2018 University of Iowa International Genetically Engineered Machine Team
Dr. Jan Fassler's Lab, Biology Department
Eleanor Bessner, Jennifer Farrel, Yishuo Jiang, Rafael Linares, Delaney Soules, Shao Yang Zhang

Improve 2017 UIOWA IGEM biobrick

MONDAY, 8/6/2018

Goal: To replicate the IGEM 2017 promoter-regulator E. coli optimized biobrick and transform it into E. *coli*

Steps:

- PCR on 2017 template amplify & confirm
  - Can use same primers
  - Protocol: Q5 pcr
- Digest with EcoR1, Pst1
- PCR clean-up reaction
  - Gel confirmation & recovery
- Generate new Lux plasmid backbone for insert:
  - Digest with PST1 &EcoR1 & gel recovery
  - Amplify outward from insert through pcr
- Ligate 2017 biobrick into plasmid
- Transform into E. coli - order new 3hp tolerant strains

Thursday August 2, 2018:

Title: PCR on 2017 g-blocks to amplify and confirm that we have DNA of expected size

Abstract: To perform Q5 pcr on our 2017 biobricks-- PmmsA and LysR to validate and prepare for ligation then transformation into E. *coli.*

Procedure:

1. G-blocks left over from last year: PmmsA already rehydrated to a concentration of 10 uM per writing on tube.
   LysR - added 100 uL ultrapure water - tube C - to rehydrate to 10 uM concentration

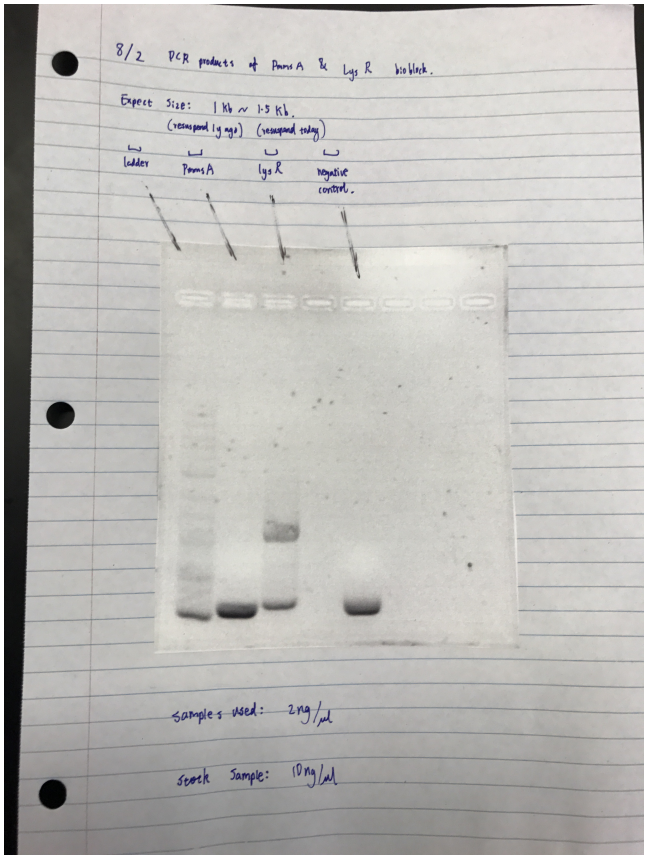
2. Create working stocks of these biobricks for PCR- gray tape on top of tube -PmmsA: add 4 uL to 16ul ultrapure h20 (tube C)
   - LysR: add 4 uL to 16ul ultrapure h20 (tube C))
3. PCR with Q5 high fidelity Polymerase (protocol) - 50 uL rxns
   three rxns prepatred: PmmsA, LysR and neg control
   - add 25 uL of Q5 mm - tube D
   - add 18 uL ultrapure H2O - tube C (20 uL to neg control)
   - 5 uL 5 uM mixed prefix & suffix primers - pink label prepared 8/1/18
   - 2uL template DNA (no DNA added to neg control)
4. Cycled under the following conditions:
   - initial denaturation 98deg C 60 sec
   - 30 cycles
     - 98 deg C 20 sec
     - 72 deg C 20 sec annealing
     - 72 deg C 20 sec extension
   - final extension 72 deg C for 120 sec
   - hold @ 4 deg C

   *note tubes were melty but intact when taken from cycler
5. Visualize on agarose gel

- gel prepared 2% .6 g agarose 30 uL Tris 3 uL gel red - note this was used only because it had been left over from a previous lab experiment. did not need a 2% gel, and age of gel might have inhibited performance, but did not want to waste.
- Ln 1: ladder Ln2: PmmsA Ln3: LysR Ln 4: empty Ln 5: neg control
- 5ul ladder and 2uL dye plus 10uL template loaded

6. Results and Discussion:

image.png



no bands of expected size seen for PmmsA and splotchy band seen at about 1-1.2kb in LysR . Seeing primer dimers at approx 200 bp in neg control and both samples. Either primer, water or leaving primers mixed in tube for several days is increasing dimer intensity. Typical of what we have been seeing in our neg control. Ladder is pretty splotchy in appearance too, which would lead me to believe the gel quality might be subpar.

In order to confirm our original oligo concentrations were as expected, I nanodrop tested our pcr stock preps (expected 1-2 ng/ul concentration. Received the following results:

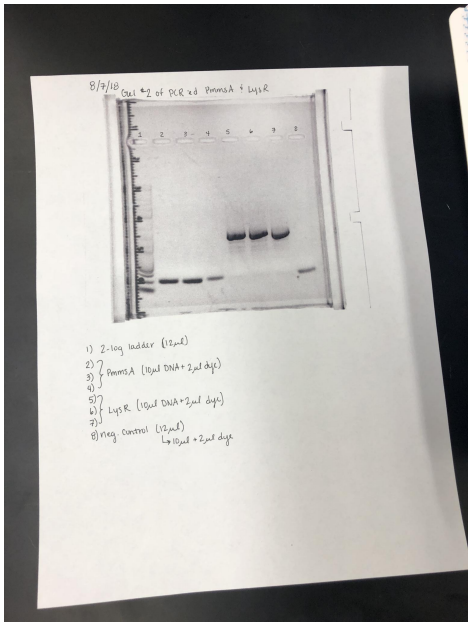
PmmsA .6 ng/uL
LysR: 3 ng/uL

neither result shows a drastic problem with our starting stock, but at only .6 ng/uL --still less than ideal for a starting concentration.
Will discuss with group if want to try another pcr with different reagents or rerun these samples on a new gel.

TUESDAY, 8/7/2018

Second gel run on the PCR products of PmmsA and LysR:

20180807_230813691_iOS.jpg



Goal: Gel extraction on PCR products PmmsA and LysR using Qiagen Gel Extraction Kit

Abstract:

Procedure:

1. Cut out the desired bands on the gel electrophoresis using the UV light and a scalpal.
2. Measured the weight of the gel extracted by putting into 1.5mL and weighing it, making sure to subtract the weight of the 1.5mL tube.
    a. PmmsA Tube 1: 0.16g
    b. PmmsA Tube 2: 0.25g
    c. LysR Tube 1: 0.48g
    d. LysR Tube 2: 0.28g
3. Add 3 volumes of QC to each volume of gel and incubate at 50^{o} for 10 minutes, vortexing occassionally.
4. Added 1 gel volume of isopropional to each sample and inverted a few times
5. Applied each sample to the QIAquick columns and spun in centrifuge for 1 minute at 12,000 rpm
6. The flow through was discarded and Step 5 was redone for tubes that had more volume left in them
7. Washed each tube by using 700μL of PE buffer and centrifuged for 1 minute at 12,000 rpm
8. Removed flowthrough and centrifuged again to remove residual wash
9. Original 2mL tubes were thrown away and QIAquick is transfered to a clean 1.5mL tube
10. Tubes were eluted in 50mL of EB buffer and centrifuged for 1 minute at 12,000 rpm. Throw away QIAquick and keep the 1.5mL tube with the DNA in it.

WEDNESDAY, 8/8/2018

11. (continuation of yesterday) DNA was taken to be nanodropped using EB:

Table1

	A	B	C	D
1		ng/uL	A260/A280	A260/A230
2	PmmsA 1	-20.1	33.91	0.04
3	PmmsA 2	-7.8	10.16	0.04
4	LysR 1	-17.0	-32.33	0.04
5	LysR 2	-19.7	78.45	0.02

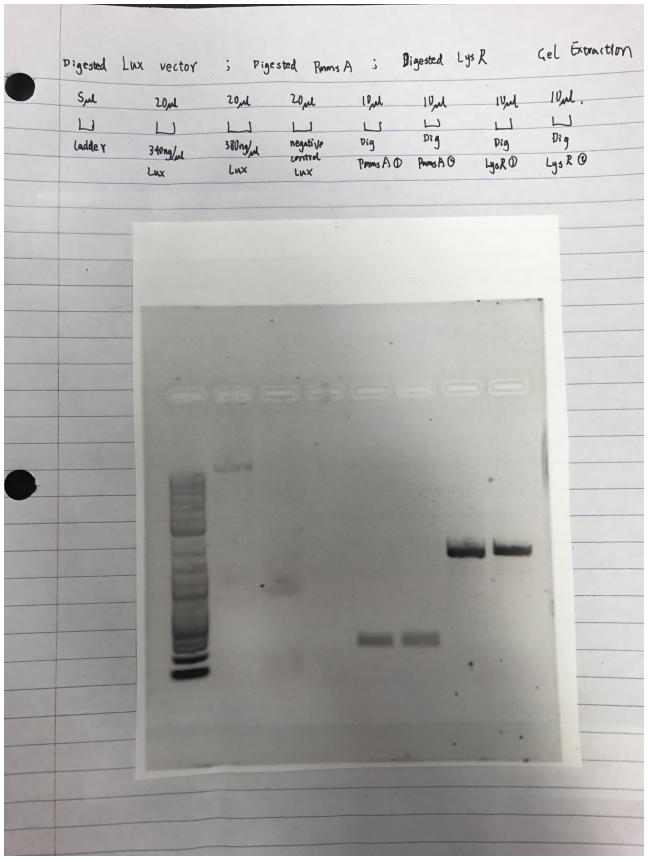
It seems from the nanodrops that there is no DNA present in each of the samples taken, but further steps will be taken in order to determine if there is DNA present, which will be discussed with lab members.

