Notebook: PgsA_MlrA

WEDNESDAY, 7/7/2021

Protocol:

Summary- Resuspension of gBlocks:

1. Centrifuge at 3000 x g
2. Add 100 uL TE buffer to reach a final concentration of 10 ng/uL
3. Vortex 1 minute
4. Incubate at 50 C for 20 minutes
5. Briefly vortex and centrifuge.

Experimental:

1. Set heat block to 50 degrees Celcius
2. Match the weight of gBlock tube and empty eppendorf tube filled with water (1.59 g)
3. Set Centrifuge at 3000 x g and centrifuged for 1 minute
4. Added 100 uL TE buffer
5. Vortexed for 20 seconds
6. Incubated on a heat block set at 50 degrees Celcius for 20 minutes
7. Vortexed for 20 seconds
8. Centrifuged for 1 minute (weight matched by control tube of water weighed at 1.68 g)

Resuspension of pGSA Forward and Reverse Primers

1. Centrifuge at 3000 x g
2. Add 32 uL TE buffer to forward primer and 37 uL to reverse primer to reach a final concentration of 100 uM
    a. Depending on amount of primer in nmol, multiply by 10 to get amount of TE buffer needed (uL)
3. Vortex 1 minute
4. Incubate at 50 C for 20 minutes
5. Briefly vortex and centrifuge.

Experimental

1. Set heat block to 55 degrees Celcius
2. Matched the weight of forward and reverse primers (1.62 and 1.63 g)
3. Set Centrifuge at 3000 x g and centrifuged for 1 minute
4. Added 32 uL TE buffer to forward primer and 37 uL to reverse primer to reach a final concentration of 100 uM
5. Vortexed for 20 seconds
6. Incubated on a heat block set at 55 degrees Celcius for 3 minutes
7. Vortexed for 20 seconds
8. Centrifuged for 1 minute

THURSDAY, 7/8/2021

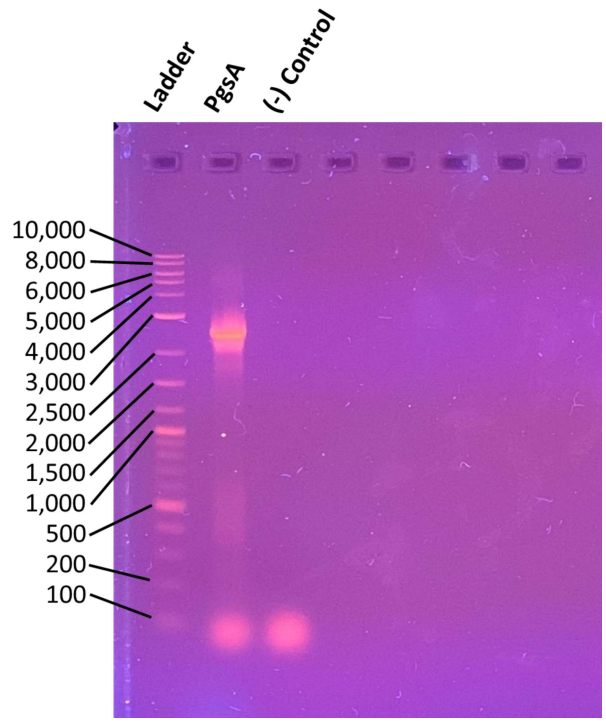
PCR

1. Diluted primers to 10 uM
    a. for primer: 100 uM * 32 uL = x * 10 uM
        I. x= 320 uL (320 -32= 288 uL needed to be added) (only 144 uL needed bc making 1/2)
    b. rev primer: 100 uM * 37 uL = x * 10 uM
        I. x= 370 uL (370 - 37= 333 uL needed to be added) (only 166.5 uL needed bc making 1/2)
2. d

Experimental

1. Dilution:
    a. Forward Primer
        I. 16ul of forward primer in 2ml eppendorf tube and then added 144 uL of nuclease free water to get the concentration to 10 uM.
    b. Reverse Primer
        I. 18.5 of reverse primer in 2ml eppendorf tube and then added 166.5 uL of nuclease free water to get the concentration to 10 uM.
    c. Add
        I. Q5 mastermix= 12.5 uL
        II. For Primer :1.25 uL
        III. Rev Primer : 1.25 uL
        IV. DNA : 2.5 uL
        V. Nuclease free water: 7.5 uL
2. Thermocycler Settings: According to NEB protocols for Q5 mastermix
    a. Initial Denaturation: 98 degrees celcius; 30 seconds; 10 cycles
    b. Denaturation: 98 degrees celcius; 5-10 seconds; 10 cycles
    c. Annealing: 70 degrees celcius; 10-30 seconds; 10 cycles
    d. Extension: 72 degrees celcius; 90 seconds
    e. Hold: 4-10 degrees celcius
3. Gel Electrophoresis:

