Metagenomic Library Time Line

May 25 2017

Made Buffers for DNA Extraction:

1M TRIS-HCL pH 8.0 buffer
1M sodium phosphate dibasic
1M sodium monobasic

DNA Extraction Buffer Recipe (Make 25 mL/ extraction):

100mM Tris HCL pH 8

100mM Sodium EDTA ph 8

100mM Sodium phosphate pH 8 (94.7% dibasic sodium phosphate and 5.3% monobasic)

1.5M NaCl

1% cetyltrimethyl ammonium bromide

Actual Measurements for 25 mL:

2.5 mL 1M Tris-HCL pH 8

2.5 mL 1M Sodium Phosphate pH8

5mL 0.5M EDTA pH8

7.5 mL 5M NaCl

250mg CTAB

Make up to 25 mL with ddh20

June 8 2017

DNA Extraction:

- weighed 5 g porcupine fecal samples

- mixed with 13.5 mL extraction buffer and 50 ul Proteinase K (20 mg/ml). Shook at 37 C at 225 rpm for 30 min

- added 1.5 ml 20% SDS and incubate in 65C water bath for 2h, gently inverting every 15 min.

- centrifuge at 6000 x g for 10 min at room temperature and transfer supernatant to centrifuge tubes

- extracted the pellet two more times using 4.5 mL extraction buffer and 0.5 ml 20% SDS, vortexing for 10s, incubating at 65 C for 10 min and centrifuging as before

- supernatants were combined, and an equal volume of chloroform isoamyl alcohol (24:1) was added in a fume hood. Tubes were spun at 15000 x g for separation of aqueous and organic layers.

- aqueous layer was moved to two 15 mL conical flasks and DNA was precipitated with 0.6 volume of isopropanol at room temperature for 1 hr.

- tubes were centrifuged at 15000 x g for 20 min at room temperature, washed with 70% cold ethanol (-20 C) and resuspended to give a final volume of 500 Ul

- This 'dirty' nucleic acid sample was analyzed using the nanodrop to give an estimate of DNA concentration: ~3000 ng/ul each

June 9th 2017

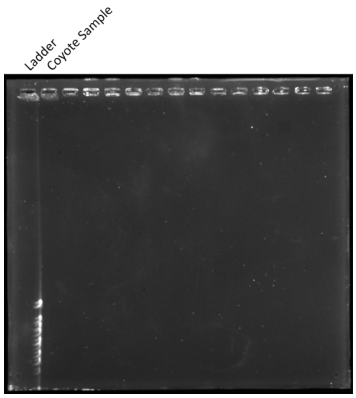
-set up test PFGE with coyote samples (conc: 6000 ng/ul) from an earlier extraction.

-used parameters for BIORAD CHEF Mapper:

- Temperature: 14 C
- Buffer: 1x TAE
- Voltage 5.5 V/cm
- Switch time: 0.5- 10 sec
- Angle: 120 degrees
- Run Time: 20 hr

June 10 2017

Pulse field from June 10th failed ☹ The protocol we were using used a different PFGE rig then the one we had access to.



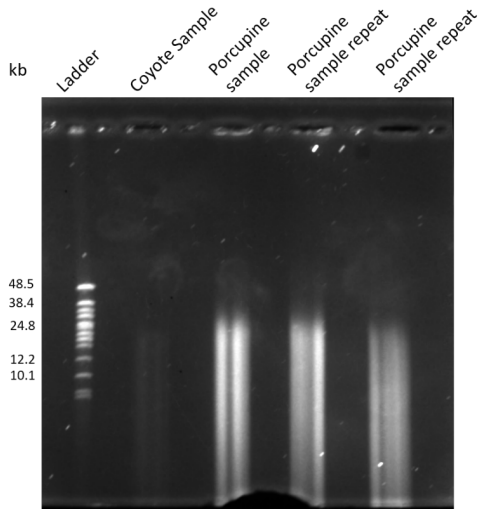
Redo:

- Tested the size of the DNA extracted via pulse field gel electrophoresis over night.
- Poured a 100 mL 1% PFGE gel.
- Used Bio rad CHEF standard in 8.3-48.5 kb because we are looking for 25kb and above fragments.
- Loaded 500 ng of nucleic acid sample

- Ran using the Bio-Rad CHEF-DR III Pulse Field Electrophoresis System using the following parameters:
    - Temperature: 14 C
    - Voltage: 6 V/cm
    - Buffer: 0.5x TAE
    - Switch Time: 1-6 sec
    - Run Time: 11 hrs
    - Angle: 120 degrees
- Also used larger combs

June 11 2017

- stained the pulse field gel with etbr for 40 min, destain for 20 min.
- viewed on UV transilluminator, some of the DNA was over 25kb and under 75 kb therefore no shearing was needed



July 12 2017

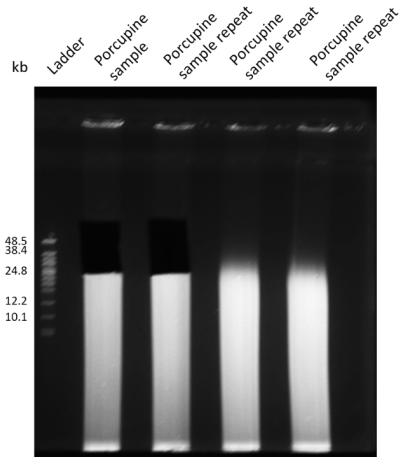
- Set up a second pulse field gel: 150 mL, 1% agarose
- Both samples were loaded twice, with a well between them to prevent cross contamination
- Gel was run with the parameters described above
- 45 minutes in, the gel was taken out of the PFGE apparatus, and all gel underneath the DNA loading dye was cut out, and the rest of the gel was put into a gel mould and re-poured.
- After the gel solidified, it was added back to the PFGE apparatus and run for the remaining 10 hrs and 15 min.

Prepped Dialysis tubing

- 2 pieces of dialysis tubing were cut to 15cm lengths and placed in distilled water for 15 min
- The membranes were heated on a hot plate up to 80 C in 10 mM sodium bicarbonate while stirring.
- Membranes were transferred to 10mM Na-EDTA solution and soaked for 30 min
- Solution was replaced with distilled water and the membranes were heated to 80C for 30 min
- Once cooled, the membranes were move to 20% ethanol solution and placed at 4C until use.

June 13 2017

- As above, the gel was stained with ethidum bromide.
- The gel was then imaged on a Versadoc, the 25kb band was marked, and the DNA above the 25kB section was cut out of the most concentrated wells (1 & 2) and placed on a petri dish.



Gel Extraction

- Each sample was placed in a prepared piece of dialysis tubing.
- The tubing was filled with 1xTAE, clamped and placed in a gel electrophoresis box
- The gel slices were electrophoresed for 2 hrs at 120 V
- After 2 hours, polarity of field was reversed and electrophoresed for 1 minute at 120 V
- The TAE in the dialysis tubing was moved using wide-mouthed pipet tips to an Amicon ultra centrifugal filter (30,000 MWCO). Tubing was rinsed twice with 0.5 mL 1x TAE and placed in filter tube.
- Amicon filter was centrifuged at 4050 x g for 20 min at 20 C until samples was ~ 0.2 mL
- The concentrated DNA was moved to a 1.5 mL microcentrifuge tube, and the membrane was rinsed twice with 0.2 mL 1x TAE and moved to the microcentrifuge tube.

DNA Precipitation

- The volume of the two samples were measured (650 ul, and 700 ul for repeat 1 and 2 respectively).
- A 1/10 th volume of 3 M sodium acetate, and 1 volume of isopropanol was added.