8/22/17
abstract: run digested parts and plasmids on gel to visually confirm and to obtain extraction of parts and plasmids free of digested waste.

procedure:
1. load 1% agarose gel as follows:(YJ)

Lane:
1 2-log ladder 5 ul
2 340 LUX plasmid digest 20ul plus 4 ul dye
3 380 LUX plasmid digest 20ul plus 4 ul dye
4. neg control LUX digest 20ul plus 4 ul dye
5 digest PmmsA_1 digest 20ul plus 4 ul dye
6 digest PmmsA_2 digest 20ul plus 4 ul dye
7 digest LysR_1 digest 20ul plus 4 ul dye
8 digest Lys R_12digest 20ul plus 4 ul dye

image.png



Gel extractions: Ln 2- Lux
Ln 4&5 PmmsA
Ln 5&6 LysR

08/24/18:
abstract: perform gel extraction purification to recover the digested parts and plasmids

Procedure: follow protocol: Extraction of DNA fragments from an Agarose Gel

(continued in "Extraction of Digested Lux vector"

Extraction of Digested Lux vector, Digested LysR and Digested PmmsA fragments from an Agarose Gel

Introduction

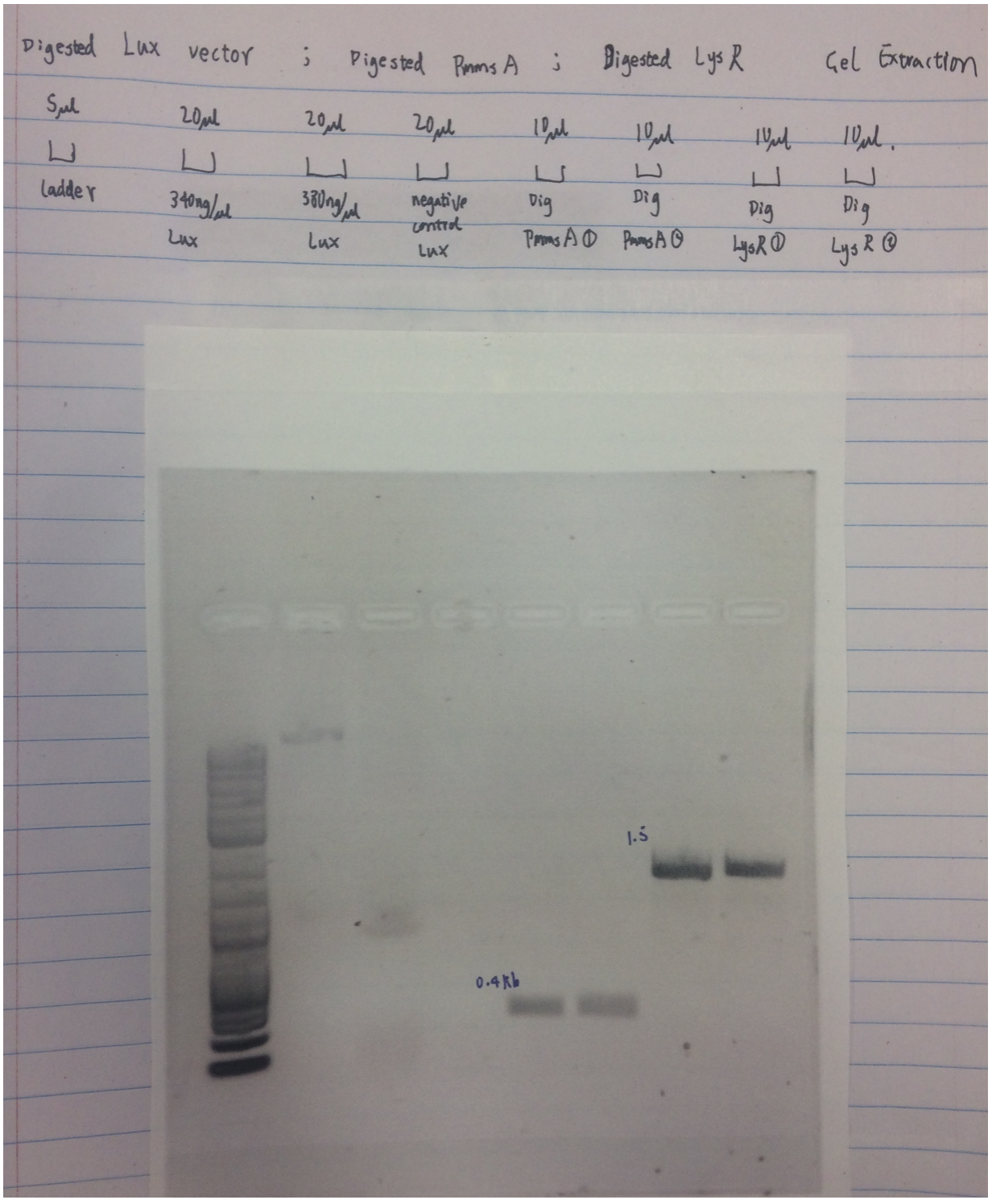
After digestion of lux vector, LysR and PmmsA with PstI and EcoRI enzymes, these samples were put into 1% agarose gel for electorphoresis. Then, gel extraction was performed to purified the digested DNA samples.

Materials

- Buffer QG (yellow at pH less than or equal to 7.5)
- Buffer PE (Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- Isopropanol (100%)
- a heating block or water bath at 50°C are required.

Procedure

Gel Image



Gel Extraction

1. Cut the fragment out of an agarose gel
2. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel on a UV box. Important: use eye and skin protection while doing this; the UV radiation is harmful.

3. Weigh the gel slice in a colorless tube whose weight has been pre-determined.

4. Add 3 volumes Buffer QG to 1 volume gel

   Note 1: 100 mg gel ~ 100 µl.
   Note 2: The maximum amount of gel per spin column is 400 mg.
   Note 3: For >2% agarose gels, add 6 volumes Buffer QG.

5. Incubate at 50°C for 10 min or until the gel slice has completely dissolved. Vortex the tube every 2–3 min to help dissolve gel.

6. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose). If the color of the mixture is orange or violet, add 10 µl 3 M sodium acetate, pH 5.0, and mix.

7. Add 1 gel volume isopropanol to the sample and mix.

8. Place a QIAquick spin column in a provided 2 ml collection tube. Apply the sample to the QIAquick column and centrifuge for 1 min.

9. Discard flow-through and place the QIAquick column back into the same tube. For sample volumes of >800 µl, load and spin again.

10. To wash, add 750 µl Buffer PE to QIAquick column and centrifuge for 1 min. Discard flow-through and place the QIAquick column back into the same tube.

11. Centrifuge the QIAquick column in the provided 2 ml collection tube for 1 min to remove residual wash buffer.

12. Place QIAquick column into a clean 1.5 ml microcentrifuge tube.

13. To elute DNA, we add 30 µl Buffer EB (10 mM Tris·Cl, pH 8.5) (protocol said 50ul) to the center of the QIAquick membrane. We let them stand for 5 min, then centrifuge the column for 1 min (13,000 rpm).