PgsA Gel.png



MONDAY, 7/26/2021

Preparation of PgsA_MlrA insert

- PgsA_MlrA gene was 2,500 base pairs and gel extracted was conducted
- Gel extraction:
    - To ensure saftey, we removed the gel piece with a gel cutter under the fumehood with gloves, lab coats, and goggles.
    - Add one to one ratio of binding buffer to gel. Mass of gel was 230 mg, thus 230 ug of binding buffer

- Incubate solution for 10 minutes at 50-60 degrees till gel is fully dissolved
- Transfer the solution to a column, centrifuge for one minute and discard the flow through
- Add 100 uL of Binding buffer to the column to increase yield, centrifuge for one minute, then discard the flow through.
- Add 700 uL of Wash Buffer, centrifuge for two minutes, and discard the flow through
- Next we transfered the column to a new centrifuge tude and added 50 uL of Elution buffer, centrifuged for 1 minute, and got our final PgsA DNA.
    - One thing we learned was to avoid using Elution buffer to prevent downstream effects on the DNA and other reactions.

- Nanodrop result: 21.2 ng/uL

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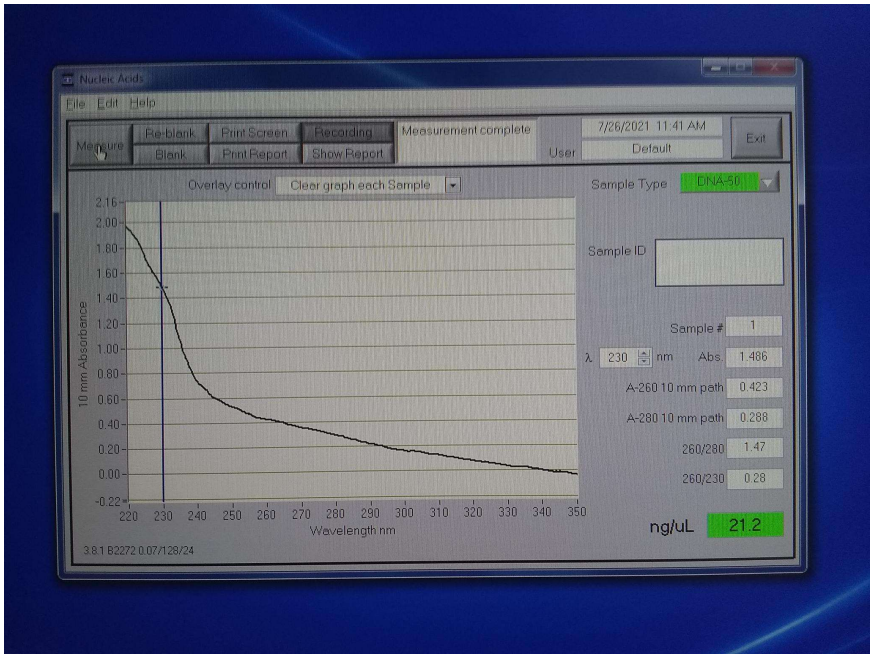

TUESDAY, 7/27/2021

Preparation of PKSI-1 Backbone

We bought the backbone PKSI-1 from addgene which came in DH5-alpha. Thus we had to plate our DH5-alpha, plasmid extract (miniprep), and RE digest before conducting HIFI with the PgsA construct.

