

All experiments in this notebook were conducted with the advice and support of Douglass Marr of Dr. Airola laboratory and Andrew Junior (AJ) Sillato.

All the protocols are saved in the google drive under their designated names.

Template:

Date

Aim:

Notes: (optional)

People:

Protocol: Exact name as in the google drive

Changes to protocol: (optional)

Results: (optional)

July 1, 2022

Aim: PCR new primers onto the ends of the gene of interest (GOI)

People: Eva and Maulik

Notes: We ordered the GOI with other primers attached to its ends. We need to use different primers, however. The new primers are compatible with YMBac II plasmid, into which the GOI (with new, compatible primers), will be PCR-ed into.

Protocol: Protocol for Q5® High-Fidelity 2X Master Mix

Changes to protocol:

25 ul reaction

The amount of template DNA is 1 ng in 0.5 ul of TE.

Our primers were diluted to 10 uM.

We went roughly by the rule that 1kb needs 30 seconds for the extension cycling step.

PCR cycling:

STEP	TEMP in degrees Celsius	TIME
Initial denaturation	98	30 seconds
30 cycles:	98	10 seconds
	55	30 seconds
	72	2 minutes
Final extension	72	2 minutes
Hold	4	infinity

Aim: PCR new primers onto the ends of the YMBac II

People: Eva and Maulik

Protocol: Protocol for Q5® High-Fidelity 2X Master Mix

Changes to protocol:

25 ul reaction

The amount of vector is 0.5 ul of ~10 uM.

Our primers were diluted it to 10 uM.

PCR cycling as above (we run both the YmBac II and the GOI above in the same PCR).

Aim: Running a DNA gel with both products of the PCR reaction (modified YmBac II and modified GOI) to see if they have been amplified and have correct weight

People: Eva

Notes: Why 1% agarose? Our products are pretty high weight, so we need a lower percentage of agarose. The higher percentage the greater separation or in other words greater resolution of the gel.

Protocol: See written out below:

Shorthand of protocol: 1% agarose gel in TAE buffer

25 minutes, 100 V

Well set up:

Well number	Contents	Amount
1.	Thermo Scientific GeneRuler 1 kb Plus DNA Ladder 100 ng/ul	2 ul
2.	GOI PCR product undiluted + 6x loading dye	5 ul + 1 ul (this makes the 6x loading dye become 1x)
3.	vector PCR product undiluted + 6x loading dye	5 ul + 1 ul

15 min of staining in SYBR Gold (5 ul of SYBR Gold + 50 ml of TAE, the same TAE the gel was running in, no need for fresh TAE)

Scanning using GE Typhoon FLA 9000 Gel Scanner. Resulting image:

Detail of protocol:

1. Making the 1% agarose gel:

Take an autoclaved plastic 250 ml screw-cap erlenmeyer. (Cannot use glass or non-screw cap because of the high temperatures it will be subjected to.) Put it on a weighing machine without the cap, tar the weight, add 1g of agarose. Take off the weighing machine, add 100 ml 1x TAE and mix thoroughly by swirling with the cap screwed on. A slightly opaque fluid will result.

Put it into the microwave with the cap barely put on top of the neck of the erlenmeyer for 2 minutes. If the cap is screwed, the erlenmeyer may explode. Warning: the contents are very hot! Take out using heat shielding glove toppers. Examine the fluid and see if there is anything floating in it. It must be completely clear. At times there are very small translucent particles of agarose floating in the fluid which are difficult to notice. If they are present or if the fluid is still opaque, microwave in 30 second increments (do not let the solution boil for longer than a few seconds).

In the meantime, prepare the gel pouring box, tray for the gel and well comb. Place the tray into the slot of the pouring box along with the comb.

Once the fluid is translucent, pour the 25 ml or 12.5 ml depending on the size of the gel (or whatever amount needed for the gel pouring box you have). Let the gel sit for 15 minutes or until it is firm (preferably do not touch it). One can put it into a 4 degree refrigerator to speed up the process, but make sure it is placed on an even surface.

2. Preparing to run the gel

Pour 1x TAE into the gel electrophoresis box up to the line. Put the firm gel in its tray into the designated spot in the box (it has to be fully covered in TAE) so that the wells are closer to the negative end and further from the positive end (cathode). Write down what you will put in each gel. Load the gel.

3. Run the gel at 100 V for 25 minutes (30 minutes is preferable). Monitor it every couple minutes to make sure that the lower weight dye (purple in the case of the 1 kb plus DNA ladder) does not run off the gel.

4. Fully thaw SYBR Gold in a dark box with room temp. water (SYBR Gold is light sensitive).

5. Once done, unplug the gel electrophoresis box. Take 5 ul of SYBR Gold (for staining DNA), measure 50 ml of the TAE which is in the gel electrophoresis box, and put it into a staining box (one that has a cover, because SYBR Gold is light sensitive). Then put 5 ul SYBR Gold into the staining box and mix until you cannot see the colored streaks. Very carefully transfer the gel by putting it with its tray into the staining box and then slide it out of the tray. (If you take it by hand, the gel will break. Not a big deal, usually imaging of the gel goes fine anyway, but you can avoid it.) Stain for 15 minutes if in a rush (30 minutes preferable).

6. Image the gel using GE Typhoon FLA 9000 Gel Scanner. The machine is in our friendly lab, it's not in any of the rooms we have access to (will ask Gergen).

Well set up:

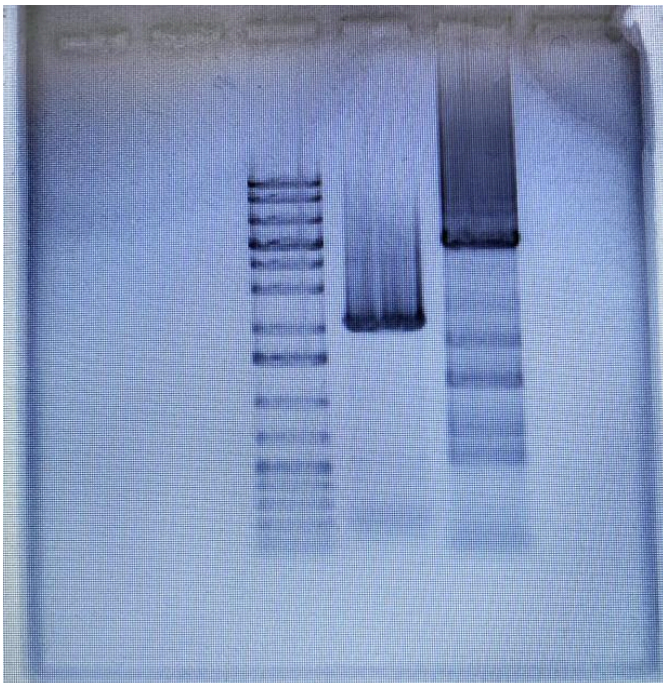
1. -
2. -
3. 1 kb Plus DNA Ladder
4. Vector
5. Gene
6. -

Results and analysis:

The first sample of 2 kb aligns with 2 kb band on ladder.

The second sample of 5 kb aligns with 5 kb band on ladder.

Intense band indicate good amplification. The dark smudges above both samples indicate a highly concentrated sample. For future gels, one would recommend using a lower amount of the sample or a diluent.



July 5, 2022

Aim: Step 6 of the LIC cloning protocol: "Use the PCR clean up kit to remove the digested DNA and do the wash step twice. The double wash is to thoroughly remove dNTPs that could otherwise interfere with the T4 resection reaction."

PCR product clean up

People: Eva and Maulik

Protocol: Protocol for DNA Cleanup and Concentration Using the Monarch® PCR & DNA Cleanup Kit (5 µg) (NEB #T1030)

July 6, 2022

Aim: Nanodrop to determine the concentration of the cleaned product

People: Eva and Maulik

Protocol: Written out below:

Equipment: Thermo Scientific™ NanoDrop™ OneC Microvolume UV-Vis Spectrophotometer

1. Wipe the pedestal of the device with a KimWipe, blank the device with 2ul of what DNA is diluted in (in our case the elution buffer from the following protocol: Protocol for DNA Cleanup and Concentration Using the Monarch® PCR & DNA Cleanup Kit (5 µg) (NEB #T1030)).
2. Put 2 ul of the sample onto the pedestal, and perform the reading.
3. Save the displayed graph.