- Solution was mixed gently by inversion
- The samples were added to the -80 C for 25 min. While chilling, a microcentrifuge was pre-chilled to 4 C
- After 30 min, the samples were removed, and the supernatant was discarded by pipetting
- 800ul cold (-20C) etoh was added to the tubes and inverted twice.
- The tubes were spun at 4C for 10 min at 13500 rpm
- All ethanol was removed via pipet tip WITHOUT disrupting the pellet.
- The pellet was dryed at room temperature, and resuspended in 20 uL of water. The DNA was stored at 4 C over night to facilitate proper resuspension.

June 14 2017

- The precipitated DNA was end-repaired by NEB Quick Blunting Kit
- After following the protocol provided, Quick Blunting Enzyme was inactivated by incubation on a 70 C heatblock for 10 minutes.
- Once cooled to room temperature, the DNA was cleaned up via phenol-chloroform extraction to remove any left over impurities.
- DNA was stored at -20 C.

June 15 2017

-Did two midi-preps of cosmid pJC8

- Achieved concentrations of 280 ng/ul and 300 ng/ul

June 16 2017

Digestion of pJC8

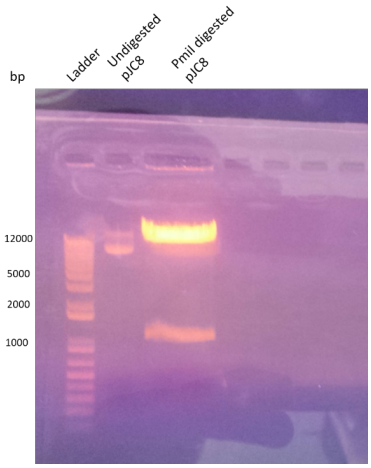
-digested 10 ug pJC8 with CIP and PmiI for 1 hr and 15 min at 37 C

-1uL of digested pJC8 was run on 0.8% agarose gel with etbr to ensure proper digestion.

- some pJC8 was still undigested so 2 uL of PmiI was added to the tube and incubated for 30 more minutes.

- The whole pJC8 digested sample was loaded into a 0.8% agarose gel and run for 1 hr at 80V.

- gel was viewed on a UV transilluminator, and the 11 kb band was excised and gel purified.



- yield was ~ 30 ng/ul

Assessment of digestion and ligation efficiency

- Reaction 1:
  - 1.67 ul DNA (50 ng)
  - 2 ul 10x T4 ligase buffer
  - 6.33 ul water
- Reaction 2:
  - 1.67 ul DNA
  - 2 ul 10x T4 ligase buffer
  - 2.5 ul T4 Ligase
  - 13.83 water
- Reaction 3:
  - 1.67 ul DNA
  - 2ul 10x T4 ligase buffer
  - 1 ul PNK
  - 12.83 ul water
  - Incubate at 37 C for 1 hr
  - Add 2.5 ul T4 ligase
- All reactions were made up and incubated at Room temperature for 2 hrs, transformed into Stbl 3 e.coli cells and incubated over night at 37 C

June 17 2017

- Plates were counted from ligation controls:
  - Reaction 1: 5 cfu
  - Reaction 2: 23 cfu
  - Reaction 3: >1600 cfu (TNTC)
  - Indication of high ligation efficiency
- Set up porcupine microbiome sample insert DNA and pJC8 digested vector:

- 20 ul pJC8
- 20 ul insert DNA
- 5 ul T4 ligase
- 5 ul T4 ligase buffer
- Incubated at 16 C o/n

June 19 2017

- Shipped ligation off to Waterloo University (Dr. Trevor Charles Lab)
- Got on a plane to Waterloo University