1. Plate DH5-alpha with backbone on an ampicilian plate, let it grow overnight.
2. We extracted a single culture and placed in LB media (containing Ampicilian), and let it incubate overnight in 37 degrees.
3. Now time for Miniprep (centrifuge speed is 13,000 Gx
    a. Extract 1.5 mL of liquid bacteria culture and add it to a centrifuge tube
    b. Centrifuge for one minute and discard the supernatant
    c. Add 200 uL of B1 buffer, vortex, mix properly
    d. Add 200 uL of B2 Buffer, DO NOT VORTEX, invert the tube 5-6 times till dark pink.
    e. Incubate at room temperature for 1-2 minutes
    f. Add 400 uL of B3 neutralization buffer and invert the tube 5-6 times.
    g. Incubate at room temperature for 2 minutes, then centrifuge for 5 minutes
    h. Transfer liquid to cleaning column and centrifuge for one minute. Discard flowthrough.
    i. Add 200 uL of wash buffer 1, then centrifuge for one minute and discard flow through.
    j. Add 400 uL of wash buffer 2, then centrifuge for one minute and discard flow through.

k. Transfer column to new centrifuge tube, add 50 uL of nuclease free water, incubate for one minute.
l. Centrifuge for one minute and flow through is final product.

4. Nanodrop:
5. RE digest:
    a. We are doing a 50 uL reaction, thus a PCR tube was used.
    b. We want 1 ug worth of backbone, thus based on our nanodrop result we used ...uL of miniprep product (backbone).
        I.
        II. 1 uL of BamHI-HF and 1 uL of HindIII-HF
    c.
    d. ****We learned that after RE digest, we should have ran it on a gel to confrirm that the linear backbone is 3002 base pairs (minus the 30 base pairs that was cute during RE digest).*
6. PCR Cleanup (13,000 RPM)
    a. 100 uL of DNA cleanup buffer to 50 uL of RE digest product.
    b. Insert Solution to column with collection tube, centrifuge for one minute, discard flow-through.
    c. Add 200 uL of DNA wash buffer, spin for one minute, discard flow through.
    d. Repeat last step
    e. Transfer Column to clean 1.5 ml microfuge tube.
    f. Add 30 uL of Nuclease free water.
    g. Centrifuge for one minute, flow through is final product (Linear backbone).
7. Nanodrop again: 48.2 ng/uL

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WEDNESDAY, 7/28/2021

HIFI: Now we combine the backbone and insert....well kinda...actually not really :(

- We used a HIFI calculator to do a 0.1 pmol reaction.
- In a PCR Tube: (negative control)
    - 3.057 uL of Nuclease Free Water
    - 10 uL of HIFI mastermix
    - 1.36 uL of backbone

    - 5.13 uL of insert
    - 20 uL total
- Postive control:
    - 5 uL of each fragment
    - 10 uL of HIFI Mastermix
    - 20 uL total
- Incubate in thermocycler at 50 degrees for 15 minutes

THURSDAY, 7/29/2021

Transformation with competent DH5-alpha

- Thaw competent cells on ice
- Transfer 50 uL of competent cells into two 1.5 mL centrifuge tube. (one for negative and postive control)
- Add 2 uL of HIFI product to each (postive and negative control respectivley)
- Mix Gently by pipetting
- Place on ice for 30 minutes
- Heat Shock for 30 seconds at 42 degrees
- Transfer to ice for 2 minutes.
- We added 950 uL of LB media. (we should of used SOC media)
- Incubate both tubes at 37 degrees and shake at 250 RPM.
- Add 100 uL of each product to two seperate warm ampicilian plates with a sterile glass spreader (use ethanol and bunsen burner)
- Incubate overnight at 37 degrees.

MONDAY, 8/2/2021

We checked our negative control was able to produce 10 colonies and therefore we transfered them into LB media with Ampicilan and let that grow over night.

TUESDAY, 8/3/2021

Conducted 10 minipreps for all colonies:

1. Now time for Miniprep (centrifuge speed is 13,000 RPM)
    a. Extract 1.5 mL of liquid bacteria culture and add it to a centrifuge tube
    b. Centrifuge for one minute and discard the supernatant
    c. Add 200 uL of B1 buffer, vortex, mix properly
    d. Add 200 uL of B2 Buffer, DO NOT VORTEX, invert the tube 5-6 times till dark pink.
    e. Incubate at room temperature for 1-2 minutes
    f. Add 400 uL of B3 neutralization buffer and invert the tube 5-6 times.
    g. Incubate at room temperature for 2 minutes, then centrifuge for 5 minutes
    h. Transfer liquid to cleaning column and centrifuge for one minute. Discard flowthrough.
    i. Add 200 uL of wash buffer 1, then centrifuge for one minute and discard flow through.
    j. Add 400 uL of wash buffer 2, then centrifuge for one minute and discard flow through.
    k. Transfer column to new centrifuge tube, add 50 uL of nuclease free water, incubate for one minute.
    l. Centrifuge for one minute and flow through is final product.

