band, with the next band being 4KB, so it looks likely we have the expected fragment size of 5.4Kb been produced. The smaller vector fragment previously seen has run off the gel, meaning it must be smaller than 1.2Kb, the smallest remaining

band of the low mass ladder than can be seen.



Also ran a 1% gel of the low mass ladder and gene fragment for 40 minutes and 90V to determine DaIRIP fragment size.



Expected fragment size is 725bp. The observed band is sitting just under the 800bp low mass ladder band, so the PCR has been successful. However, we have again observed the streaking of the band, which will to be investigated.

To do

Determine vector batch concentration for gel intensity (Can't use nanodrop, I've tried, too much interference)
Determine how much volume of the above we need to have a total of 50 ng of vector.
Carry out the calculation to find out now much DaIRIP needed to have a 3 fold molar excess, using the formula on the gibson reaction protocol.

Make up a batch of ampicillin plates for use following transformation.

Gibson reaction - 10μL total.
50ng vector PCR fragment
3 molar excess gene fragment
5μL of master mix for gibson reaction
?μL to make it up to 10μL
Cover surface with mineral oil to prevent evaporation.

110917

Seed Harvesting

Before you begin bring the harvesting, bring the bins over to the table. Also pull out the tray so that it slightly overhangs the table, to ensure that no floor is exposed when working. Make sure the paper is clean and stacked.

Label the eppendorf tubes on both the top and sides with the sowing number, type and date.

Firstly, tightly grab the bottom of the bag and stake, and cut the inflorescences at the base. Carefully pull the stake out of the rock wool, and move the bag and stake over the tray, being careful not to spill any seed.
Twist the stake and pull to remove it from the bag, holding the top of the bag closed as you do so. Place the stake directly into the stake bin.
Holding the bag closed, but still allowing air to escape, crush the bag to make the inflorescences release their seeds.

Place the sieve on top of the papers, and turn the bag upside. Create a small opening and shake/tap the seeds into the sieve. Discard bag and chaff into the bin. Shake the seeds through the sieve onto the paper, then discard the chaff.
Gently tip the seeds off the paper and through the sieve after placing it on the sheet below. Again discard the chaff. Repeat until clean, then pour into the eppendorf tube.

120917 Tuesday
Seed sterilisation
Used Q585 4 tube, sterilised ~10000 seeds using the aforementioned protocol. Due to the larger volume of seeds, 1mL of water and bleach was used throughout. Placed in the fridge ready for use on Friday.

150917 Friday

Sowing number Q 629, 2 blocks.
Split seeds between two tubes, diluted with extract water and pipetted rows using 1 ml pipettes. Had quite large amounts of seed per row. Next time might be better to dilute more, pipette quicker or spread the seeds out more with a tooth pick following the pipetting.
Seeds will need to be first sprayed at 7-10 days.

180917 Monday
Watered plants.

200917 Wednesday
Watered Plants and lifted the plastic to reduce humidity.

210917 Thursday

Woe be to the PCR. Woe be, woe be…

Hypothesis: To PCR amplify the vector at a higher temperature to try and remove the faint, secondary bands.
To PCR the gene fragment so we have a larger stock. Making a dilution to try and reduce streaking.

Methods:

Made up a 1/100 dilution of the DaIRIP4 protein expression gene fragment. 1uL of 19.3ng/µL and 99µL of nuclear free water.
Final concentration 0.193ng/µL.

Mixed up a 50µL reaction with the following volumes.

	µL
5x Phire Buffer	10
10uM dNTP	1
10uM gene primer inwards from His	1
10uM gene primer inwards from pelB	1
Phire TAQ	1
Water	35
0.193ng/µL DaIRIP4	1

Ran at the following settings:
Initial denaturation- 98.0 $\circ C$, 00:30 minutes
Denaturation- 98.0 $\circ C$, 00:15 minutes
Annealing- 60.8 $\circ C$, 00:10 minutes
Extension- 72.0 $\circ C$, 00:20 minutes
Final extension- 72.0 $\circ C$, 01:00 minute
Storing conditions- 15.0 $\circ C$, Indefinitely/ until use

Mixed a vector PCR amplification:

	µL
5x Phire Buffer	10
10uM dNTP	1
10uM primer pet22b+ outwards from His	1
10uM primer pet22b+ outwards from pelB	1