Results:

gene concentration - 132.4 ng/uL

vector concentration - 251.4 ng/uL

Calculations:

molecular weight 1,314,076.6 g/mol

mole weight 1.3×10^6 g/mol

$\text{mol} = \text{g} / (\text{g/mol})$

$0.03 \text{ pico mol} = x / (1.3 \times 10^6 \text{ g})$

$x = 39,000 \text{ g}$

Aim: Calculations to find the volume of cleaned up PCR product needed for LIC

Protocol: LIC Cloning (Douglass Marr, Michael Airola)

Steps 7 and 8 of the protocol

People: Eva and Maulik (Ya Jing, Divleen, Steph, Ahmed)

July 7, 2022

Aim: Making Ampicillin LB agar plates to grow the transformants on

People: Eva

Protocol: Making plates, protocol shown below (provided by Douglass Marr)

15 g Luria Broth or Terrific Broth

9 g Agar

600 ml water milliQ

1. Add the above to a 1 L bottle with a cap and autoclave in a tray filled with water with the bottle cap semi-loose. After the autoclave, keep the lid semi-closed until the bottle has cooled off a bit.
2. Put a stirring rod and spin on a heat plate at 60 C for 1h.
3. Add 300 ul of antibiotic per 300 mL of agar media mix. This should result in a 50 ug / ml working concentration of

the antibiotic. (I believe it is ug not mg.)

The same working concentration can be used for Ampicillin or Carbenicillin. We used Ampicillin in this experiment.

4. Set up the bench for pouring: use ethanol to disinfect the surface, and lay out the plates in three plates per stack and pour them in a lift-pour-close technique to minimize contamination. No speaking during pouring to minimize contamination.

5. Pour.

6. Let them set and dry overnight. They can be used the next day.

7. If you are making them for storage, store them for 2 days in a dark place. Then they are dry enough to be wrapped into their original plastic with the plastic taped shut, and stored in 4 C.

Changes to protocol:

Step 2. - No stirring rod needed (esp. b/c it might get contaminated). We just kept swirling the bottle to dissolve the forming agar layer.

Step 6. - next time we may not have to wait overnight because the plates set quickly.

Step 7. - no need to let them sit for 2 days before storing in 4 C. We parafilmmed them in the morning and stored in 4 C.

July 8, 2022

Aim: Cloning and plating the Nova Blue *E. coli* with the plasmid+gene of interest cloned into them

People: Eva, Maulik, Ya Jing, Nami, Steph

Protocol: Transformation of *E. coli*

Changes to protocol:

We are doing 3 samples total: experimental sample, positive control, and negative control.

We are also not incubating overnight at 37 C, but rather incubating for 2 days at 24 C in a dark drawer.

We are using Nova blue strain of *E. coli* donated from Dr. Michael Airola's lab.

experimental sample - Nova blue + gene of interest + plasmid + 200 ul LB

positive control - Nova blue + 2 ul plasmid+ 2 ul water + 200 ul LB

negative control - Nova blue + 200 ul water

July 10, 2022

Aim: Moving the experimental plate, and the positive and negative control plates from 24 C dark incubator into 37 C dark incubator.

People: Ya Jing

Results: No growth present after 2 days of 24 C dark incubation.

July 11, 2022

Aim: Running a positive control using intact YMBacII plasmid: transforming Nova Blue with intact plasmid and plating them

Notes: No cultures grew (not any in experimental sample or positive or negative control. Thus, we decided to run a positive control by transforming and plating Nova Blue with intact YMBac plasmid.

People: Eva, Maulik, Ya Jing

Protocol: *E. coli* Transformation

July 12, 2022

Aim: We redid adding primers to the gene and to the vector by PCR. We did not make any changes to the protocol to see if it was a procedure mistake that caused no cultures to grow.

Notes: Positive control of plating Nova Blue with intact YMBac plasmid showed positive results - a lawn of small, light colored, dot-shaped cultures.

People: Eva, Maulik, Ya Jing

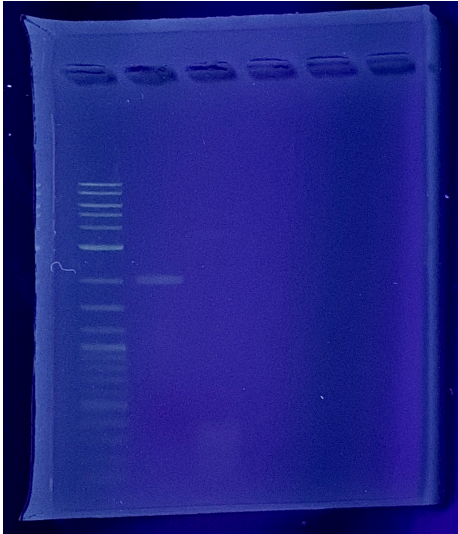
Protocol: PCR protocol and DNA gel protocol

Changes to protocol: -

Wells:

1. 1kb Plus DNA ladder
2. gene of interest after miniprep (G)
3. vector after miniprep (V)

Running a gel of V and G. Only G band shows with extremely faint V band. Some background noise of low weight visible in the V well.



July 13, 2022

Aim: Redo PCR the vector using a new annealing temperature 62 C.

People: Eva, Maulik, Ya Jing, Nami

Protocol: NEB PCR protocol and the same PCR program as that in July 1, 2022 except for the annealing temperature.

Changes to protocol:

We ran two tubes of 50 uL reaction. We named them Va and Vb.

PCR cycling:

STEP	TEMP in degrees Celsius	TIME
Initial denaturation	98	30 seconds
30 cycles:	98	10 seconds
	62	30 seconds
	72	2 minutes
Final extension	72	2 minutes
Hold	4	infinity

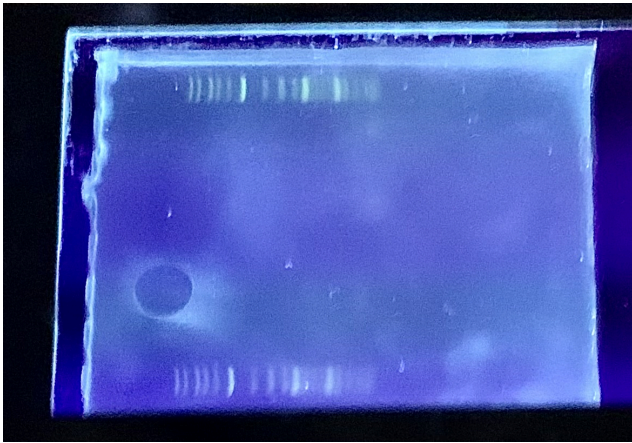
Wells:

1. 1 kb Plus DNA Ladder
2. Va PCR product
3. Vb PCR product leaked out so this well should not be considered
4. Vb PCR product
5. -
6. -

7. -

8. 1 kb PLus DNA Ladder

Running a gel of the PCR product:



The PCR reaction or gel imaging failed completely. The circle visible is a bubble of air.



July 14, 2022

Aim: Redo the vector PCR with 55 and 62 annealing temperatures and several corrected steps and new vector from stock

People: Eva, Maulik, Ya Jing, Ahmed

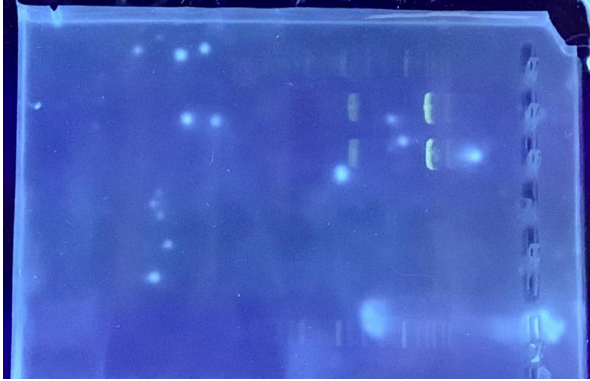
Protocol: NEB PCR protocol and DNA gel protocol

Changes to protocol:

STEP	TEMP in degrees Celsius	TIME
Initial denaturation	98	30 seconds
30 cycles:	98	10 seconds
	62	30 seconds
	72	2.5 minutes
Final extension	72	5 minutes
Hold	4	infinity

Gel well set up:

1. 1kb Plus DNA ladder
2. Vector with 55 C annealing temperature
3. Vector with 62 C annealing temperature
4. -
5. -
6. -
7. -
8. Ladder



There is a significant laddering effect, but nonetheless the amplification is clear, the bands are bright and sharp. It seems however that the laddering effect may not be easy to overcome. The vector was amplified successfully enough for a gel extraction, which removes the factor of laddering effect.

July 15, 2022

Aim: Running all 90 ul of PCR product left (the vector) on 0.7 % agarose gel. Then performing a gel extraction. The hope is that we get at least 400 ng (0.4 ug) of product to be able to carry out transformation.