WEDNESDAY, 8/4/2021

Conducted Nanodrop on all 10 samples

1. k

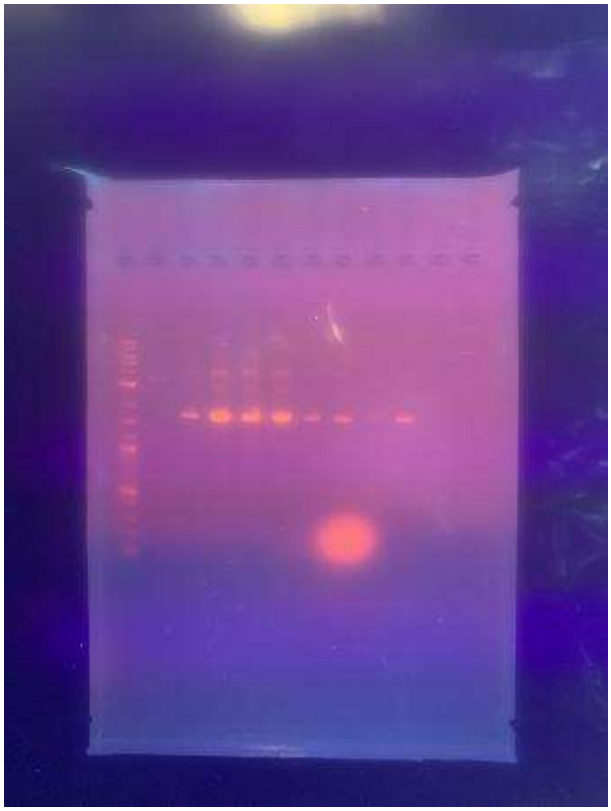
THURSDAY, 8/5/2021

***All 10 samples sent to sequencing

MONDAY, 8/9/2021

- Sequencing results arrived and were poor. Though samples 2, 4, 5, and 6 had sequences that matced PKSI-1 (backbone), there was no evidence of the insert being ligated to the backbone.
- Thus we ran a gel to see if the assembled plasmid was over 5000 base pairs and got this result:

image.png



- Because all 10 samples were not 5000 base pairs we initally thought our past experiments were a failure, but we had a difficult time properly troubleshooting what we did wrong.
- Howver we realized that cirucular DNA can undergo supercoiling which results in the DNA traveling further down the gel then expected. Thus we decided to conduct an RE digest the next day and run a gel.

TUESDAY, 8/10/2021

- New PgsA insert with Overhangs came in the mail.

- Backup plan: Though our sequencing results were poor, there is a chance our plasmid could be good and the correct size. However, incase today's gel is failure, we will redo HIFI and transformations. Therefore, we replated our DH5-alpha with our PKSI-1 backbone.

- We plated more DH5-alpha containing the backbone PKSI-1. (Ampicilian plate was used)
- We let that incubate overnight

- RE Digest of samples 5 and 6
  - Learning from yesterday's mistake we did RE digest to test our gel results. We only conducted this on samples 5 and 6 since the gel from august 9th implied, all the plasmids are the same. (Also to avoid wasting samples)
  - We conducted an additional nanodrop on 5 and 6.
    - Sample 5: 96.2 ng/uL *(not pictured)*
    - Sample 6: 65.3 ng/uL