Result

lux vector	16.1ng/ul
PmmsA	11.2ng/ul
LysR	2,4ng/ul

T4 ligation

14. Thaw the T4 DNA Ligase Buffer and resuspend at room temperature.

15. Use NEBioCalculator (http://nebiocalculator.neb.com/#%21/) (http://nebiocalculator.neb.com/#!/ligation) to calculate molar ratios for insert and vector DNA of specific sizes. **The desired molar ratio is 1:3 vector to insert**

16. Set up the following reaction in a microcentrifuge tube on ice:

    Set up the following reaction in a microcentrifuge tube on ice:

Pmmsa (2 rxn)

17. 10x ligation buffer 2ul
18. lux vector 3ul
19. insert pmmsa 2 ul
20. dH2O ultrapure 12ul
21. t4 ligase 1ul
22. 20 ul total rxn labeled pmmsa1 and pmmsa2

LysR (2 rxn)

23. 10x ligation buffer 2ul
24. lux vector 3 ul
25. insert LysR 2.5 uL
26. dH2O ultrapure 11.5 uL
27. T4 ligase 1ul
28. 20 ul total rxn labeled LysR1 and LysR2

cycled in thermocycler for 16C for 900 min then 65C for 10 mins

Igem style transformation

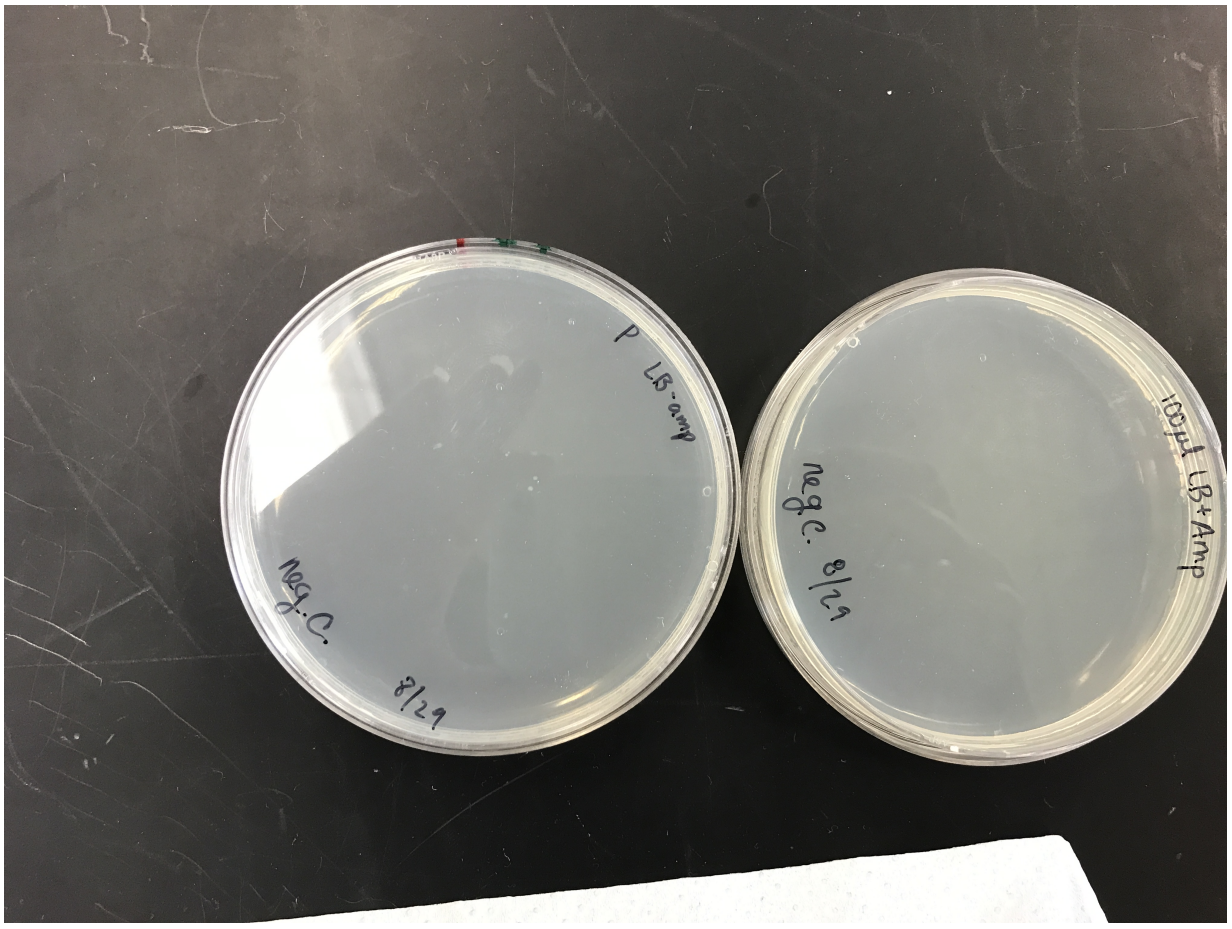
29. IGEM style transformation 5 transformations:

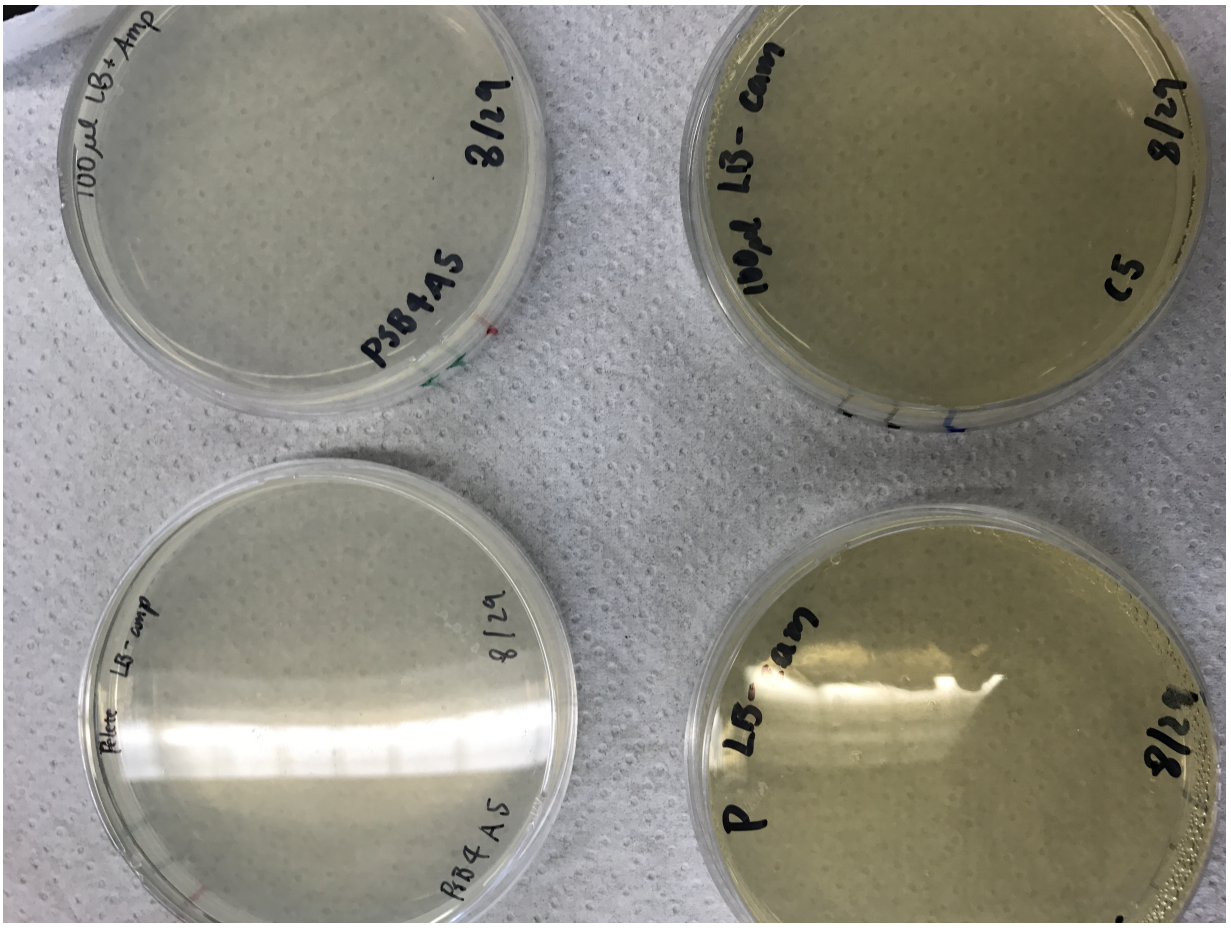
    PmmsA1
    Pmmsa2
    LysR1
    LysR2
    pos (lux)
    psb4A5
    psb4C5 (CAM)
    neg