Phire TAQ	1
Water	33
Vector Dilution 0.137ng/μL	3

Ran at the following settings, 35 cycles

Initial denaturation- 98.0 $\circ C$, 00:30 minutes
Denaturation- 98.0 $\circ C$, 00:15 minutes
Annealing- 61.5 $\circ C$, 00:10 minutes
Extension- 72.0 $\circ C$, 01:30 minutes
Final extension- 72.0 $\circ C$, 01:00 minute
Storing conditions- 15.0 $\circ C$, Indefinitely/ until use

Ran a 1% mini agrose gel with a low mass DNA ladder, gene fragment dilution, the newly PCR'd gene fragment and the previous PCR attempt which resulted in high mass streaking.

Loaded 4μL of 5x Blue Juice loading dye with each 2μL of samples.
Ran for 40 minutes at 90V, 400A.

Results: No fragment on the new PCR. Amplification failed.

Ran a 1% mini agrose gel with a High Mass DNA ladder, along with todays vector amplification and vector pcr 3.
Ran at 90V, 400A for 70 Minutes.

Results: No fragment on the new PCR. Amplification failed.

Ran another DaIRIP amplification trial. Made up a master mix with the following volumes:

	μL
5x Phire Buffer	50
10uM dNTP	5
10uM gene primer inwards from His	5
10uM gene primer inwards from pelB	5
Phire TAQ	5

This was aliquoted into 5 tubes, 14μL each.
Into the 5 tubes was added.

Tube	1	2	3	4	5

0.193ng/µL DaIRIP4 (µL)	30	20	10	5	1
Water (µL)	6	16	26	31	35

Began a run at the following settings, 35 cycles:
Initial denaturation- 98.0 $\circ C$, 00:30 minutes
Denaturation- 98.0 $\circ C$, 00:15 minutes
Annealing- 60.8 $\circ C$, 00:10 minutes
Extension- 72.0 $\circ C$, 00:20 minutes
Final extension- 72.0 $\circ C$, 01:00 minute
Storing conditions- 15.0 $\circ C$, Indefinitely/ until use

Realised at 45 minutes in that the likely reason the PCR was failing was due to insufficient annealing time. Aborted run, restarted for 25 cycles with a 20 second annealing time.

Was running low on time when the second run finished, so ran a 1% gel at 120V for 18 minutes. 4µL of low mass ladder. 4µL of 5x BlueJuice, 2µL sample.

INsert Picture

Results: Samples 30, 20, 10 and 1µL showed extensive high mass streaking. 5µL showed major band plus minor bands. Unsure of why.

220917 Friday

Sprayed plants with Basta to select for transformants.

Mixed up 500mL of spray using a 200g/L stock to a final concetration of 120mg/L.
So that is 300µL of stock into 500mL of miliQ water. Swirl to mix, and pour into spray bottle.

When spraying the plants, do it out in the plant hall away from other cultures. Keep the bottle close when spraying to get good coverage and prevent contamination of surfaces. If you are unsure of your coverage, go over the rows again.

Plants were sprayed at 7 days old, will spray again on monday at 10 days old.

250917 Monday

Sprayed plants again to continue selection. Did a single pass.

Mixed a vector PCR amplification:

	μL
5x Phire Buffer	10
10uM dNTP	1
10uM primer pet22b+ outwards from His	1
10uM primer pet22b+ outwards from pelB	1
Phire TAQ	1
Water	35
Vector Dilution 0.137ng/μL	1

Ran at the following settings, 25 cycles

Initial denaturation- 98.0 $\circ C$, 00:30 minutes
Denaturation- 98.0 $\circ C$, 00:15 minutes
Annealing- 61.5 $\circ C$, 00:20 minutes
Extension- 72.0 $\circ C$, 01:30 minutes
Final extension- 72.0 $\circ C$, 01:00 minute
Storing conditions- 15.0 $\circ C$, Indefinitely/ until use

Also reran the vector PCR from Thursday because might as well.

Also ran the DaIRIP4 PCR that failed on Thursday at
Initial denaturation- 98.0 $\circ C$, 00:30 minutes
Denaturation- 98.0 $\circ C$, 00:15 minutes
Annealing- 61.0 $\circ C$, 00:15 minutes
Extension- 72.0 $\circ C$, 01:30 minutes
Final extension- 72.0 $\circ C$, 01:00 minute
Storing conditions- 15.0 $\circ C$, Indefinitely/ until use

When finished placed all in freezer box.