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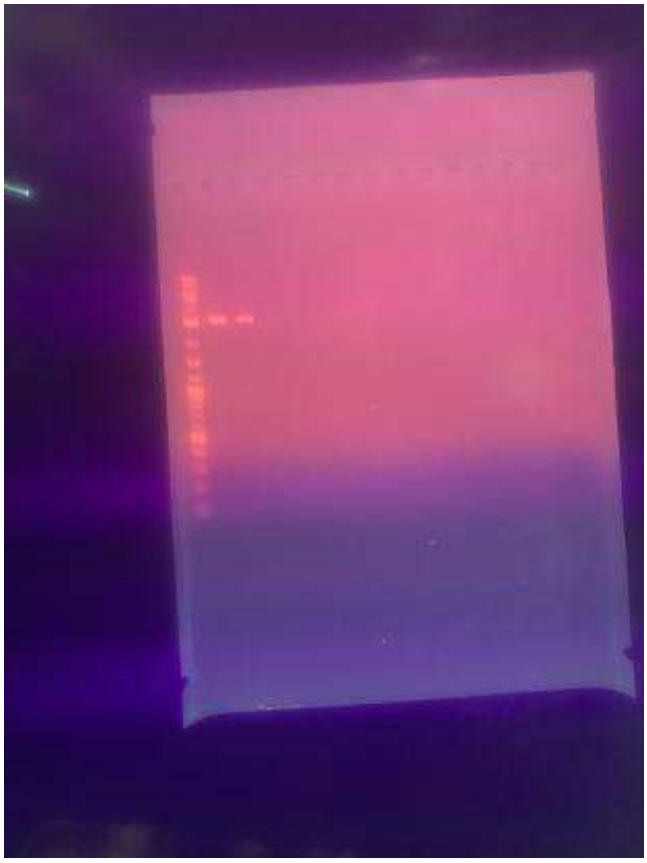
- RE Digest was conducted (50 uL reaction)
  - Based on Nanodrop results we need 1 ug worth of both samples 5 and 6
    - Sample 5--> .0962 ug/uL * V1 = 1 ug --> V1 = 10.395 uL of sample 5
    - Sample 6--> > .0653 ug/uL *V2 = 1 ug --> V2 = 15. 314 uL of sample 6
  - Sample 5:
    - 1 uL of BamHI and 1 uL of HindIII
    - 5 uL of cutsmart buffer
    - 32.61 uL of Nuclease free water
  - Sample 6:
    - 1 uL of BamHI and 1 uL of HindIII
    - 5 uL of cutsmart buffer
    - 27.69 uL of Nuclease free water
  - Incubate at 37 degrees (thermocycler) for 15 minutes

- Gel was conducted.

image.png



Expected result: For each column, there should be two bands. One band is 3000 base pairs (backbone) and the second band is 2,500 base pairs (insert).
Actual result: Both samples have one band that is 3000 base pairs which is the length of the backbone.
Likley explanation: So it turns out that the insert does not show up on the gel. This explains why the sequencing results only showed the backbone. This could mean two things:

1. PCR of the PgsA_MlrA insert introduced a mutation on the homology arms and it did not properly ligate to the backbone. Ordering the insert with the homology arms already attached reduce the risk of mutation during PCR.
2. We messed up during HIFI (though we beleive we did it correctly).

What now? We have to redo HIFI and transformation with the new ordered insert.

WEDNESDAY, 8/11/2021

- We placed our colonies of DH5-alpha containg our backbone in 5 small LB tubes and let it incubate overnight at 200 RPM.
- We put our plate in the 4 degree fridge.

THURSDAY, 8/12/2021

- We conducted 4 Minipreps of our DH5-alpha bacteria to extract our backbone PKSI-1.
    a. We named our samples 1, 2, X and Y. We did this for each sample:
        I. Extract 1.5 mL of liquid bacteria culture and add it to a centrifuge tube
        II. Centrifuge for one minute and discard the supernatant
        III. Add 200 uL of B1 buffer, vortex, mix properly
        IV. Add 200 uL of B2 Buffer, DO NOT VORTEX, invert the tube 5-6 times till dark pink.
        V. Incubate at room temperature for 1-2 minutes

VI. Add 400 uL of B3 neutralization buffer and invert the tube 5-6 times.
VII. Incubate at room temperature for 2 minutes, then centrifuge for 5 minutes
VIII. Transfer liquid to cleaning column and centrifuge for one minute. Discard flowthrough.
IX. Add 200 uL of wash buffer 1, then centrifuge for one minute and discard flow through.
X. Add 400 uL of wash buffer 2, then centrifuge for one minute and discard flow through.
XI. Transfer column to new centrifuge tube, add 50 uL of nuclease free water, incubate for one minute.
XII. Centrifuge for one minute and flow through is final product.

- Next we nanodropped all 4 of our samples
  - Sample 1: 170.2 ng/uL

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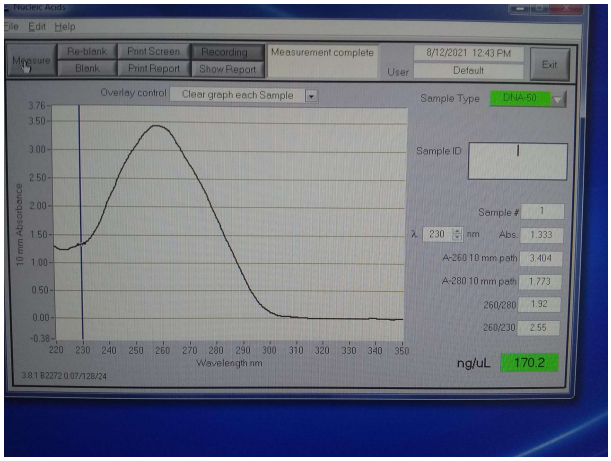