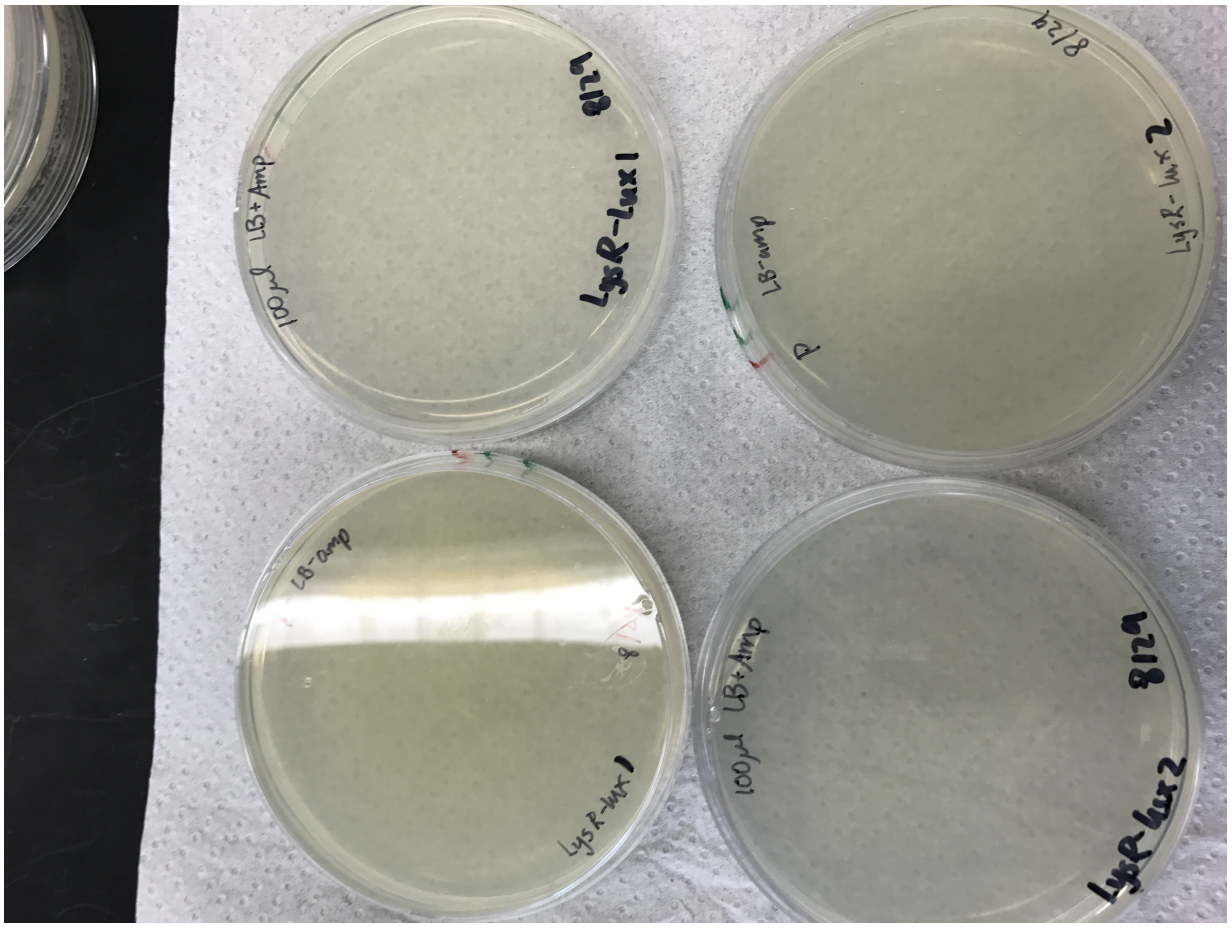
30. labaled tubes as above and chilled on ice
31. added 50 uL MG1655 competent cells to each tube
32. added 2 uL ligate to each tube
33. chilled on ice 30 minutes
34. heat shock 45 sec @42C
35. chilled on ice for 5 min
36. added 400 uL Xymo broth
37. incubated @35C for 1 hour
38. plated on AMP agar plates 100 ul each direct and 100ul each pelletted (psb1C5 plated on CAM)
39.
40. incubate overnight @ 35C

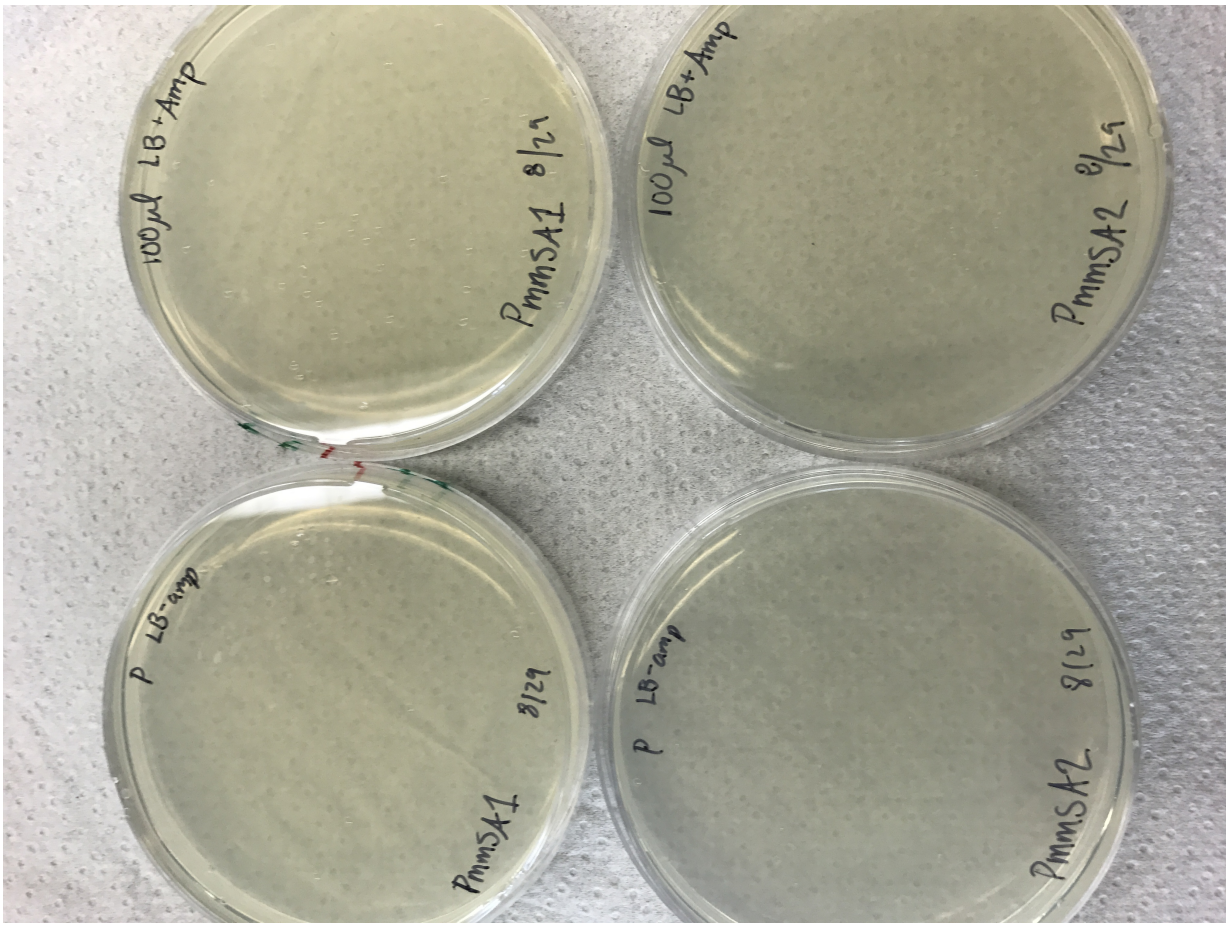
Results:

41.


P LB-amp
neg.C.
8/29
neg.C. 8/29
100µL LB+Amp


100µl LB+Amp
8/29
P384A5
100µl LB-cam
C5
8/29
Plate LB-amp
8/29
P384A5
P LB-am
8/29


100µl LB+Amp
8/29
LysR-Lux1
LB-amp
8/29
LysR-lux2
LB-amp
LysR-lux1
100µl LB+Amp
8/29
LysR-lux2



42. Innoculated transformants into Amp lb broth (C5 into CAM lb)

iGEM Regulators/Promoters into iGEM plasmids

Introduction

Materials

-
  - Inserts: CE3010 PmmsA; CE 3011 Pxyl-MMSR; CE3012 PhpdH-lux
  - Plasmid Psb1C3 - linear from IGEM kit
  -
  -

