



*Cosmid Library Generation**

2017 Protocols

* Based off the protocol from Dr. Trevor Charles Lab created by Dr. Jiujun Cheng

Purpose:

Purpose of this protocol is to generate a library of cosmid clones that are representative of the microbiome of the porcupine for screening for novel enzymes.

Required Materials:

- Stbl 3 competent *E. coli*
- Cosmid Vector pJC8
- *E. coli* HB101 strain
- Plasmid Mini-Prep Kit
- Gel Extraction Kit
- Agarose
- *Pml*I
- NEB CutSmart Buffer
- CIP or SAP
- Electrophoresis Chamber
- TAE
- NEB T4 Ligase and Ligase Buffer
- NEB T4 polynucleotide kinase
- Tetracycline
- Streptomycin
- Proteinase K
- 5M NaCl
- 20% SDS
- Extraction Buffer*
- Chloroform
- Isoamyl alcohol
- Isopropanol Alcohol
- Ethanol
- PFGE certified agarose
- Bio-Rad CHEF Ladder (8-48kb)
- PFGE Gel rig
- 12 000 MWCO Dialysis Tubing
- Dialysis clamps (no metal!)
- 30 000 MWCO Amicob Ultra-Centrifugal Concentration Filter
- 3M Sodium Acetate pH 5.3
- NEB Quick Blunting Kit
- Phenol: chloroform: isoamyl alcohol (25:24:1)
- LB with 10mM MgSO₄, 0.2% Maltose
- Phage Dilution Solution*

Solutions

Extraction Buffer

- 100mM Tris-HCl pH 8.0
- 100mM Sodium EDTA pH 8.0
- 100mM Sodium Phosphate pH 8.0
- 1.5M NaCl
- 1% CTAB
- 100uL proteinase K (10mg/ml)

Phage Dilution Solution (1L)

- 5.8g NaCl
- 2.0g MgSO₄·7H₂O
- 50 mL 1M Tris-HCl pH 7.5
- 5.0 mL 2% (w/v) gelatin
- Up to 1L H₂O

Autoclave

Procedure:

Preparation of cloning ready vector

- *E. coli* STBL3 carrying pJC8 was streaked on LB-Tc plate and incubated overnight at 37 °C.
- Three colonies were inoculated in 50 ml of Terrific broth (TB) medium with tetracycline (15 µg/ml), and grown overnight at 37 °C with shaking.
- Cosmid was isolated using Plasmid Miniprep kit
- DNA was quantified using NanoDrop spectrophotometer and run 50 ng DNA on 0.8% agarose gel to check the presence of RNA, as RNA will reduce the dephosphorylation efficiency of linearized pJC8 later.
- In order to digest and dephosphorylate vector simultaneously, 10 µg of pJC8 was incubated at 37 °C for 1 hr with 10 units *Eco*72I (*Pm*II), 10 units of CIP and 1 × CutSmart buffer in a final volume of 50 µl. Five microliters of reaction solution was

loaded on 0.8% TAE agarose gel to ensure the complete restriction of pJC8. If the digestion is incomplete, add 2 μ l *Eco*72I to the sample and incubate for 30 min.

- *Eco*72I- and CIP-treated pJC8 DNA was Loaded onto 0.8% TAE agarose gel and run at 5 V/cm for 1 h. Out sides of the gel containing DNA marker and small portion of pJC8 was cut off, and stained with gel red at room temperature for 1 hr. DNA was visualized under UV light and the position of pJC8 backbone (11 kb) was marked. The gel was reassembled and the region of gel containing 11 kb pJC8 was excised. DNA was isolated using Gel Extraction kit and quantified using NanoDrop spectrophotometer.
- DNA (30 ng) was run on 0.8% gel to confirm the amount and size of isolated DNA compared with 1 kb DNA ladder.
- Totest the efficiency of dephosphorylation and ligation of the prepared pJC8, three reactions were carried out.
- Reaction 1 contained 50 ng DNA in 1 \times ligation buffer (20 μ l).
- Reaction 2 consisted of 50 ng DNA and 5 Weiss units of T4 DNA ligase in 1 \times ligation buffer (20 μ l).
- Reaction 3 was composed of 50 ng DNA , 5 Weiss units of T4 DNA ligase and 10 unit of T4 polynucleotide kinase in 20 μ l of 1 \times ligation buffer.
- The reactions were incubated at room temperature for 2 hr or overnight at 16 °C. Five microliters of ligation reaction was transformed into 100 μ l of DH5 α competent cells by heat-shock. Following adding 1ml of LB and incubating at 37 °C for 1 hr, transformants were selected on LB-Tc plate overnight at 37 °C. The number of colonies

on the plates were counted and the efficiency of *Eco72I* restriction and SAP dephosphorylation was calculated.