  - Sample 2: 217.4 ng/uL

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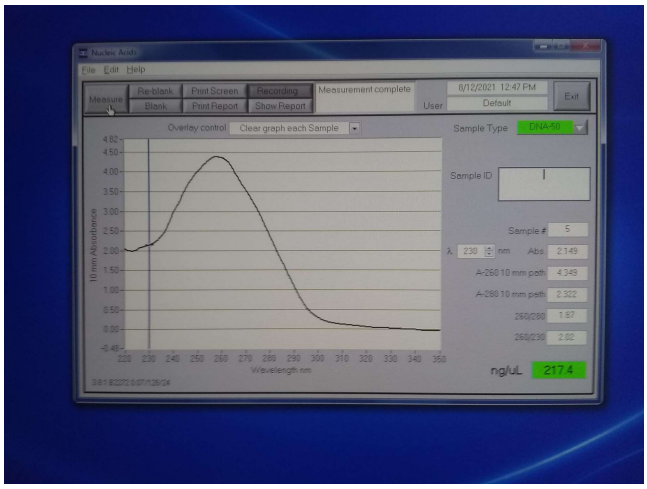

  - Sample X: 152.2 ng/uL

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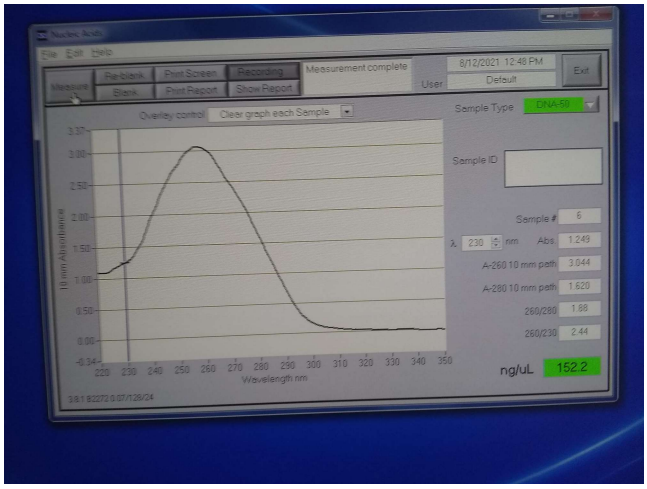

- Sample Y: 113.9 ng/uL

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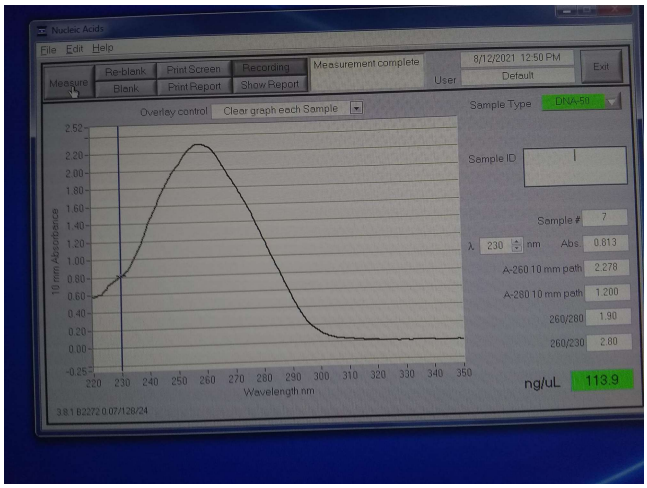
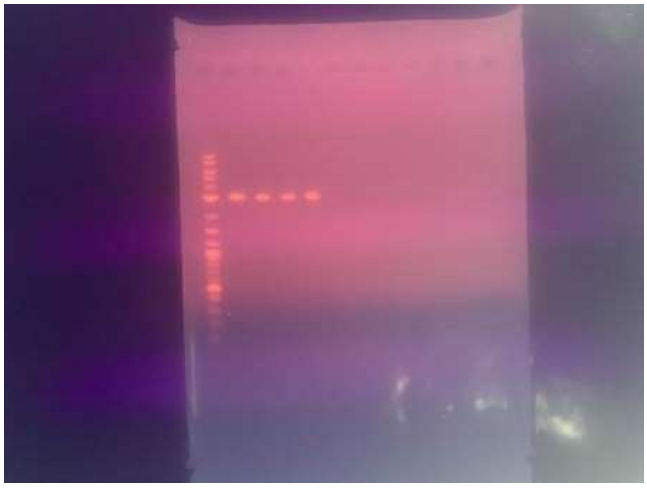