260917 Tuesday

Ran a 1% gel for both the gene fragment and vector fragments PCR'd yesterday.

2/10/17 to 20/10/17
During this time we were attempting to characterise and create biobricks. For creation of our biobrick we followed this protocol:

Digest

§ Enzyme Master Mix for Plasmid Backbone (25ul total, for 5 rxns)

§ 5 ul NEB Buffer 2

§ 0.5 ul BSA

§ 0.5 ul EcoRI-HF

§ 0.5 ul PstI

§ 0.5 ul DpnI (Used to digest any template DNA from production)

§ 18 ul dH20

§ Digest Plasmid Backbone

§ Add 4 ul linearized plasmid backbone (25ng/ul for 100ng total)

§ Add 4 ul of Enzyme Master Mix

§ Digest 37C/30 min, heat kill 80C/20 min

§ Digest Insert

COMPONENT	50 µl REACTION
DNA	1 µg
10X NEBuffer 3.1	5 µl (1X)
EcoRI	1.0 µl (or 10 units)
PstI	1.0 µl (or 10 units)
Nuclease-free Water	to 50 µl

- Incubate at 37°C for 5-15 minutes

Ligation

§ Add 2ul of digested plasmid backbone (25 ng)

§ Add equimolar amount of EcoRI-HF PstI digested fragment (< 3 ul)

§ Add 1 ul T4 DNA ligase buffer. **Note:** Do not use quick ligase

§ Add 0.5 ul T4 DNA ligase

§ Add water to 10 ul

§ Ligate 16C/30 min, heat kill 80C/20 min

This protocol was followed once, and the product was transformed using electroporation into E. coli strain DH5alpha. This produced no growth, and we hypothesised that the ligation buffer we used (which posed some concerns at the time as it appeared crystallised rather than liquid) was not stored appropriately and thus was not good for use.
We repeated the same protocol using ligation buffer that had been previously used by one of our team members in an unrelated (non-iGEM) experiment that was successful.

We also changed to using a heat-shock method due to concerns about the salt content being too high for successful electroporation and concerns about arcing electricity killing the bacteria.
We began characterisation experiments on the 4th of October, resuspending the provided kit plate DNA in wells 1O and 17D with 10uL of nuclease free water as recommended on the iGEM Kit Plate Use protocol.
We transformed the DH5alpha bacteria using (separately) 2uL of each of these provided DNA samples, and with 4uL of our created biobrick, as well as transforming strain b221 with 4uL of our created biobrick (in an attempt to determine if it was simply not expressing in DH5alpha).
These were plated onto CAM plates with a concentration of 30ug/mL chloramphenicol.
All plates were incubated at 37C overnight.
No growth was observed here.

We repeated the experiment, transforming this time with 8uL of provided DNA sample, and 8uL of our created biobrick, each into DH5alpha.
These were plated onto freshly poured CAM plates with a concentration of 25ug/mL, in case our previous concentration had been too high for the strength of the promoter for the CAM resistance on pSB1C3.
Again all plates were incubated at 37C overnight.
Once again no growth was observed. At this point, there was insufficient time to repeat the experiment, and thus the characterisation results were written up at this point, and a raw ligation product was sent in as our 'biobrick submission'.

Monday 23rd to Friday 27th October

Electrolyte Leaky Assay

From our 5 healthiest DaIRIP4 transgenic plants, along with 3 control plants, three young leaves of approximately the same size were taken. These leaves were allocated to 1 of 3 conditions; 25 o C, 0 o C and -20 o C.
The leaves were washed, and then placed into individual tubes.
For the 25 o C and 0 o C trials, the leaves were immersed in 3mL miliQ water and left in their respective conditions for 24 hours.
For the -20 o C trial the leaves were exposed for 1 hour in dry conditions. This was to prevent
confounding damaged cause by the freezing of the water surrounding the leaves. The samples were then soaked in 3mL of miliQ for 30 minutes to allow the electrolytes from the damaged cells to diffuse.

The electroconductivity of each solution was measured, and then the samples placed in a 90 o C water bath for 30 minutes to blanch and release the total electrolytes into solution. The

electroconductivity was measured again, the results for each leaf normalised as a percentage loss of total electrolytes.

Thank you for reading our lab notes.
We hope you had fun.
For inquiries about the what happened in our lab this year, please contact
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