People: Eva, Ya Jing, Nami

Protocol: DNA gel as written up above

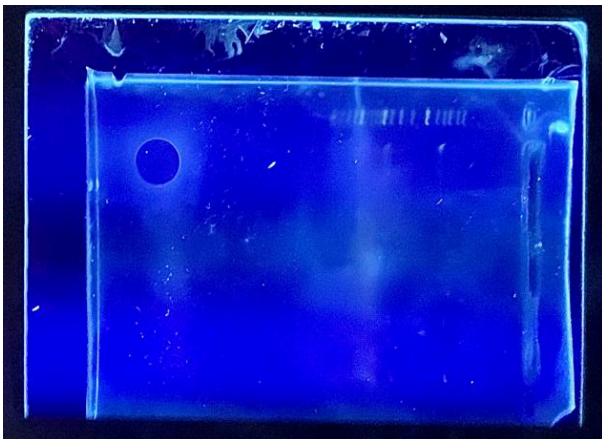
Well set up:

1. 1 kb Plus DNA Ladder

Wells #2, 3, 4, 5, 6, and 7 were fused together - PCR product

8. -

Results:



Protocol: DNA gel and MAN0012661_GeneJET_Gel_Extraction_UG

Changes to protocol: We used a blue light, orange glasses and orange shield to cut out the gel pieces.

We first tried putting the entire gel into one tube and adding binding buffer to it, but halfway through the process we

realized the volume of the tube is not sufficient. Thus we divided the gel into two tubes:

Tube 1 - 0.30 g of gel + 430 ul of binding buffer

Tube 2 - 0.37 g of gel + 530 ul of binding buffer

We ended up with two vials of 800 uL of binding buffer solution. As an end product, we eluted two vials.

July 18, 2022

Aim: Running PCR of the Gel extraction sample of the Vector and the gene, both are at very low concentrations than the ones required for LIC cloning.

1. We combined the two tubes with product of gel extraction (the vector) into one 1.5 ml tube labeled “7/15 gel extraction”

2. We performed a nanodrop of the gel extraction. **The results are 1.7 ng/ul.** These results are low for the purposes of the LIC protocol. We needed a minimum of 400 ng of the vector, and the volume of our gel extraction product was between 100 and 150 ul. We decided to PCR the (gel extraction) product. However, the annealing temperature of the primers is high, because now we need to consider the entirety of the primer sequence since the primer is already incorporated into the vector. The current annealing temperature is 79 C or so, unfortunately, and the maximum temperature for the Q5 polymerase is 72 C. We decided to run two 50 ul PCRs: one with 66 C and one with 72 C annealing temperature, for both the vector (gel extraction sample) and the gene (29 ng/uL). We arrived at these conclusions with the help of Douglass Marr (Airola Lab).



nanodrop results

We also decided to re-PCR our gene sample. The gene sample we had was at a concentration of 29 ng/uL, and we did not have enough of this for LIC as well.

3. Protocol for PCR reaction (2 annealing temps, 66 and 72, everything else the same)

A total of 4 PCR tubes/reactions:

Gene (66 C annealing temp.)

Gene (72 C annealing temp.)

Vector (66 C annealing temp.)

Vector (72 C annealing temp.)

STEP	TEMP in degrees Celsius	TIME
Initial denaturation	98	30 seconds
35 cycles:	98	10 seconds
	66 or 72	30 seconds

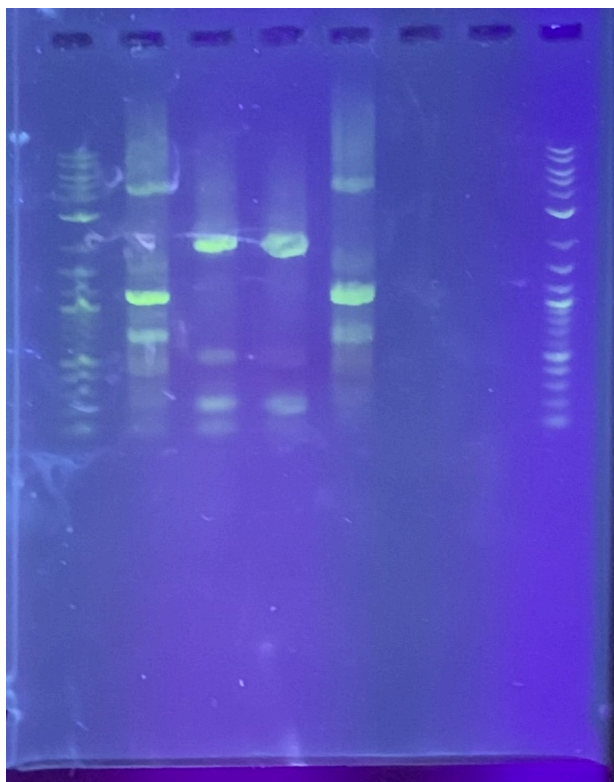
	72	2.5 minutes
Final extension	72	5 minutes
Hold	4	infinity

4. Running a gel of the PCR products

Error: tube labels got scratched off, but we can infer based on the gel which tube is which, since the temperature labels did not get scratched off

Well set up:

1. 1 kb Plus DNA Ladder
2. Sample 1 (gene - lower temp. 66 C) **We have reevaluated and concluded that this is the vector.**
3. Sample 2 (vector - lower temp 66 C) **We have reevaluated and concluded that this is the gene.**
4. Sample 3 (vector - higher temp 72 C) **We have reevaluated and concluded that this is the gene.**
5. Sample 4 (gene - higher temp 72 C) **We have reevaluated and concluded that this is the vector.**
6. -
7. -
8. 1 kb Plus DNA Ladder



Analysis: Because the gel has so much non-specific annealing represented by the ladder effect in both the gene and the vector, we decided to try GC enhancers as well as trying several different temperatures alongside each other. We have taken into account that we may not be able to get clean bands without laddering and may have to gel extract.

July 19, 2022

Aim: PCR experimental runs for gene and vector. Then, 0.8% agarose gel of the PCR products

People: Maulik, Ya Jing, Eva

Protocols: PCR and DNA gel

PCR 7/19 reaction set up: 4 - 50 uL tubes.

Gene Reaction Set up (With GC enhancer) -

- Q5 Master Mix - 25 uL (1X final conc)
- Forward Primer 10mM - 2.5 uL
- Reverse Primer 10mM - 2.5 uL
- GC enhancer - 10 uL
- H2O - 9.5 uL
- DNA Template - 0.5 uL (1 ng), IDT sample concentration is **2 ng/uL**

We split this reaction into two 25 uL tubes (one with 60 C annealing temp and another with 55 C)

Gene Reaction Set up (Without GC enhancer) -

- Q5 Master Mix - 25 uL (1X final conc)
- Forward Primer 10uM - 2.5 uL
- Reverse Primer 10uM - 2.5 uL
- H2O - 19.5 uL
- DNA Template - 0.5 uL (1 ng), IDT sample concentration is **2 ng/uL**

We split this reaction into two 25 uL tubes (one with 60 C annealing temp and another with 55 C)

Vector Reaction Set up (With GC enhancer) -

- Q5 Master Mix - 25 uL (1X final conc)
- Forward Primer 10mM - 2.5 uL
- Reverse Primer 10mM - 2.5 uL
- GC enhancer - 10 uL
- H2O - 9.5 uL
- DNA Template - 0.4 uL (10 ng), sample concentration is **27 ng/uL**

We split this reaction into two 25 uL tubes (one with 62 C annealing temp and another with 55 C)

Vector Reaction Set up (Without GC enhancer) -

- Q5 Master Mix - 25 uL (1X final conc)
- Forward Primer 10mM - 2.5 uL
- Reverse Primer 10mM - 2.5 uL
- H2O - 19.5 uL
- DNA Template - 0.4 uL (10 ng), sample concentration is **27 ng/uL**

We split this reaction into two 25 uL tubes (one with 62 C annealing temp and another with 55 C)

PCR set up of 8 samples total:

1 ul gel stain + 5 ul gene + GC enhancer	annealing T: 55
1 ul gel stain + 5 ul gene	annealing T: 55
1 ul gel stain + 5 ul gene + GC enhancer	annealing T: 60
1 ul gel stain + 5 ul gene	annealing T: 60
1 ul gel stain + 5 ul vector + GC enhancer	annealing T: 55
1 ul gel stain + 5 ul vector	annealing T: 55
1 ul gel stain + 5 ul vector + GC enhancer	annealing T: 62
1 ul gel stain + 5 ul vector	annealing T: 62

Amount of GC enhancer: 10 ul in a 50 ul PCR reaction.