June 20 2017

- Met the Charles Lab!
- Streaked E.coli HB 101 on a LB agar with streptomycin

June 21 2017

- Inoculated 3 mL of culture with one clone, placed in 37 C shaker over night

June 22 2017

- Took inoculum out of shaker and placed at 4C until Monday

June 26 2017

- 100 uL of the HB101 culture was added to 20 mL of LB (no antibiotic!) and was placed in a 37 C shaker
- 2.5 hr after, OD600 was checked, once an OD600 of 0.6 was reached (needs to be between 0.6 and 1) the culture was removed from the incubator
- While waiting, 15 cm LB agar plates were poured with tetracycline at a final concentration of 20 ug/ml
- Phage Dilution solution was made according to Agilent Giga-pack III XL
- Just after OD600 was read at 0.6, phage was prepared by adding 4ul of ligation reaction to phage packaging solution that had JUST begun to thaw from the -80 C
- Phage reaction was incubated at room temperature for 2 hr, then 500 ul phage dilution solution was added.
- 25 ul of chloroform was added, and the tube was spun down briefly
- 10 ul of phage solution was mixed with 100 ul of HB101 at OD600 of 0.6, mixture was incubated for 30 min at room temperature
- 400 ul of lb was added and the olution was incubated at 37 C for 1 hr, inverting gently every 15 min
- Cells were pelleted for 3min at 13 000 rpm
- Bacteria + Phage were resuspended in 200 ul LB and spread on the TC_{20} LB agar plates. The plates were incubated overnight at 37 C

June 27 2017

- No colonies were found on the plates ☹
- Redid the HB101 infection with phage
- Incubated plates overnight at 37 C

June 28 2017

- Still no colonies ☹
- Got some great feedback from Dr. Cheng in the Charles Lab.
    - Never expose your DNA to UV or ethidium bromide
    - Put guanosine in your DNA gels to protect the DNA

July 14 2017

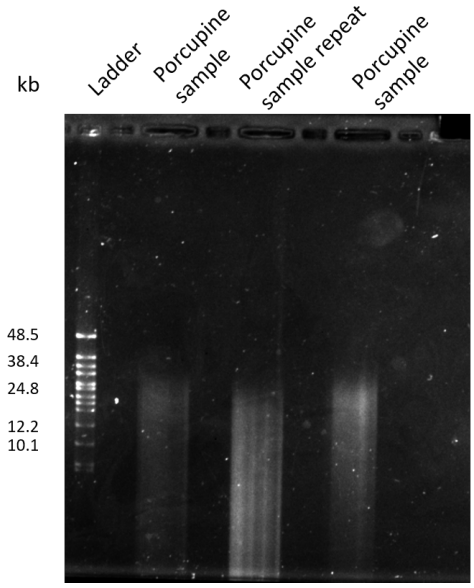
- Redid the DNA extraction from 5g of porcupine fecal samples, with the below changes
- 1. Ground the sample with a pre-chilled mortar and pestle with a small amount of liquid nitrogen in the mortar. This increases surface area where extraction buffer can work
- 2. Used a hybridization oven at 65 C rotating slowly instead of water bath. This kept the tube horizontal therefore more surface area exposed to extraction buffer
- Concentration was estimated on nanodrop ~5700 ng/ul

July 24 2017

- 500 ng was run via the pre-described parameters on a Bio-Rad CHEF DR III to test size of DNA

July 25 2017

- The PFGE gel was stained with etbr and imaged on a Bio-Rad Versadoc

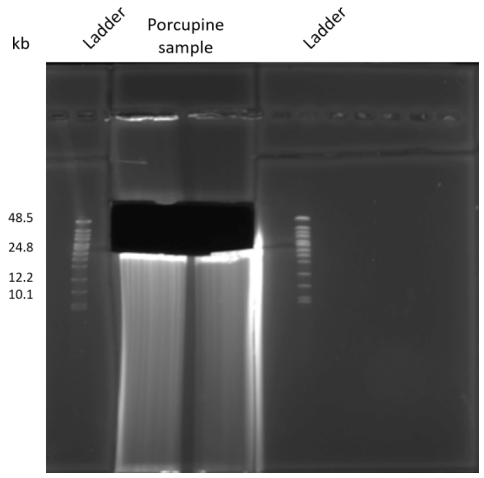


July 26 2017

- A 150 mL PFGE gel was set up, and two wells in the comb were taped together twice to form two larger wells. These were loaded with DNA from the porcupine microbiome extraction. The PFGE was set up with parameters previously described.
- The DNA ladder was placed on both sides of the sample
- 45 minutes into the run, the gel was cut off at the loading dye front and re-poured as previously described