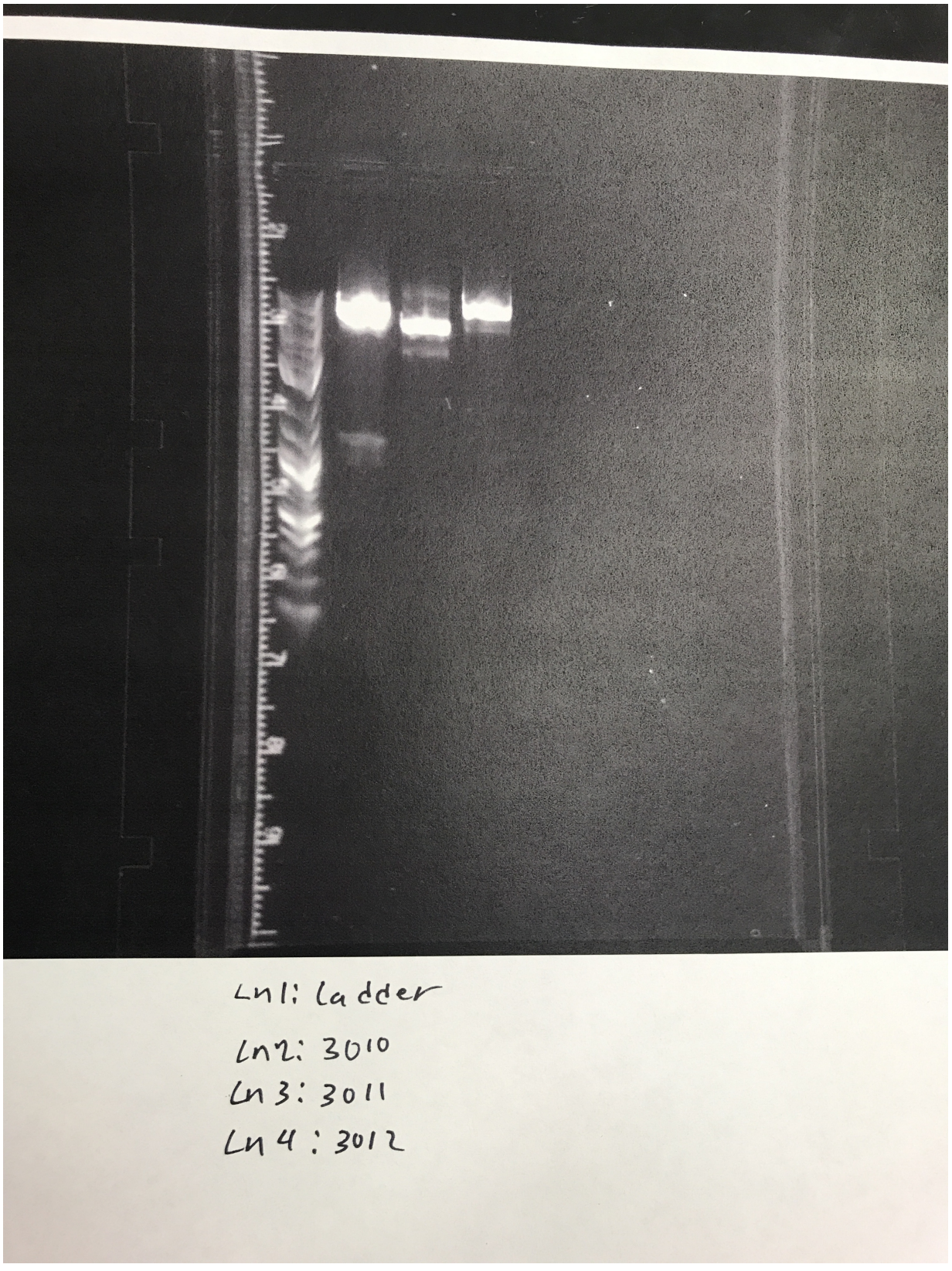
Procedure

Inserts

1. Qiagen Miniprep (COMPLETED)
2. Nanodrop (completed 09/24/18)

	ng	260/280	260/230
3010--	363.8 ng/ul	1.88	1.44
3011	127.2 ng/ul	1.77	.88
3012	287.1 ng/up	1.80	1.39

3. Gel verify



4. EcoRI/PstI Digest (Restriction Digest, IGEM style)

    2 ul of cutsmart
    4 ul of DNA
    .5 uL EcoR1-HF

.5uL Pst1
13 ul of up h2O
digest 37 deg C 180 min heat kill enzyme 80 deg C 20 min

5. Gel Purify

-9-25-18 9:00 AM - Made 1.2% Agarose gels. Mixed 1.2 g of agarose powder into 100 mL of TAE buffer. - Microwaved until dissolved, then added 6 uL of Gel Red.
-Cast three gels. Moved over to electrophoresis machine, added more TAE buffer carfully pulled comb out
-Added 4 uL of Gel Loading Dye Purple (#B7025S NEB) to each tube and gently pipetted up and down to uniformly mix.
- Transferred volume (~24 uL) carefully to each lane and ran at 75 V for ~ 45 minutes. Only added 2.5 uL of the 2log DNA Ladder.
- Note the image below was taken on my cell phone and is not the highest quality. There is a band in 3011 that looks like the size of the part. Lower down and much fainter are bands in 3012 and 3010 that should correpond to the part. These parts were not separated from the dye so Fassler recommended running them a bit longer.

6. (EcoRI/PstI) iGEM Restriction Digest with higher DNA Concentration (26Sep2018)

6uL Cutsmart NEB Buffer
24uL DNA
28uL dH2O
1uL EcoRI-HF
1uL PstI
iGEM Restriction Thermocycler Protocol: 37 deg C 180 min, heat kill enzyme 80 deg C 20 min

Plasmid

7. PSB1C3 linearized

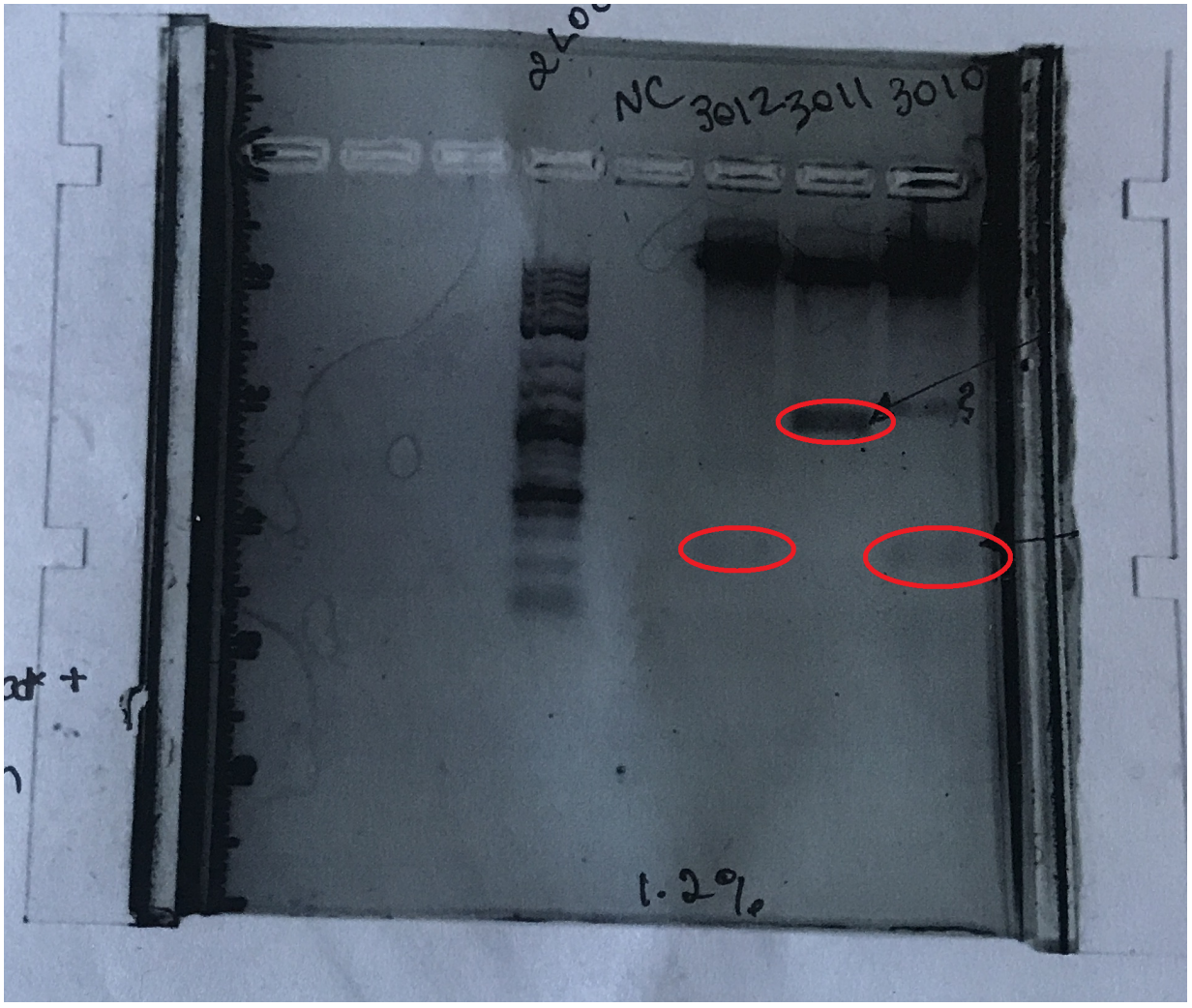
8. Digest (done 9/24/18)

Enzyme MM
5 uL NEB buffer (sub cutsmart)
.5ul BSA
.5 uL EcoR1-HF
.5uL Pst1
.5 uL Dpn1

18uL up H2O

add 8ul Enzyme MM to 8 uL linearized plasmid (doubled recipe)
digest 37 degC 180 min heat kill enzyme 80 deg C 20 min

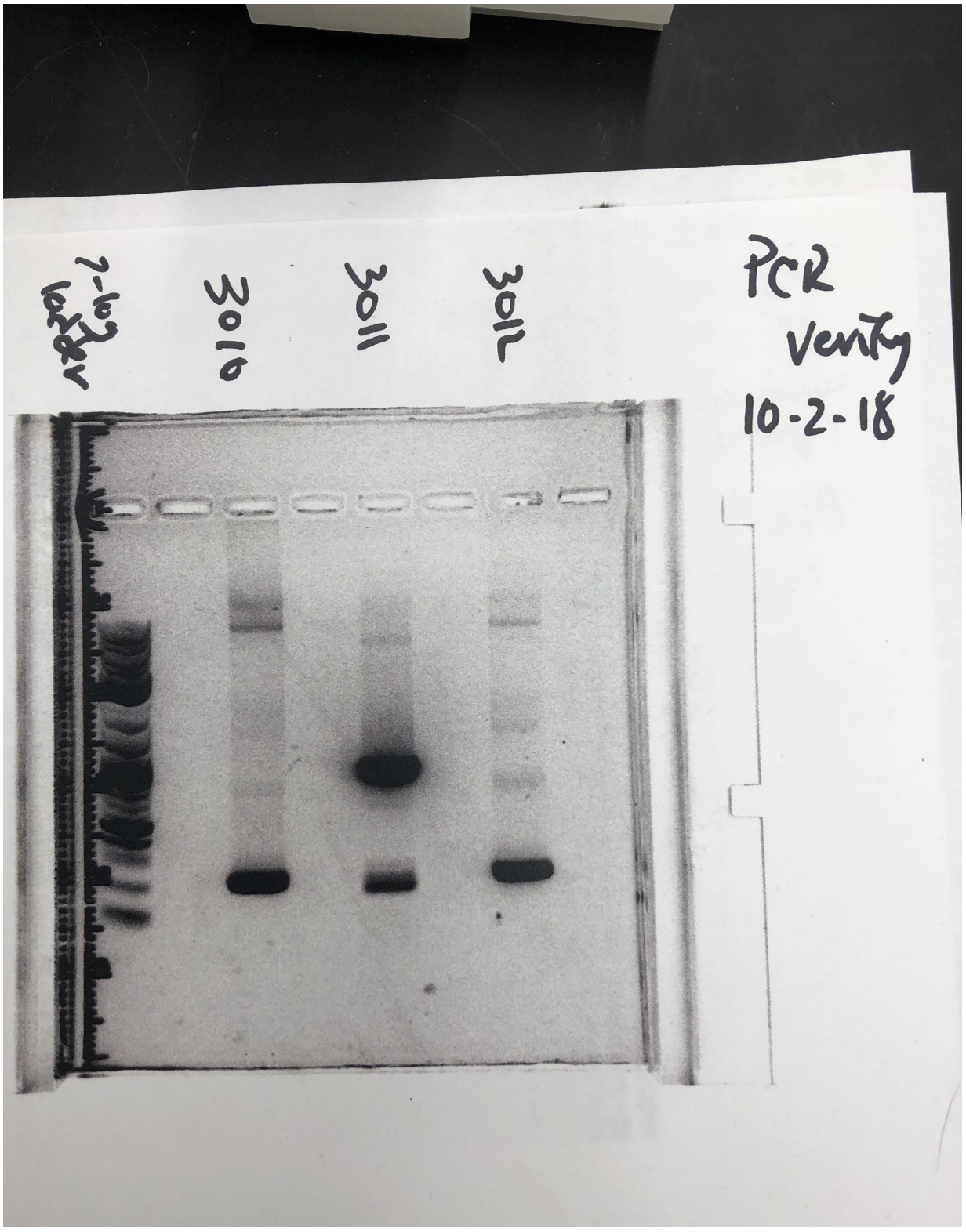
Gel Purification (9/25/18)