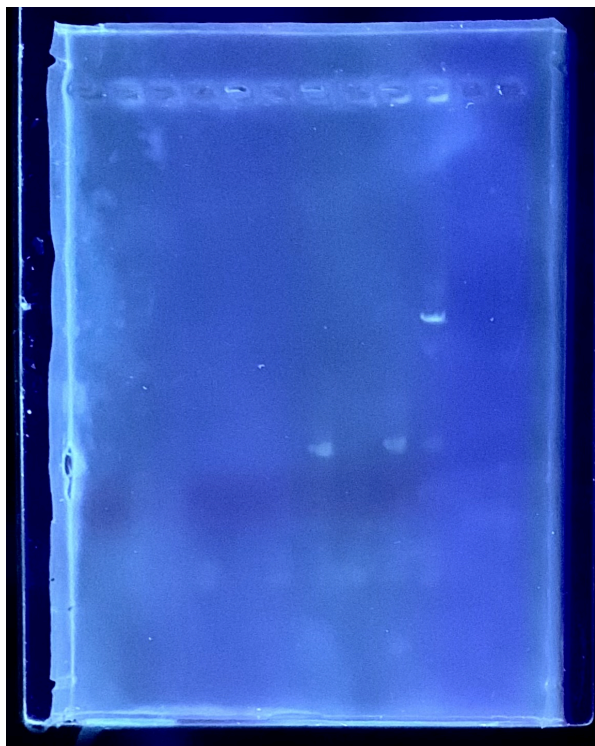
STEP	TEMP in degrees Celsius	TIME
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Initial denaturation	98	30 seconds
35 cycles:	98	10 seconds
annealing	55 or 60 or 62 depending on sample	30 seconds
extensions	72	2.5 minutes
Final extension	72	5 minutes
Hold	4	infinity

Well set up:

- | | |
|---|-----------------|
| 1. - | |
| 2. 1 kb Plus DNA Ladder 2 ul | |
| 3. 1 ul gel stain + 5 ul gene + GC enhancer | annealing T: 55 |
| 4. 1 ul gel stain + 5 ul gene | annealing T: 55 |
| 5. 1 ul gel stain + 5 ul gene + GC enhancer | annealing T: 60 |
| 6. 1 ul gel stain + 5 ul gene | annealing T: 60 |
| 7. 1 ul gel stain + 5 ul vector + GC enhancer | annealing T: 55 |
| 8. 1 ul gel stain + 5 ul vector | annealing T: 55 |
| 9. 1 ul gel stain + 5 ul vector + GC enhancer | annealing T: 62 |
| 10. 1 ul gel stain + 5 ul vector | annealing T: 62 |
| 11. - | |
| 12. 1 kb Plus DNA Ladder 2 ul | |

Result:



Analysis:

The only result on the gel that had good amplification (and decent purity indicated by relatively light laddering effect) is well 10 - vector with 62 C annealing temperature. All other amplifications have failed. We made sure to take a note of the successful annealing temperature for the vector and the lack of GC enhancer as preferable conditions.

However, we noticed that the imaging method may be at fault this time or possible previous times, so we decided to use a different imaging method to achieve a clearer image, namely imaging using SYBR Gold and, and imaging in Typhoon scanner lended to us by Dr. Ed Luk, Stony Brook University Life Sciences.

July 20, 2022

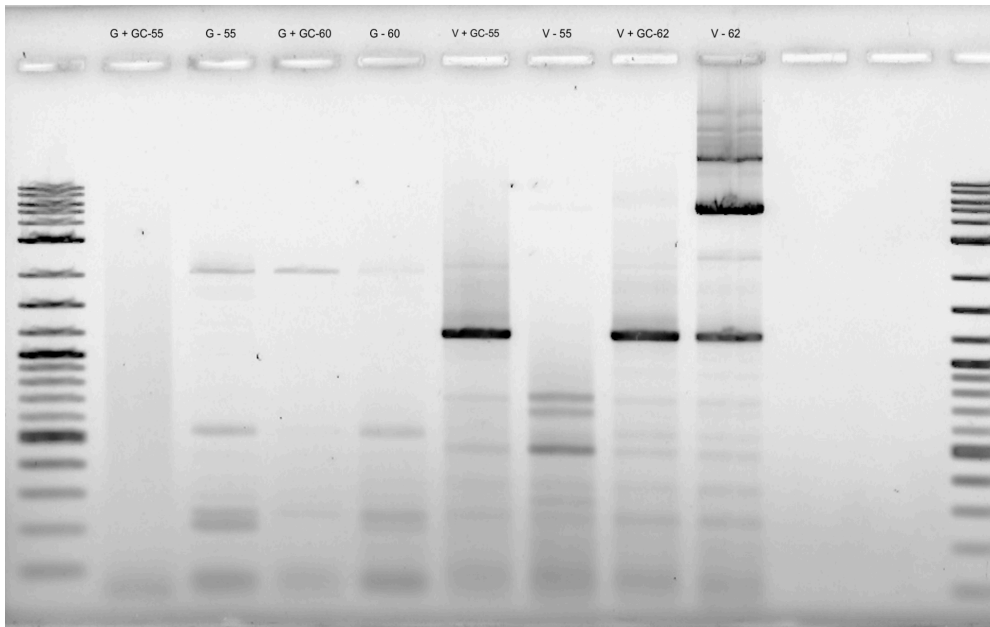
Aim: Run the gel for evaluating the PCR results from 7/19 (using Luk Lab Typhoon scanner and SYBR Gold)

People: Eva, Maulik, Ya Jing

Protocol: Gel protocol above

We performed the same protocol as on July 19, 2022 for gel electrophoresis, except that we did not add the SYBR Safe directly into the agarose gel mix. Instead we stained the gel for 30 minutes in a 1:10 000 dilution of SYBR Gold and TAE.

Result:



Analysis:

All samples with gene are very low concentration and faint. We realized that the reason is that we only used 1 ng of gene for the entire reaction. In the subsequent PCR we are planning to use 10 ng for gene + GC enhancer with 60 C annealing temperature because the band in lane 4 is the clearest with the least non-specific binding.

As for the vector, the sample without GC enhancer and 62 C annealing had the most amplification and could be easily gel extracted. We could perform ethanol precipitation and concentration of DNA if needed.

Aim: Concentrating DNA from gel extraction by ethanol precipitation (protocol provided by Cindy Converso, Luk Lab)

We estimated the volume of the 1.7 ng/ul gel extracted product.

Protocol:

1. Add 0.1x volume of 3 M NaOAc. Add 2.5x volume of 100% EtOH. Vortex.
2. Spin: 4 C, 15k rpm, 15 min. Remove supernatant by pipetting.
3. Wash pellet two times with 500 ul 100% EtOH.
4. Wash with 70% EtOH. Remove all liquid.
5. Allow pellet to air dry for at least 30 minutes (until completely dry).
6. Redissolve in 5 ul of molecular H₂O (or in greater amounts of water depending on the amount and concentration goal). Usually there is a loss of about 30% of the product which has to be considered.

Changes to protocol:

- We could not see the pellet, so we had to work under the assumption of its placement. We made sure to always centrifuge with the eppendorf in the same position relative to the center of the centrifuge and then assumed the pellet is on the opposite side of the tube.

- After each wash and before discarding the wash fluid, we centrifuged the eppendorf for about 1 minute to make sure that the pellet went back to its position.

Results: After completing the procedure, we used 1 ul to nanodrop (and after nanodrop we collected the nano dropped sample back into the tube).

Concentration: 3.8 ng/ul

A260/A280: 1.25

A260/A230: 0.63



July 21, 2022

Aim: PCR reactions:

2x 50 ul PCRs of gene at 60 C,

2x 50 ul PCRs of gene at 60 C + GC enhancer,

2x 50 ul PCRs of vector at 62 C.