July 27 2017

- To prevent the DNA from being exposed to UV light and ethidum bromide, the outer sides of the gel containing the ladder and a slice of sample were cut off and stained in ethidum bromide.
- The ends of the gel were imaged on a UV transilluminator, and the 25kb ladder band was marked with a razor, as was the area where the largest DNA was found (~75kb).
- The gel was reassembled and the razor marks were matched up to cut out the section of gel containing the DNA of interest.
- The rest of the gel was stained and imaged to ensure proper cutting of the gel



Gel Extraction

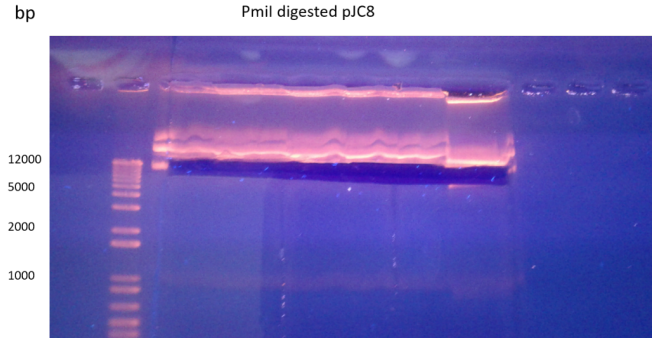
- Gel extraction was preformed via the dialysis method described above
- Volume of concentrated DNA was 625 ul, therefore 62.5 ul of 3M sodium acetate and 625 ul of isopropanol to precipitate

August 1 2017

- DNA extracted from the PFGE gel was end-repaired via the NEB Quick Blunting kit as previously described
- After blunting, DNA was cleaned up via the phenol chloroform method.
- DNA concentration was measured by nanodrop: 75ng/ul

August 14 2017

- 10 ug of PJC8 was digested with CIP and PmiI:
  - 33.3ul pJC8 (300 ng/uL)
  - 5uL Cutsmart
  - .5uL PmiI
  - 1ul CIP
  - 10.2 uL H2O
  - Incubate at 37 C for 1 hour and 15 min
- 1 uL of this digestion was run on a 0.8% agarose gel to assess efficiency
- Incomplete so 2 uL of PmiI was added and the sample was incubated at 37 C for 30 min
- Entire sample was loaded in a 0.8% agarose gel without ethidium bromide
- After running for 1 hour at 80V, the ladder and a slice of sample was cut off the gel and stained in etbr, as described above,
- Gel was reassembled, 11kb band marked, and area of interest was cut out and the rest of the gel was stained for confirmation



- a Qiagen Gel purification kit was used to obtain the DNA

Comment [m1]: get real name

Ligation efficiency test

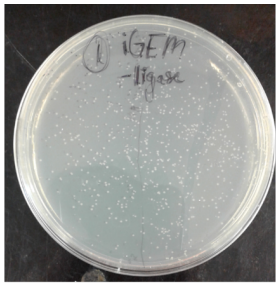
- Reaction 1:
  - 1.67 ul pJC8
  - 2 ul T4 ligase buffer
  - 15.83 water
- Reaction 2:
  - 1.67 ul PJC8
  - 2ul T4 ligase buffer
  - 2.5 ul T4 ligase
  - 13.83 water
- Reaction 3:
  - 1.67 ul pJC8
  - 2 ul T4 ligase buff
  - 1ul PNK
  - Incubated at 37 C for 1 hour
  - 2.5 ul T4 ligase was added
  - Incubated at 16 C overnight

August 15 2017

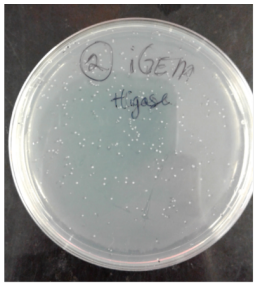
- Ligation reactions were transformed into STBL 3 E.coli and were incubated overnight at 37 C