October 2, 2018

9. Ran an agarose gel to verify pcr product made by KH 10/2/18

PCR:
25 uL Q5
5 uL Primer
2 uL DNA
18 ul H2O



10. Ran a pcr Qiagen kit column purification (see IGEM protocol - Clean up for PCR)

11. Restriction digest: 3010, 3011, 3012

    6 ul Cutsmart
    24 uL DNA
    28 dH2O ultrapure

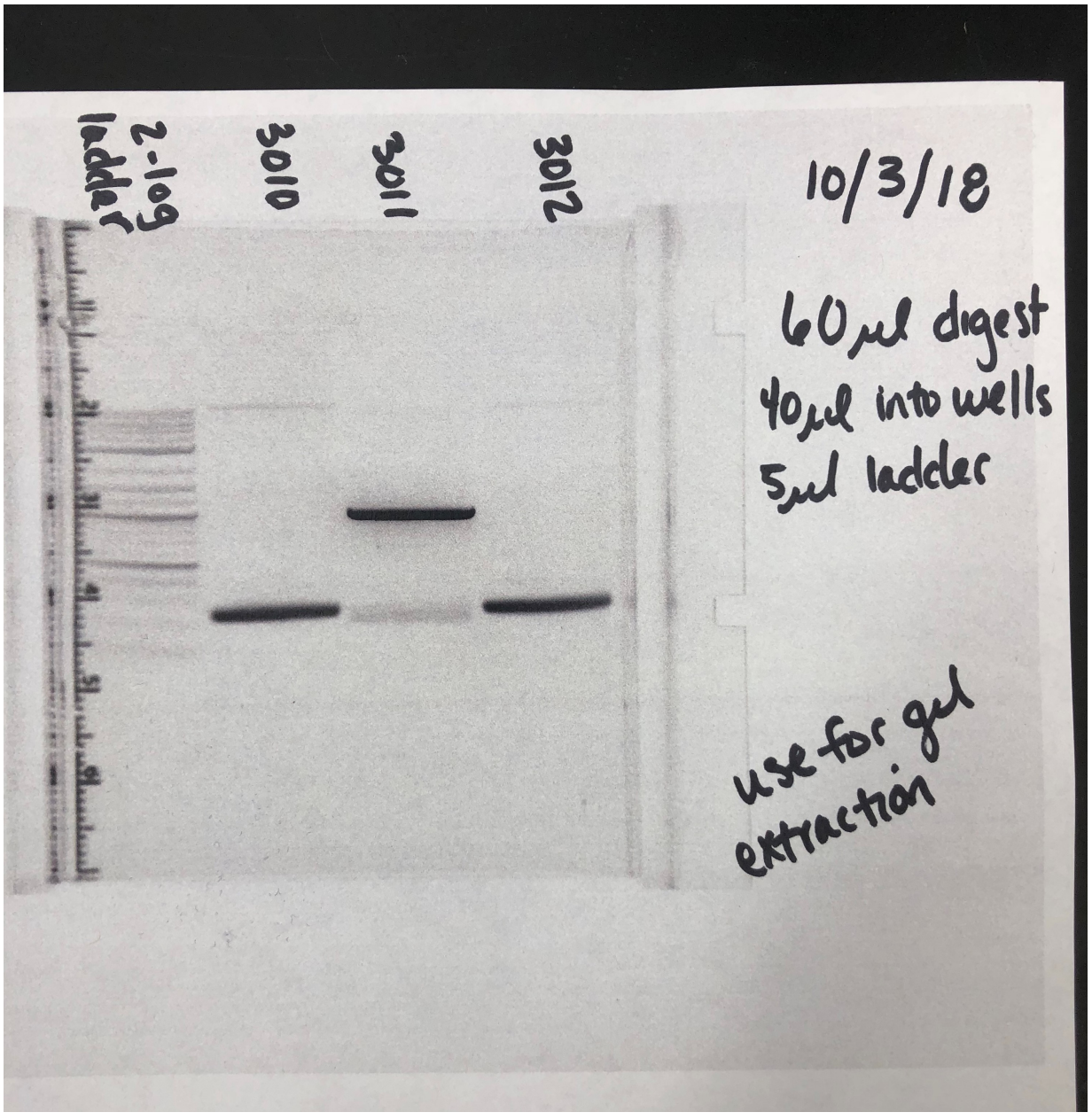
1ul ECOr1
1ul PST1

12. Ran restriction digest protocol on thermocycler (180 mins @ 37C and 20 min @80C hold @ 4C) by JF & DS 10/02/18

October 3, 2018

13. Gel electrophoresis for gel extraction (10/03/18)

    4 well gel with 40 uL DNA samples
    Mixed 12 uL dye with ~60 uL digest from 10/02/18 and added 40 uL to wells
    5 uL ladder



14. Gel Extraction by DS (10/03/18)

Followed Benchling protocol on "Extraction of DNA fragments from gel"

Table1

	A	B	C	D
1		3010	3011	3012
2	Weight	.27 g	.34 g	.29 g
3	QG (uL)	810	1020	870
4	Isoprop (uL)	270	340	290

Results from nanodrop:

Table2

	A	B	C	D
1		ng/uL	A260/A280	A260/A230
2	3010	16.4	1.83	0.07
3	3011	27.0	1.78	0.06
4	3012	24.2	1.78	0.04

15. Ligation Protocol with T4 by DS (10/03/18)

    Ligation is in the right thermocycler set for 900 minutes at 16^{o}, 65^{o} for 10 minutes and stored at 4^{o} until ready for next step