People: Eva, Maulik, Ya Jing

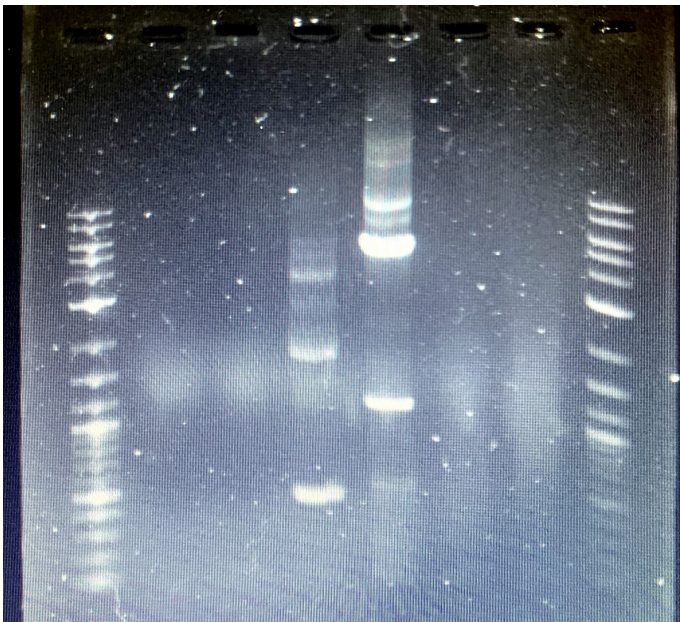
Protocol: PCR

We made sure to use 10 ng of gene for the PCR reaction.

Well set up:

G1	gene sample 1
G2	gene sample 2
V1	vector sample 1
V2	vector sample 2
G1+GC	gene sample 1 plus GC enhancer
G2+GC	gene sample 2 plus GC enhancer

Results:



Analysis:

The only well amplified sample on this gel was vector sample 2, which is intriguing because vector sample 1 and vector sample 2 are identical. The equipment may be at fault or possibly the assembly of the master mix could have been faulty: we may have forgotten to add primers or other reagents. Vector sample 2 band is clear and bright, though there is significant laddering. However, samples like this could still be used for gel extraction.

Conclusions for future experiments:

We decided to do a gel extraction of the highly-amplified vector samples acquired over the past weeks:

V2, 21 July

V (no GC) 66 C, 19 July

Sample 2 and 3, 18 July

July 22, 2022

Aim:

People: Eva, Ya Jing

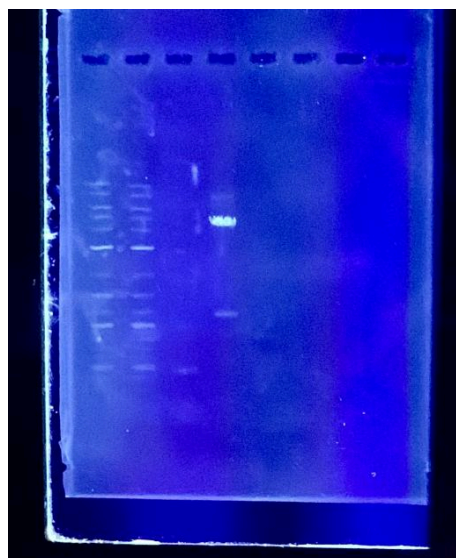
Protocol: DNA gel

Well set up:

1. Ladder
2. Negative control for gene (PROS gene)
3. Gene sample after PCR (sample 2, July 18)
4. Gene sample after PCR (sample 3, July 18)
5. Negative control for vector (YMBac II)
6. Vector sample after PCR Sample 1, July 18
7. Vector sample after PCR Sample 4, July 18
8. Vector sample after PCR July 21

We didn't have enough wells for the vector sample after PCR, July 19. However, we used it for gel extraction. We combined it with July 21 sample, because the July 19 sample had a clear, thick band in a previous gel.

Results:



Analysis:

The gene sample after PCR (sample 2 from July 18) has very good amplification indicated by a clear thick band. The gene sample after PCR (sample 2 from July 18) has very good amplification indicated by a clear thick band, so we felt confident using it for gel extraction.

Aim: 200 ul PCR of gene to prepare for gel extraction.

Reactant	Amounts for 200 ul reaction
Q5 High-Fidelity 2x Master Mix	100 ul
10 uM Forward Primer	10 ul
10 uM Reverse Primer	10 ul
Template DNA	20 ul = 40 ng of DNA b/c 0.5 ul = 1 ng of DNA
Nuclease-Free Water	60 ul

STEP	TEMP in degrees Celsius	TIME
Initial denaturation	98	30 seconds
35 cycles:	98	10 seconds
	72	30 seconds
	72	2.5 minutes
Final extension	72	5 minutes
Hold	4	infinity

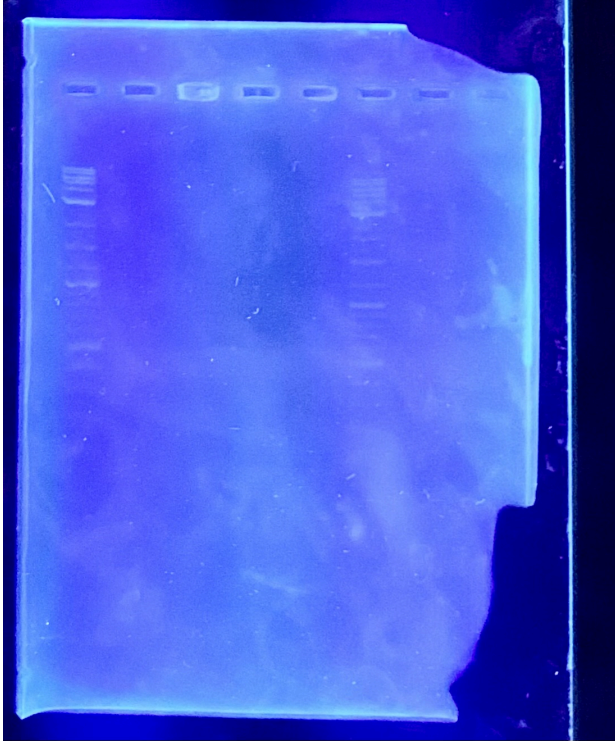
We ran an agarose of the products of this PCR.

Well set up:

- 1 kb Plus DNA Ladder
- Tube 1

3. Tube 2
4. Tube 3
5. Tube 4
6. 1 kb Plus DNA Ladder
7. -
8. -

Results:



Analysis:

We run a sample from each of the tubes as in previous experiments (at the same conditions and with the same reactants). However, this gave us different PCR results than before. This was likely due to human error or due to imaging error, or due to low concentration of samples.

There are no visible sample bands on the gel, which suggests that the PCR reaction either did not run at all or was ineffective. We decided to run another PCR reaction the following day, this time with annealing temperature of 55 C among other changes listed below.

July 23, 2022

Aim: Nanodrop

People: Eva, Ya Jing

Protocol: Nanodrop protocol

Results:



Analysis:

The vector is highly pure as shown by the graph (a clear curve around the 260 nm wavelength) and the A260/A280 is only 0.03 away from the ideal 1.80 value. The concentration of the sample is 47.3, which is more than enough for the purposes of LIC, especially that we have about 20 ul of the gel-extracted vector.

Aim: Re-reading gel from July 22 using a gel scanner

People: Eva, Ya Jing

Results: The gel looked exactly the same, either because of the results or because the DNA have diffused overnight in the gel. Either way, we need to redo the PCR reaction.

Aim: PCR reaction of the gene at favorable conditions to prepare enough sample for gel extraction.

People: Eva, Ya Jing

Protocol: PCR

Changes to protocol:

2x for 50uL PCR (one with old master mix and one with new master mix)

Reactants	Amount for 50 ul reaction
Q5 High-Fidelity 2x Master Mix	25 ul
10 uM Forward Primer	2.5 ul
10 uM Reverse Primer	2.5 ul
Template DNA	5 ul of 2 ng/ul (10 ng)
Nuclease-Free Water	15 ul

STEP	TEMP in degrees Celsius	TIME
Initial denaturation	98	30 seconds
40 cycles:	98	10 seconds
	55	30 seconds
	72	2.5 minutes
Final extension	72	10 minutes

Hold	4	infinity
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Dear SF9 Magician Wizarding Team:

Next steps are:

- 0.5) Don't do touchdown PCR. Let's scale up gene PCRs and gel extract. Let's get to LIC within a day!
- 1) Run a gel of the above PCR product (3 ul of water, 2 ul sample, 1 ul of loading dye). Image the gel wherever I guess, but Airola or AJ's imaging machines are preferable (less UV-induced cancer for us and better image). Run 2 ul of sample from each tube in case one of the tubes doesn't have product for whatever reason.
- 2) If the gel turns out good, put all of the darn product into two wells taped together. 43 ul + 43 ul
- 3) Gel extraction (make sure to cut real close to the band and DO NOT keep the DNA under UV for more than 15 seconds. Use razor to cut (next to UV machine in our lab or blue light in Airola lab). Ask AJ to help if needed (he will have to bring the UV masks in anyway if you use our UV lamp) Also, warm up the binding buffer before using it if you can (not critical, but AJ says it helps). Make sure all gel is dissolved!!!! Use 21 ul of eluting buffer (so we have 20 left after nanodrop). Keep binding buffer at 50 C or 52 C !!not 60!! when incubating for 10 minutes in the first step.
- 4) Nanodrop (1 ul or 2 ul, you decide)
- 5) Be happy if it worked, be happy if it didn't. You're working hard and it's something to be proud of. I appreciate you guys so much
- 6) LIC maybey? :3 We need competent cells tho (AJ offered his cells if anything and he can grab them for us even during a weekend)