- Next we conducted RE Digest on all 4 samples to linearize them before HIFI
  - Based on Nanodrop results we need 1 ug worth of both samples 5 and 6
    - Sample 1--> .1702 ug/uL * V1 = 1 ug --> V1 = 5.88 uL of sample 1
    - Sample 2--> .2174 ug/uL *V2 = 1 ug --> V2 = 4.60 uL of sample 2
    - Sample X--> .1522 ug/uL *V3 = 1 ug --> V3 = 6.57 uL of sample X
    - Sample Y--> .1139 ug/uL *V3 = 1 ug --> V4 = 8.78 uL of sample Y
    - Sample 1:
      - 1 uL of BamHI and 1 uL of HindIII
      - 5 uL of cutsmart buffer
      - 37.12 uL of Nuclease free water
    - Sample 2:
      - 1 uL of BamHI and 1 uL of HindIII
      - 5 uL of cutsmart buffer
      - 38.40 uL of Nuclease free water
    - Sample x:

        - 1 uL of BamHI and 1 uL of HindIII
        - 5 uL of cutsmart buffer
        - 36.43 uL of Nuclease free water
    - Sample Y:
        - 1 uL of BamHI and 1 uL of HindIII
        - 5 uL of cutsmart buffer
        - 34.22 uL of Nuclease free water
    - Incubate at 37 degrees (thermocycler) for 15 minutes

- Unlike last time, we conducted a gel of our RE digest product to make sure the backbone properly linearized and is the correct size.
    - Our expected result should be one band that is 3000 base pairs to represent our backbone.
    - The 30 basepairs that was cut from RE digest may not appear since it is small and run off the gel.
    - The total length of the backbone is about 3032 base pairs.

image.png



Our result is exactly how we expect it and thus we are ready to conduct HIFI assembly.

FRIDAY, 8/13/2021

- Conducted PCR cleanup on samples 1, 2, X, and Y
    a. 100 uL of DNA cleanup buffer to 50 uL of RE digest product.
    b. Insert Solution to column with collection tube, centrifuge for one minute, discard flow-through.
    c. Add 200 uL of DNA wash buffer, spin for one minute, discard flow through.
    d. Repeat last step
    e. Transfer Column to clean 1.5 ml microfuge tube.
    f. Add 30 uL of Nuclease free water.
    g. Centrifuge for one minute, flow through is final product (Linear backbone).

MONDAY, 8/16/2021

1. Nanodropped samples 1, 2, X, and Y
    a. sample 1--> 33.7 ng/uL
    b. (pic)
    c. Sample 2--> 33.2 ng/uL

d. Sample x--> 22.3 ng/uL
e. Sample y --> 24.4 ng/uL

2. Conducted HIFI (we used sample 1)
    a. Experimental control
        I. Vector (sample 1)--> 5.936 uL
        II. Insert--> 3.263 uL
        III. water--> 0.801 uL
        IV. HIFI master mix--> 10 uL
        V. TOTAL= 20 uL
    b. Positive control
        I. Vector (7 uL)
        II. Insert--> 3 uL
        III. Positve control mix--> 10 uL
        IV. TOTAL= 20 uL

TUESDAY, 8/17/2021

HIFI was conduced again and transformed onto DH5-alpha bacteria.