October 5, 2018

16. IGEM style transformation 5 transformations:

    3010
    3011
    3012
    pos (PSB1C3 red)
    neg

17. labaled tubes as above and chilled on ice

18. added 50 uL MG1655 competent cells to each tube

19. added 2 uL ligate to each tube

20. chilled on ice 30 minutes

21. heat shock 45 sec @42C

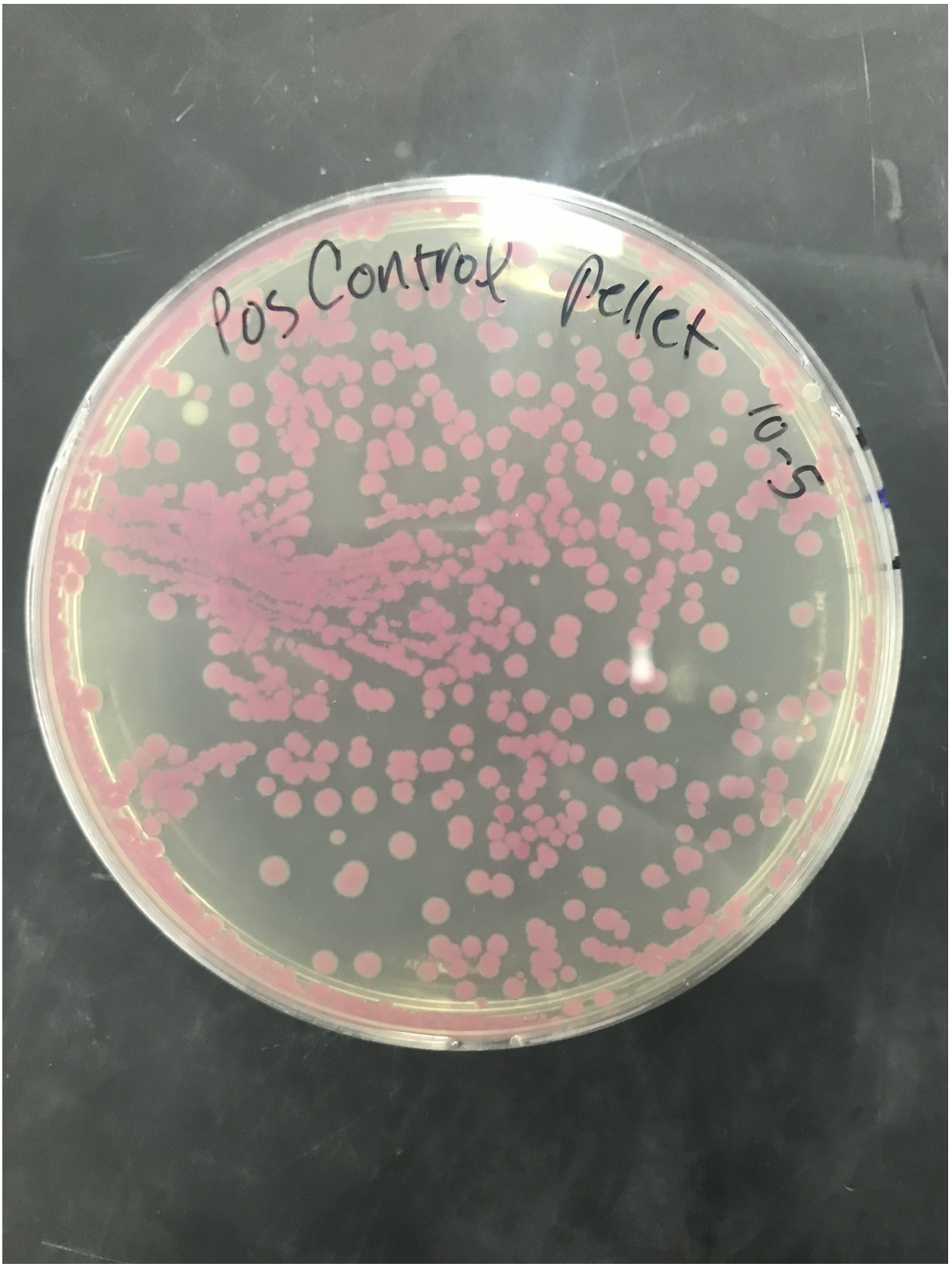
22. chilled on ice for 5 min

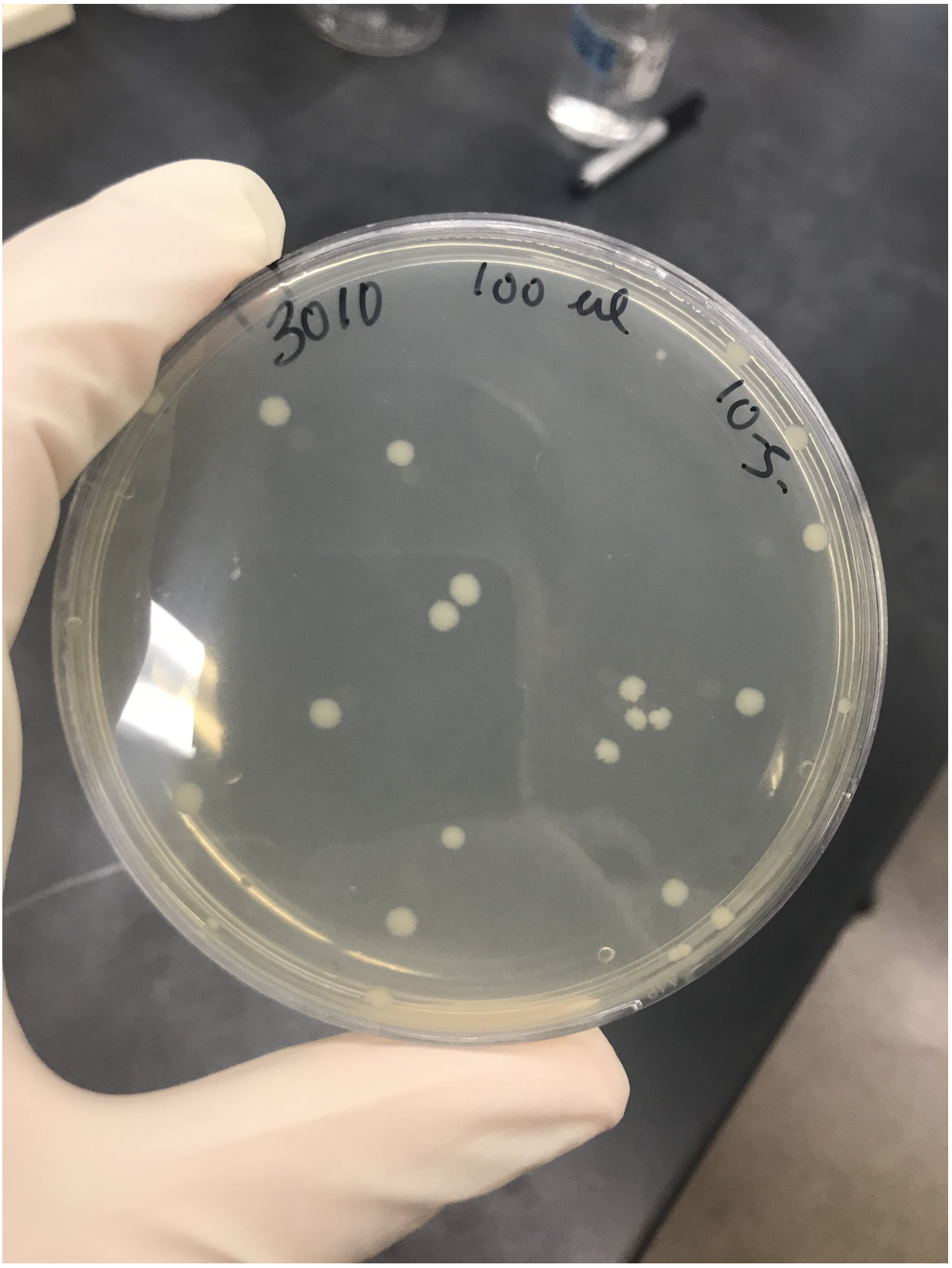
23. added 400 uL Xymo broth

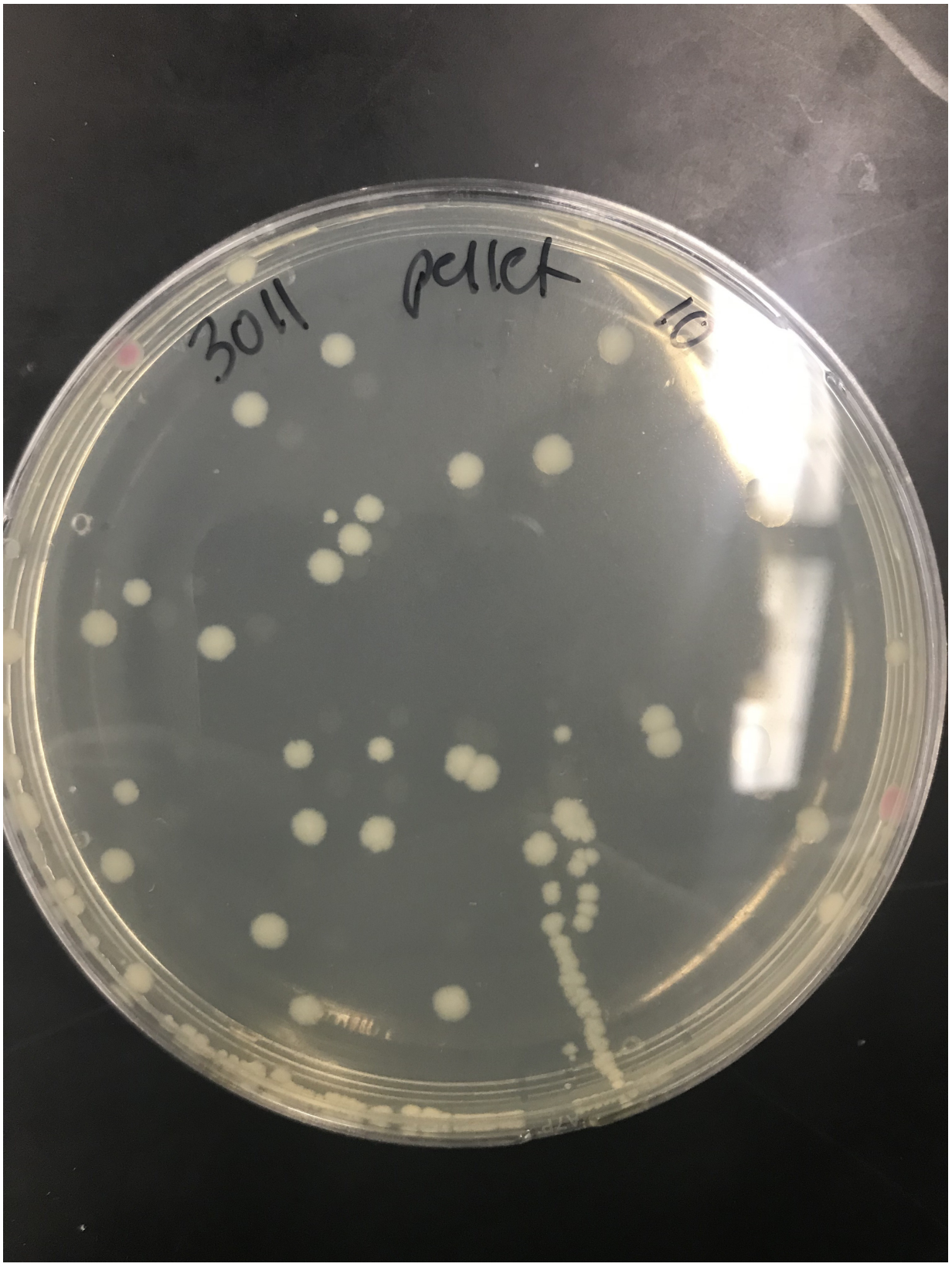
24. incubated @35C for 1 hour
25. plated on CAM agar plates 100 ul each direct and 100ul each pelletted
26. incubate overnight @ 35C