TLDR:

- 1) Pilot gel of gene PCR product
- 2) Large scale gel of gene PCR products for gel extraction
- 3) Gel extraction
- 4) Nanodrop

July 25, 2022

Aim: Pilot gel of PCR products from 7/23/22

People: Ya Jing

Protocol: DNA Gel

0.7% agar gel used

DNA in wells was used in a ratio of 1 dye : 2 DNA : 3 water

Well set up:

1. Ladder
2. Gene sample with new master mix after PCR, July 23
7. Gene sample with old master mix after PCR, July 23

Bands appeared for both samples. However, old master mix created a much brighter band of DNA. Old master mix

will be used for future PCR reactions.



Aim: Repeat of PCR reaction (7/23) of the gene for more product to use in DNA extraction

People: Ya Jing

Protocol: PCR

Changes to protocol:

150uL PCR (with old master mix)

Reactants	Amount for 150 ul reaction
Q5 High-Fidelity 2x Master Mix	75 ul
10 uM Forward Primer	7.5 ul
10 uM Reverse Primer	7.5 ul
Template DNA	15 ul of 2 ng/ul (10 ng)
Nuclease-Free Water	45 ul

PCR mixture was made in one tube and separated into 3 tubes before being placed into the PCR machine.

Aim: Pilot gel of PCR products (repeat of 7/23 PCR) above

People:

Protocol: DNA Gel

Results: Bands have appeared for the gene PCR created on 7/25.



Analysis: All samples can be used for gel extraction.

Aim: Gel of PCR products (repeat of 7/23 PCR) above in preparation for gel extraction

People:

Protocol: DNA Gel

The band was clear. We loaded 150 ul of product onto the gel.

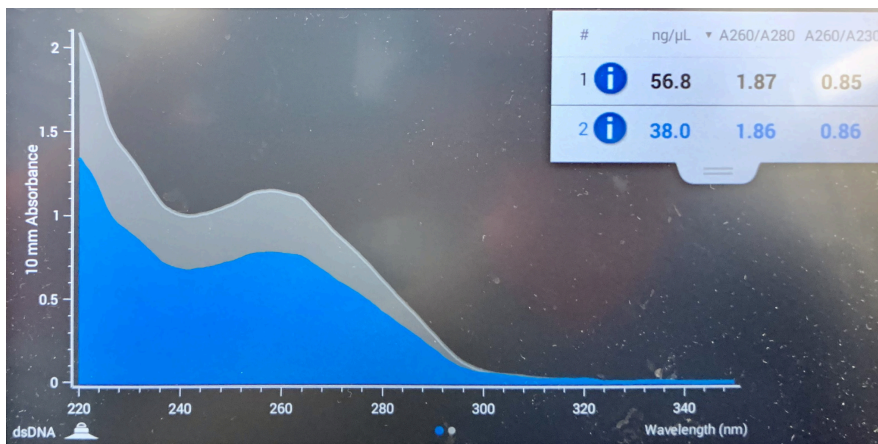
July 26, 2022

Aim: Gel extraction and nanodrop of the gel-extracted DNA

People:

Protocol:

Nanodrop results:



Analysis:

We ended up with two tubes of gel extracted gene: gel extracted gene sample 1 and gel extracted gene sample 2. As shown on the curve, the concentration of the samples ideal, the A260/A280 value and the A260/A230 values are nearly ideal.

We now have both highly pure gene and vector samples ready to use for LIC reaction.

Aim: LIC (cloning insert into the vector)

People: Eva, Ya Jing, Maulik

Protocol: LIC (Douglas Marr, Airola lab)

<https://docs.google.com/document/d/1FsF-akq21wCFQxsqdpfxpuYtuTHOnZvEMP5PnkpyyqQ/edit>

We skipped the Dpn1 incubation and PCR cleanup since the insert and vector samples were gel extracted and purified, to be used for LIC.

Digest the insert PCR reaction with T4 DNA Polymerase and dATP (no other nucleotides). Simultaneously, digest the vector PCR reaction with T4 DNA Polymerase and dTTP (no other nucleotides).

We used the following formula for pmol to ng/uL conversion (found in a different LIC protocol, link below)

$$\text{number of base pairs} \times 0.65 = \text{ng/pmol}$$

<https://www.helmholtz-muenchen.de/fileadmin/PEPF/Protocols/LIC-cloning.pdf>

Reagents	Insert Resection Reaction	Vector Resection Reaction
DNA	0.03 pmol (1 uL of sample, concentration 38 ng/uL)	0.01 pmol (1 uL of the vector, concentration 14 ng/uL)
DNTP (Singular, not all of them. 25 mM)	2 uL dATP	2 uL dTTP
T4 Polymerase	0.4 uL	0.4 uL
T4 Buffer	2 uL	2 uL
water	12.6 uL	12.6 uL
DTT (50 mM)	2 uL	2 uL
Total	20 uL	20 uL

We mixed the table contents in two different tubes, one for the insert and one for the vector, and did the following steps before transformation:

1. Incubate at 24 C for 30 minutes, then 75 C for 20 minutes to kill the T4 polymerase
2. Mix 2 uL of vector and 2 uL of insert together at the bottom of a PCR tube. Vortex and let sit for 10 minutes at room temperature.
3. Pipette the mixture to the bottom of a transformation tube on ice.

Aim: Transforming the LIC assembled vector into *E Coli* NovaBlue cells

People: Eva, Ya Jing, Maulik

Protocol: LIC (Douglas Marr, Airola lab)

https://docs.google.com/document/d/1LITxgP_RjGgLiISF6B4qv_xqKO3Sh-kAPXQoPV_HqU/edit

We did two transformations, one using the LIC assembled vector (with the insert) and one with the empty vector YMBac-II for positive control.

Before plating we spun down the cells at 3000 rpm for 1 minute, discarded the supernatant and resuspended. Then we plated the cells. This way we made sure the cells are more concentrated. However, the solution after 1 hour of incubation was not cloudy at all, suggesting that the concentration of cells is low or the cells were dead or have died during the procedure.

We concluded to use a different strain of cells (DH5 alpha) and a different protocol that works for the said strain, and increase the amounts of insert and vector (while leaving the ratio of the two the same for now).

Results of plating the transformants:

Analysis:

Experimental plate - no cells have grown
Positive control with intact vector - several colonies

July 27, 2022

Aim: repeating LIC with new conditions to troubleshoot the lack of cells on the experimental plate

People:

Protocol: DH5 alpha transformation protocol provided by

Combine 5uL LICed insert + 5uL LICed vector + 10uL ddH2O (20uL total volume), incubate 30 min at room temperature to anneal. Transform 10uL into XL-1 Blues or similar cloning strain:

1. 30 minutes on ice
2. Heat shock at 42°C for 30 seconds
3. 2 minutes on ice
4. Add 1mL 2xYT (or LB) and shake at 37°C for 1hour
5. Plate 150uL on appropriate antibiotic plate

Controls:

Vector: 47.3 ng/ul -> use 1 ul (47.3 ng)
Insert 1: 56.8 ng/ul -> use 2.5 ul (142 ng)
Insert 2: 38 ng/ul -> N/A

Insert : Vector
142 ng : 47 ng
3:1 ratio

experiment - make a new LIC using the new protocol, higher concentrations and new cells

1. Negative Control

LIC-ed vector (linearized vector)
5 ul vector + 5 ul water to transform 100 ul cells

2. Experimental

5 ul LIC-ed vector + 5 ul LIC-ed insert + 10 ul water = 20 ul total. Use 10 ul of the total to transform 100 ul cells.

LIC

Substance	Insert	Vector
DNA	2.5 ul (142 ng)	1 ul (47.3 ng)
dNTP	2 ul dATP	2 ul dTTP
T4 Pol	0.4 ul	0.4 ul
T4 Buffer	2 ul	2 ul
Water	11.1 ul	12.6 ul
DTT	2 ul	2 ul
Total	20 ul	20 ul

Before we prep them it's 0.3 OD, right before we aliquot the DH5 alpha.