August 16 2017

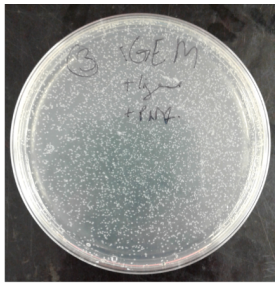
- Plate counts:
    - Reaction 1: 600 cfu
    - Reaction 2: 336 cfu
    - Reaction 3: TNTC



Reaction 1:
- pJC8
- T4 ligase buffer



Reaction 2:
- pJC8
- T4 ligase buffer
- T4 Ligase



Reaction 3:
- pJC8
- T4 ligase buffer
- T4 Ligase
- PNK

- Low efficiency, Reaction 1 and 2 should have very low to no colonies. Have to re-do.

August 23 2017

- 10 ug of pJC8 was digested as above expect 1 uL of PmiI was used rather than 0.5 uL
- Ligation control reactions were set up as previously described and incubate at 16 C overnight

August 24 2017

- Ligations were transformed into STBL 3 e.coli cells, plated, and incubated overnight at 37 C

August 25 2017

- Plates counts:
    - Reaction 1: 25 cfu
    - Reaction 2: 12 cfu
    - Reaction 3: TNTC
- I am unclear if this is considered a good ligation efficiency, will check with Faculty about it

August 29 2017

- Set up ligation with digested pJC8 from August 25th and the insert DNA from August 1st
    - 3.34 uL insert DNA (0.25 ug)
    - 5.40 uL digested pJC8 (0.75 ug)
    - 2 ul 10 x T4 ligation buffer

- 2 ul T4 ligase
- 7.26 ul water
- Incubate at 16 C overnight

September 2 2017

- HB101 *E. coli* was streaked on a streptomycin LB agar plate and incubated at 37 C overnight

September 3 2017

- 3 mL of LB was inoculated with one colony of HB101 and grown over night at 37 C

September 4 2017

- As previously described, 100 ul of HB101 culture was added to 20 ml of LB and placed in a 37 C shaker for 2.5 hr. OD 600 was check every half hour after that until it hit a range between 0.6-1 (3 hrs)
- Phage extract was prepped with 4 ul ligation reaction as previously describe and used to infect HB101
- Phage and bacteria were plated onto 15 cm Tc20 LB agar plates and incubated overnight at 37 C

September 5 2017

- No colonies were seen ☹

September 11 2017

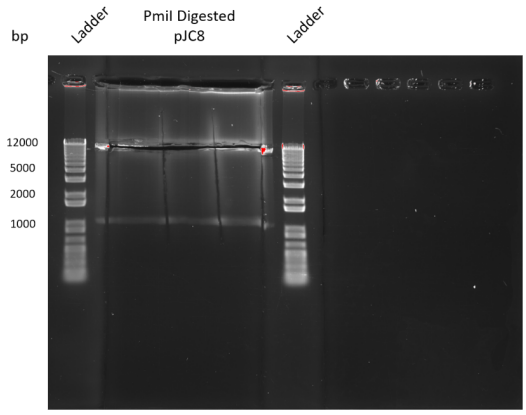
- Midi-prepped pJC8: 800 ng/ul

September 16 2017

- Decided to use the bacteria strain provided by Agilent in Giga-pack III XL packaging instead of HB101 e.coli strain gifted to us by the Charles Lab
- Plated the VCS257 e .coli strain on antibiotic free LB agar plates

September 20 2017

- 10 ug of pJC8 was digested with 1 uL of CIP and 1 ul of PmiI as previously described
- 1 ul was run out on a 0.8% gel to ensure proper digestion, then the entire sample was run out n a 0.8% gel without ethidium bromide in it. The 11kb band was excised without exposing to UV as previously described. DNA was isolated by gel extraction (Qiagen)