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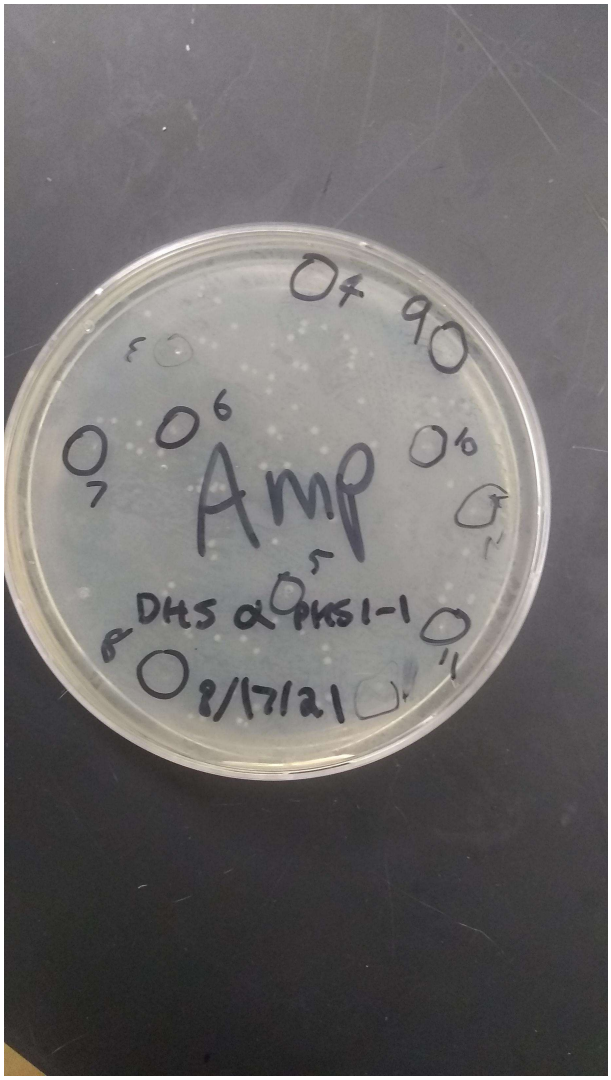


MONDAY, 8/23/2021

- Colonies picked out and transferred to LB media for Mini prep the next day (8/24/2021).

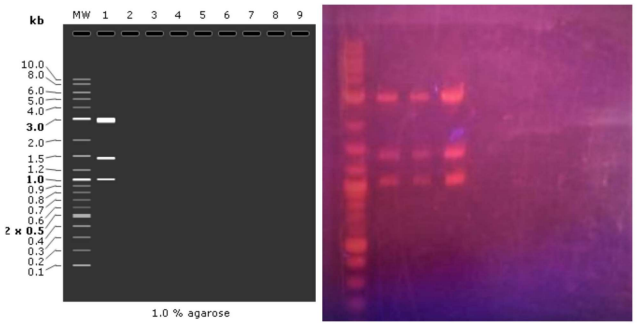
TUESDAY, 8/24/2021

- LB media was miniprepped and nanodropped to isolate the plasmid.

WEDNESDAY, 8/25/2021

- Re digest was conducted with the isolated plasmid to check if it is the correct length. The restriction enzymes SpeI and Kpn1 were used. Since there are two Kpn1 restriction sites and one SpeI restriction site, there should be three bands on a gel electrophoresis should appear. We conducted a Gel electrophoresis simulation on snapgene to confirm our gel results. Based on the snapgene simulation, our actual gel results are consistent! The plasmid is the correct size of 5427 base pairs.

BeFunky-collage.jpg



Snapgene simulation vs experimental gel

MONDAY, 8/30/2021

Since the plasmid was the correct length, we transformed the plasmid into BL-21 E.coli.

1. Take BL-21 competent cells out of -80°C and thaw on ice;
2. Warm up agar plates warm up to 37°C in the incubator;
3. Add of 2 µL vector into 50 µL competent cells, and GENTLY mix by flicking the bottom of the tube;
4. Incubate on ice for 20 min;
5. Heat shock each transformation tube in the 42°C water bath for 30 s;
6. Put the tubes back on ice for 2 min;
7. Add 950 µL SOC media (without antibiotic) to the bacteria and grow in a 37°C shaking incubator for 60 min;
8. Plate the transformation and incubate plates at 37°C overnight.

TUESDAY, 8/31/2021

BL-21 culture plate.jpg

The colonies were picked out and placed into LB media ready for testing.

THURSDAY, 10/14/2021

To inducee bacterial protien expression, we needed to induce it with IPTG

Stock IPTG (powdered form) had to be put in solution to make a 100 mM stock. .....

1. Transform expression plasmid into BL21(DE3). Plate on antibiotic selection plates and incubate overnight at 37°C.
2. Resuspend a single colony in 10 ml liquid culture with antibiotic.
3. Incubate at 37°C until OD_{600} reaches 0.4–0.8.
4. Induce with 4 or 40 µl of a 100 mM stock of IPTG (final concentration of 40 or 400 µM) and induce for 3 to 5 hours at 37°C.