Result of the transformation:

No colonies formed in either negative control or the experimental control:



July 28, 2022

Aim: Do a new LIC reaction for both insert and the vector, increase the concentrations for both

People: Eva, Ya Jing, Maulik

Protocol

No colonies showed up from the transformations we did yesterday, for both the negative control and the experimental control.

Goal for the new LIC reaction: Increase the insert and vector concentration in the reaction to 20 ng/uL. Therefore, add 400 ng of both the insert and the vector to the 20 uL LIC reaction mix to make the final DNA concentration 20 ng/uL.

Insert DNA sample concentration: 56.8 ng/uL

Vector DNA sample concentration: 47.3 ng/uL

LIC

Substance	Insert	Vector
DNA	7.2 ul (~400 ng)	8.5 ul (~400 ng)
dNTP	2 ul dATP	2 ul dTTP
T4 Pol	0.4 ul	0.4 ul
T4 Buffer	2 ul	2 ul
Water	6.4 ul	5.1 ul
DTT	2 ul	2 ul
Total	20 ul	20 ul

Annealing Reaction:

Pipette 5 uL of the insert LIC mix and 5 uL of the vector LIC mix in a separate eppendorf tube, followed by a 30 min incubation at room temperature, before transforming in *E coli* DH5alpha

Mix 5 uL of vector and 5 uL H₂O for a negative control transformation.

Followed Aj's *E Coli* transformation protocol.

Aim: Repeat Gene and Vector PCR reactions for gel extraction

People: Eva, Ya Jing, Maulik

Protocol

150ul Vector Reaction Set up (Without GC enhancer) -

- Q5 Master Mix - 75 uL (1X final conc)
- Forward Primer 30mM - 2.5 uL
- Reverse Primer 30mM - 2.5 uL
- H₂O - 58.5 uL
- DNA Template - 1.2 uL (10 ng), sample concentration is **27 ng/uL**

150uL Gene PCR (with old master mix)

Reactants	Amount for 150 ul reaction
Q5 High-Fidelity 2x Master Mix	75 ul
10 uM Forward Primer	7.5 ul
10 uM Reverse Primer	7.5 ul
Template DNA	15 ul of 2 ng/ul (10 ng)
Nuclease-Free Water	45 ul

Aim: Gel of PCR products above in preparation for gel extraction

People: Eva, Ya Jing

Protocol: DNA Gel

The band of vector was clear. We loaded 150 ul of product onto the gel into separate wells. We cut out the product on a UV plate, and total contact of the gel with UV light was 10 seconds or less.

July 29, 2022

Aim: Looking at plated transformants from July 28 transformation

People: Eva, Ya Jing

Results: Negative control



Experimental



No colonies present in negative control. This suggests that the plasmid does not re-ligate to itself in the process, though protocol error is also possible.

In a previous experiment we conducted a positive control: we transformed E. coli with an intact plasmid with Amp resistance and colonies grew.

No colonies present in the experimental sample plus the above information suggest that the cloning process is erroneous.

Aim: Gel extraction of Vector

People: Eva, Ya Jing

Protocol: Gel extraction

Changes to protocol: 21 ul of elution buffer instead of 50 ul

Aim: Repeat Gene PCR reactions for gel extraction

People: Ya Jing

Protocol: PCR

150uL Gene PCR (with old master mix)

Reactants	Amount for 150 ul reaction
Q5 High-Fidelity 2x Master Mix	75 ul
10 uM Forward Primer	7.5 ul
10 uM Reverse Primer	7.5 ul
Template DNA	15 ul of 2 ng/ul (10 ng)
Nuclease-Free Water	45 ul

STEP	TEMP in degrees Celsius	TIME
Initial denaturation	98	30 seconds
40 cycles:	98	10 seconds
	55	30 seconds
	72	2.5 minutes
Final extension	72	10 minutes
Hold	4	infinity

Aim: Running a gel of the above PCR products

People: Eva, Ya Jing

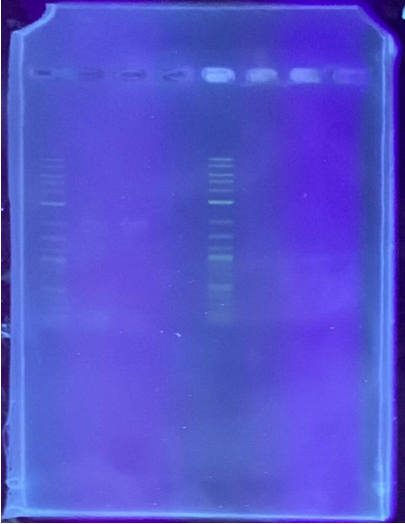
Protocol: DNA gel

Well set up:

1. 1 kb Plus DNA Ladder 1 ul
2. Gene PCR tube 1 2 ul + 3 ul H₂O + 1 ul 6x Loading Dye

3. Vector PCR tube 2 2 ul + 3 ul H₂O + 1 ul 6x Loading Dye

Results:



August 1, 2022

Aim: Nanodrop of July 29 gene gel extraction

People: Eva

Protocol: Nanodrop

Results:

Aim: Gel extraction of the Vector Sample and nanodrop

People:

Protocol:

Results:

August 2, 2022

Aim: PCR gene and insert from Doug's lab, the sample he uses for LIC

We borrowed insert and vector samples along with their respective primers from Douglas Marr (Airola lab). Doug normally uses these samples for LIC, and it works. Therefore, we want to try the DNA samples he uses as a positive control for our LIC reaction.

Initially, we had to PCR both the insert and the vector with their respective primers, PCR conditions stated below:

Reactants	Amount for 25 ul reaction
Q5 High-Fidelity 2x Master Mix	12.5 ul
10 uM Forward Primer	1.25 ul
10 uM Reverse Primer	1.25 ul
Template DNA	1 uL (20 ng/uL)
Nuclease-Free Water	9 ul

STEP	TEMP in degrees Celsius	TIME
Initial denaturation	98	30 seconds
35 cycles:	98	10 seconds
	61 insert, 65 vector	30 seconds
	72	50 sec (insert), 2 min 50 sec (vector)
Final extension	72	10 minutes
Hold	4	infinity

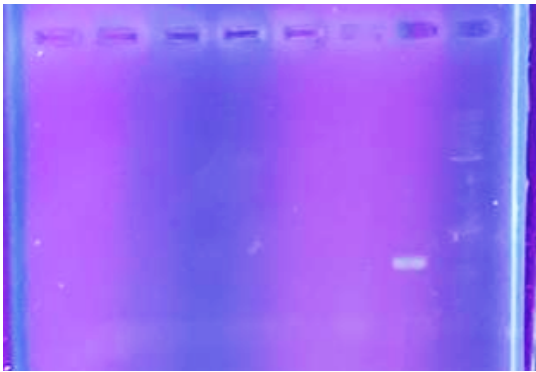
Aim: Running a DNA gel of the PCR results above

0.8% agarose

Well set up:

1. -
2. -
3. -
4. -
5. -
6. vector 2 ul + 3 ul H₂O + 1 ul 6xLoading Dye
7. gene 2 ul + 3 ul H₂O + 1 ul 6xLoading Dye
8. 1 kb Plus DNA Ladder 2 ul

Results:



Analysis:

Clear amplification for the gene, thick, bright band visible in row 7.

No results for the vector (row 6). Vector PCR assumed to be unsuccessful.

Aim: LIC reaction with gel extracted insert and vector samples.

People: Maulik, Ya Jing, Eva

Protocol: LIC cloning by Douglas Marr

Substance	Insert	Vector
DNA	6.6 (~400 ng)	7.9 (~400 ng)
dNTP	2 ul dATP	2 ul dTTP

T4 Pol	0.4 ul	0.4 ul
T4 Buffer	2 ul	2 ul
Water	7 ul	5.7 ul
DTT	2 ul	2 ul
Total	20 ul	20 ul

Final DNA concentration in a 20 uL mix should be 20 ng/uL.