- DNA concentration was measured by nanodrop: 75ng/ul
- Ligations were set up to test efficiency:
- Reaction 1:
    - 1.67 ul pJC8
    - 2 ul T4 ligase buffer
    - 15.83 water
- Reaction 2:
    - 1.67 ul PJC8
    - 2ul T4 ligase buffer
    - 2.5 ul T4 ligase
    - 13.83 water
- Reaction 3:
    - 1.67 ul pJC8
    - 2 ul T4 ligase buff
    - 1ul PNK
    - Incubated at 37 C for 1 hour
    - 2.5 ul T4 ligase was added
    - Incubated at 16 C overnight
- The insert and digested pJC8 were also set up in ligation at this time:
    - 10 uL of pJC8 (0.75 ug)
    - 3.33 uL of insert (0.25 ug)
    - 2 ul T4 ligase buffer
    - 2 uL T4 ligase
    - 2.67 ul water
- All ligations were incubated at 16 C overnight

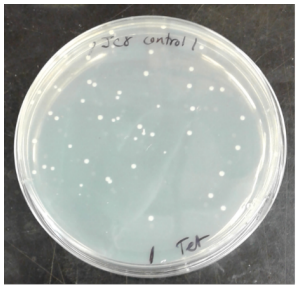
September 21 2017

- The three control ligations were transformed into STBL 3 e.coli, plated, and incubated overnight at 37 C
- VCS257 was plated on Tc5 B agar plates to ensure suscetpibility

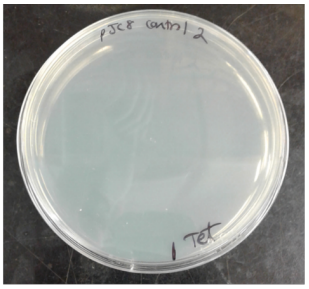
- 3 ul of the insert and pJC8 ligation was added to JUST thawed phage packaging extract and mixed by stirring until entire tube was thawed.
- This was incubated at room temperature for 2 hours
- 500 ul phage dilution solution and 20 ul chloroform were added and the tube was spun briefly to sediment debris
- Stored at 4 C

September 22 2017

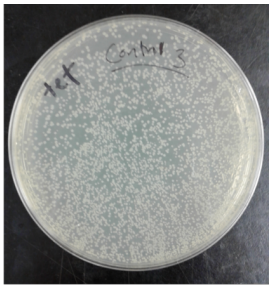
- Plate counts:
    - Reaction 1: 43 cfu
    - Reaction 2: 0 cfu
    - Reaction 3: TNTC



Control 1
- pJC8
- T4 ligase Buffer

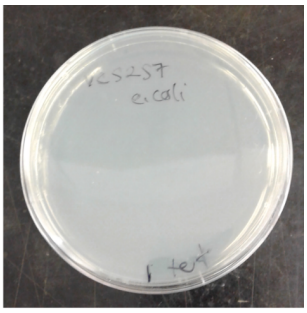


Control 2
- pJC8
- T4 Ligase Buffer
- T4 Ligase



Control 3
- pJC8
- T4 Ligase Buffer
- T4 Ligase
- T4 PNK

- Great ligation efficiency
- VCS257 is susceptible to Tc5



E. coli strain VCS257 streaked on tetracycline plate (5 µg/ml)

September 25 2017

- 5 mL LB with Mg SO4 was inoculated with one colony from the VCS257 e.coli plate and placed in a 37 C shaker for 4 hours
- OD600 was checked : OD600= 1.0
- Bacteria was pelleted at 500x g for 10 min and resuspended in 10 mL MgSO4 to achieve a OD600 of 0.5
- Using the previously prepared phage extract, a 1:10 and 1:50 dilution was made in phage dilution solution
- 25 ul of each dilution was mixed with 25 ul of prepared VCS257 and incubated at room temp for 30 min
- 200 ul of LB was added to each sample, and they were incubated at 37 C for 1 hour, inverting gently every 15 min
- Samples were spun for 1 min, resuspended in 50 ul LB and plated on 15 cm LB agar plates containing Tetracycline at a concentration of 5ug/mL
- Plates were incubated at 37 C overnight

September 26 2017

- No colonies ☹