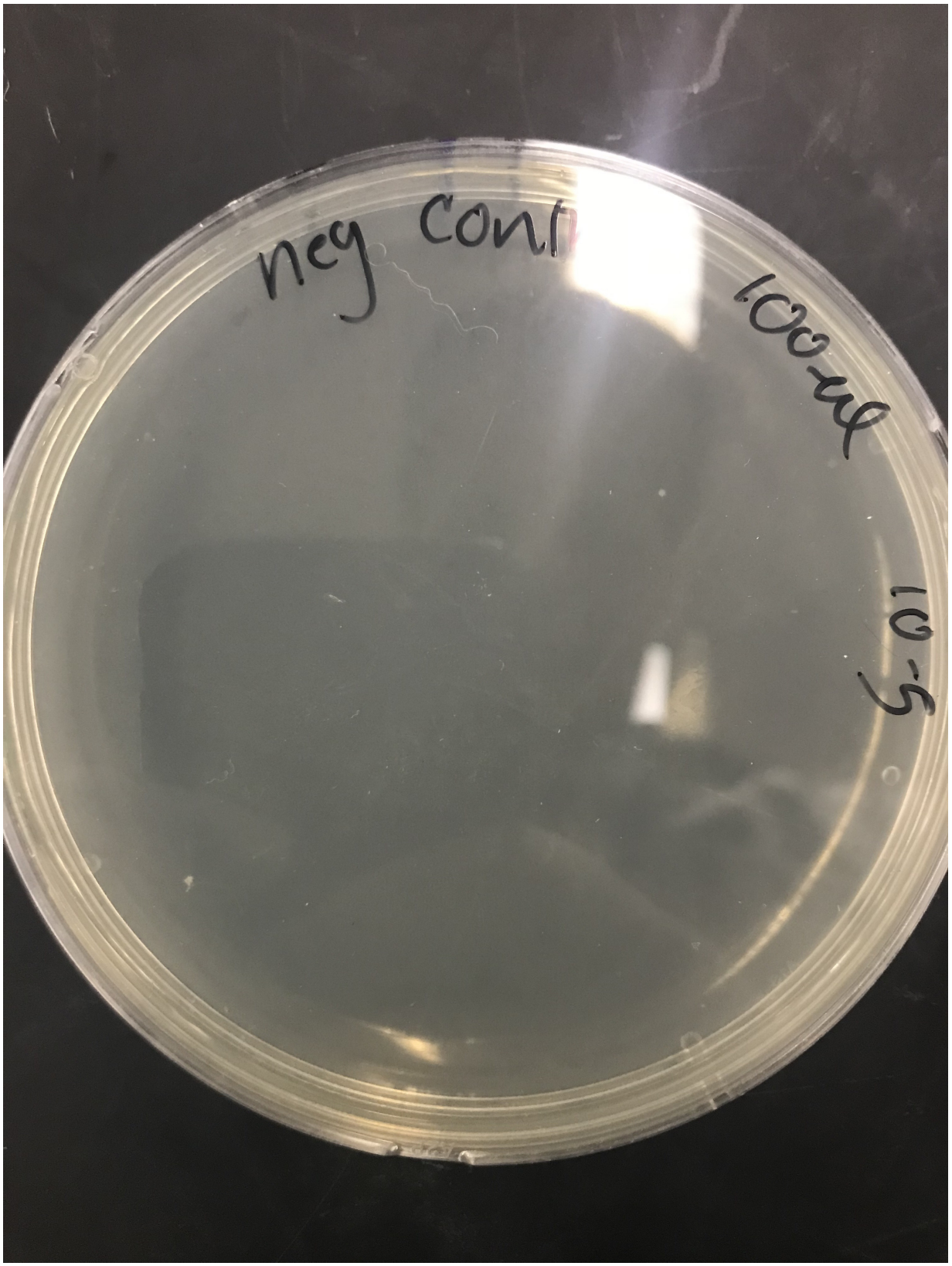
Results

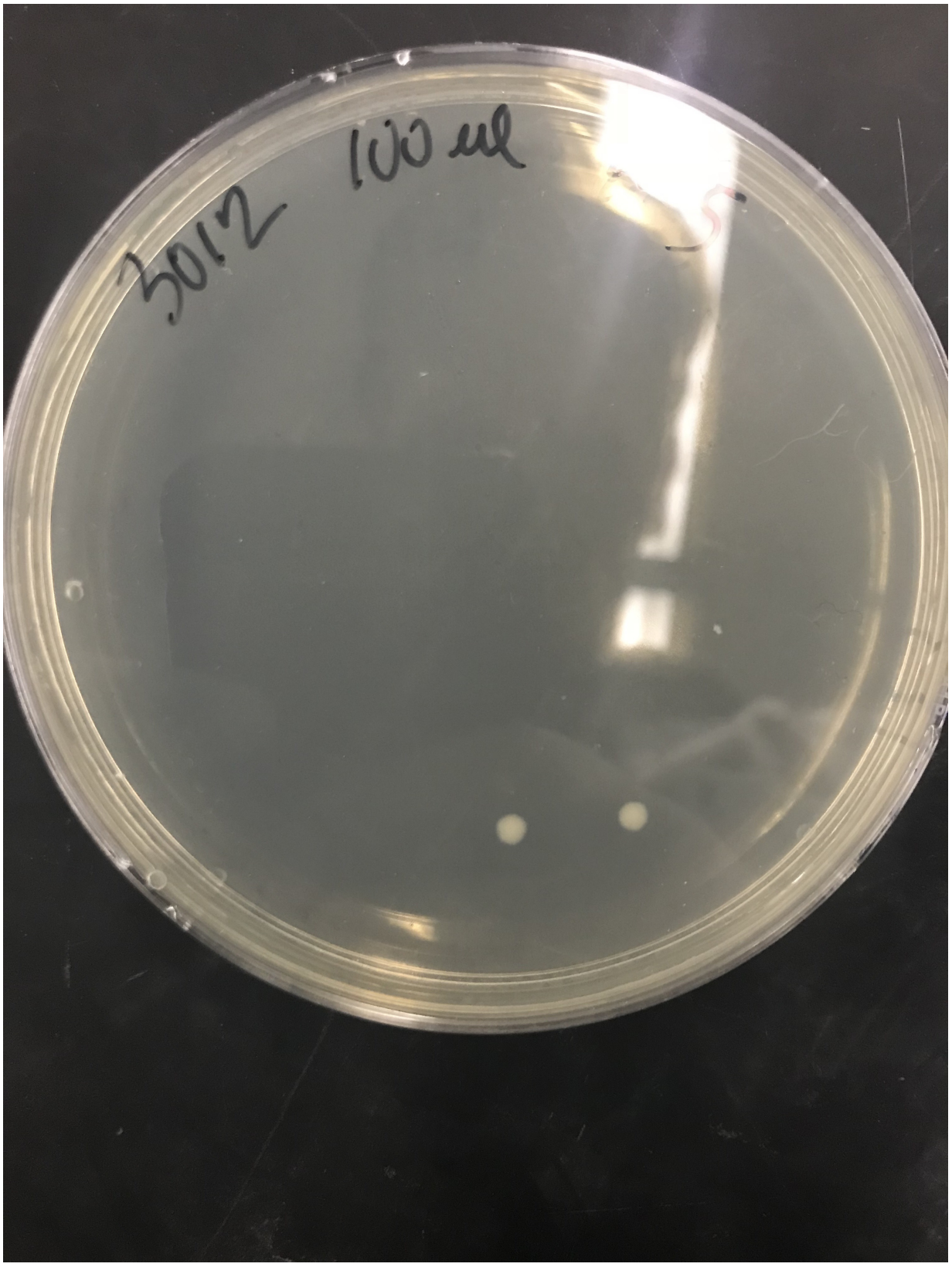
27.


Pos Control Pellet
10-5


3010
100 ul
10-5


3011
pellet
10


neg cont
100 ul
10-5



Colony PCR to verify inserts: 10/8/18

28. see IGEM protocol Colony PCR

29. prepared mastermix:
30. 266 ul sterile dH2o
31. 35 uL 10x colony pcr mix
32. 21 uL 25 mM $MgCl_2$
33. 7ul 10 mM dntps
34. 14 ul 20 uM forward & reverse primers
35. 1.4 ul Taq polymerase
36. total volume 350 uL
37. separate out mm into 7 pcr tubes 50 uL each
38. innoculate with transformants:

    2x 3010
    2x3011
    2x3012
    nc

39. Run in thermocycler 5 min @ 95C 30 cycles (1 min 95C, 1 min 55C, 1 min 72C) 5 min 72C