1. Incubate at 24 C for 30 minutes, then 75 C for 20 minutes to kill the T4 polymerase
2. Combine 5uL LICed insert + 5uL LICed vector. Vortex and let sit for 30 minutes at room temperature.
3. Pipette the mixture to the bottom of a transformation tube on ice.
4. Transform 10uL into DH5alpha. 100 uL vial, OD = 0.3

30m on ice

Heat shock at 42°C for 30 seconds

2m on ice

Add 1mL 2YT and shake at 37°C for 1 hour

Plate 150uL on appropriate antibiotic plate



Aim: Culturing one of the transformants from the plate

People: Eva, Ya Jing

Protocol: Culturing (as written out below)

1. Dip into a chosen and marked (with a sharpie marker) colony using a pipette tip.
2. Swirl the pipette tip into 5 ml of LB.
3. Let grow overnight in a 220 rpm, 37 C shaker.

Notes:

Result: Unfortunately, the temperature was only 27 C in the incubator overnight, so once we have realized that in the morning, we set it to 37 C for it to incubate for longer. The solution was not cloudy at all.

August 3, 2022

Aim: PCR-ing primers onto Doug's vector (to be used as positive control of our entire LIC protocol)

People: Ya Jing

Protocol: PCR

Reactants	Amount for 25 ul reaction
Q5 High-Fidelity 2x Master Mix	12.5 ul
10 uM Forward Primer	1.25 ul
10 uM Reverse Primer	1.25 ul
Template DNA	1 uL (20 ng/uL)
Nuclease-Free Water	9 ul

STEP	TEMP in degrees Celsius	TIME
Initial denaturation	98	30 seconds
40 cycles:	98	10 seconds
	65	30 seconds
	72	50 sec (insert), 2 min 50 sec (vector)
Final extension	72	10 minutes
Hold	4	infinity

Aim: DNA gel of the above PCR product

People: Eva

Protocol: DNA Gel

For results and analysis please see below.

Aim: Colony PCR

People: Eva

Protocol:

1. Dip into a chosen and marked (with a sharpie marker) colony using a pipette tip.
2. Swirl the pipette tip into 10 ul of molecular grade water. [We have 2 colonies we want to test, so we place them in two separate tubes of 10 ul of water.]
3. Perform a PCR protocol using the "colony water" from step one as a substitute for template.

Component	25 µl Reaction	50 µl Reaction	Final Concentration
Q5 High-Fidelity 2X Master Mix	12.5 µl	25 µl	1X
10 µM Forward Primer	1.25 µl	2.5 µl	0.5 µM
10 µM Reverse Primer	1.25 µl	2.5 µl	0.5 µM

Template DNA	variable	5 ul	< 1,000 ng
Nuclease-Free Water	to 25 µl	to 50 µl	

Set up: (50 ul reactions each):

Tube 1 - Colony 1 + original primers, annealing temp: 62 C

Tube 2 - Colony 1 + control primers, annealing temp: 55 C

Tube 3 - Colony 2 + original primers, annealing temp: 62 C

Tube 4 - Colony 2 + control primers, annealing temp: 55 C

Tube 1 and 3 were run in a PCR machine under the following conditions:

Step	Temperature	Time
1	98 C	30 sec
2	98 C	10 sec
3	62 C	30 sec
4	72 C	1 min 20 sec
5	72 C	10 min
6	4 C	infinity

Tube 2 and 4 were run in a PCR machine under the following conditions:

Step	Temperature	Time
1	98 C	30 sec
2	98 C	10 sec
3	55 C	30 sec
4	72 C	1 min 20 sec
5	72 C	10 min
6	4 C	infinity

Aim: DNA gel of the above colony PCR product and the PCR of the control vector provided by Douglass Marr

People: Eva and Nami

Protocol: DNA gel

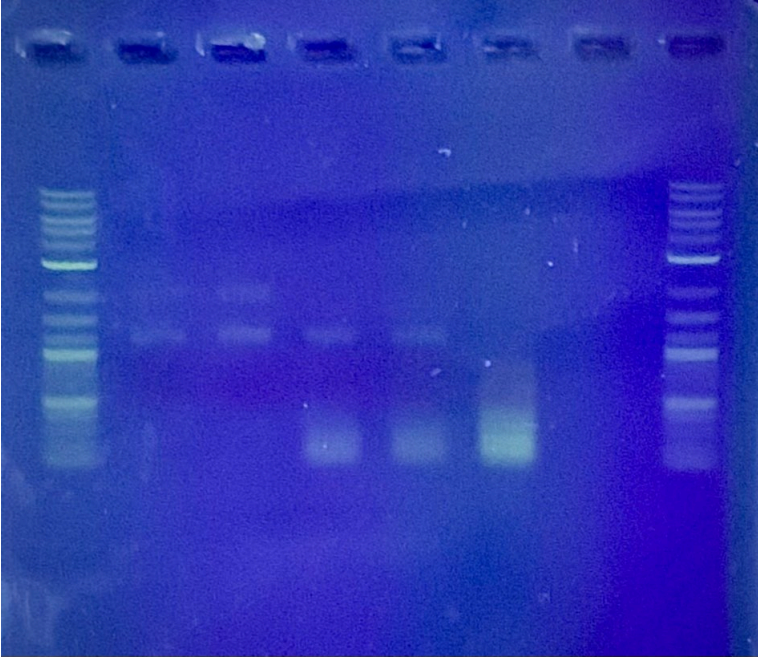
0.8% agarose gel, 110 V for about 30 minutes

Well set up:

- 1 Kb Plus DNA Ladder
- Tube 1 - Colony 1 + original primers, annealing temp: 62 C
- Tube 2 - Colony 1 + control primers, annealing temp: 55 C
- Tube 3 - Colony 2 + original primers, annealing temp: 62 C
- Tube 4 - Colony 2 + control primers, annealing temp: 55 C
- PCR amplification of control plasmid provided by Douglass Marr
-

8. 1 Kb Plus DNA Ladder

Results:



Analysis:

Colony 1 seems to be a successful transformation because of the bands in row 2 and 3 at 2 kb (or rather barely above 2 kb which is the exact weight of the gene in the vector that was supposed to be amplified by our plasmid picked from the plate). Colony 2 has seems to not have the correct construct, since there is no band right above 2 kb. The band present throughout all of the colony samples may be the untransformed vector which remained under the grown colony, but was picked up along with the colony. **This supports the claim that we achieved engineering success in creating a construct optimized for the furthering of SF9 protein S expression.**

We however do not have a definitive proof since we discontinued the experiment due to lack of time and resources, and were thus not able to perform sequencing. We have discontinued the progress for the sake of focusing on performing successful expression in *E. coli* cell lines, which we achieved a month and a half later. However, our work with SF9 shows that if only we are given enough time, reactants and financial resources, we would be able to achieve the successful expression in SF9 cells. This would be the first step in industrializing the process and bringing another option except for Origami B2 expression for creating an injectable protein S therapy.

The next step would be to purify enough of this vector by mini-prep to be able to sequence it. Once we would sequence it, we would be sure about the success of the transformation. Further steps could include checking if we need to design primers for sequencing, and making a new 5 ml LB colony so we can sequence, since we would need to get 300-400 ng of vector to sequence.

The flow of further experiments is depicted below.



Pick a colony and grow it
(5 ml LB + 5 ul 1000x Amp)



Mini-prep to purify
the construct



Restriction digest



Chew back to uncover
the overhang sequence



Ligase Independent Cloning (LIC)
and moving on to transforming cells

Aim: Miniprep transformation colony

People:

Protocol:

Results:

TO DO:

- SF9 - check with Doug if we need to design primers for sequencing
- SF9 - make a new 5 ml LB colony thing so we can sequence (we need to get 300ish-400 ng of vector to sequence)
- SF9 - ask Gergen if we can do sequencing
- SF9 - do we parailm?
- E. coli - make primers for e coli 2Bc-t vector (so we can pcr linearize and amplify)
- E. coli - order primers^
- E. coli - pick a colony 2Bct-t intact vector plate, culture that, and miniprep to have a vector stock ready for multiple LICs.
- E. coli - let the experimental grow for a couple more days in case something shows
- The reason our cells may be growing so slow is b/c of too low T in the incubator and shaker?

AJ meeting notes

- E. coli - We're making new primers for E. coli vector
- SF9 - Grow and mini-prep one of the small colonies (successful LIC)
- E. coli - Experimental did not work. Leave it till Monday just to make sure nothing grows.
- E. coli - Grow more (four preps worth) 2BCT in case we don't end up getting colonies from this^ to prepare for a new LIC - E. coli - Get a ton of linearized plasmid.
- E. coli - Multiple digests on multiple gels to brute force it?

1. pick a colony
2. plate
3. mini-prep
4. digest
5. chew back
6. LIC

jal view sequence alignment

<https://blog.addgene.org/plasmids-101-colony-pcr>

https://openwetware.org/wiki/%27Round-the-horn_site-directed_mutagenesis