Insert Preparation

- Weigh 5 g soil sample
- Mix with 13.5 ml of extraction buffer in 50 ml a Falcon tube by shaking at 37 °C and at 225 rpm for 30 min
- Add 1.5 ml of 20% SDS, and incubate in a 65 °C water bath for 2 h with gentle end-over-end inversions every 15 min
- Collect supernatants after centrifugation at $6,000 \times g$ for 10 min at room temperature and transfer to 50 ml centrifuge tubes
- Extract the soil pellets two more times by adding 4.5 ml of the extraction buffer and 0.5 ml of 20% SDS, vortexing for 10 s, incubating at 65 °C for 10 min, and centrifuging as before
- Combine supernatants and extract DNA with chloroform isoamyl alcohol (24:1)
- Precipitate DNA with 0.6 volume of isopropanol at room temperature for 1 h; or
- Obtain crude nucleic acids by centrifugation at $16,000 \times g$ for 20 min at room temperature, washed with cold 70% ethanol. Resuspend DNA in water to give a final volume of 500 μ l

DNA quality control

It is essential that the majority of random DNA fragments are >30 kb in size for successful construction of a cosmid library.

Size fractionation

- Pour 100 ml of 1% pulse field certified agarose (BIO-RAD, cat#162-0137) gel in 1 × TAE buffer
- Load Bio-Rad CHEF PFGE Ladder as recommended by company
- Load ~500 ng of metagenomic/genomic DNA
- Run the gel with parameters dictated by the PFGE rig utilized

Visualize DNA

- Stain in ethidium bromide
- Place the gel on the top of UV transilluminator
- Take a photo of gel
- A high-quality cosmid library will be generated if the majority of DNA migrates in the >25 kb range.

Size Fractionation

- Turn on pulse field gel apparatus and add 1 × TAE Buffer (~2.6 L)
- Pour 150 ml of 1% agarose in 1 × TAE to the top of the gel casting tray, and choose a comb giving you sufficient volume to load your DNA
- When the TAE buffer reaches 14 °C, place the gel in the electrophoresis chamber (wipe off any gel pieces around and underneath the black plate)

- Add loading dye solution to the DNA
- Mix and spin down the sample briefly
- Using a wide mouth pipette tip load the entire sample(s) onto a large well (the different DNA samples must be run at least one lane away from each other to prevent cross-contamination)
- Load Bio-Rad CHEF PFGE ladder (leave a small well between each sample and markers).
- Run the gel with the parameters dictated by the PFGE apparatus manual
- Pause gel running after 1.5 h, cut off the lower portion of gel containing humic acids (below the blue dye); put the upper portion back to gel cassette, fill with 1% TAE agarose, and then resume running the gel.

Visualize gel and cut out DNA

- Cut off the outer sides of gel containing marker and a small portion of DNA sample
- Stain the gel stripe with ethidium bromide, visualize DNA using blue light transilluminator and mark the position of 25-75 kb DNA
- Reassemble the gel and excise the gel slice positioned at 25-75 kb with a clean blade and place in a clean petri dish
- Take a photo of the remaining gel after excision of gel slice and save photo

Electroelution Preparation

Prepare dialysis tubing

- Prepare dialysis tubing for sensitive application based on the distributors manual

Electroelution

- Rinse the prepared dialysis tubing three times with $1 \times$ TAE
- Clamp one end of tubing
- Fill the tube with $1 \times$ TAE, place one gel slice in the tubing, and add more $1 \times$ TAE until the gel slice is completely submerged
- Remove all air bubbles, clamp the open end, and cut off excess tubing on ends
- Fill electrophoresis chamber with fresh $1 \times$ TAE
- Place the tubing in electrophoresis chamber perpendicular to current. Make sure it submerged and completely covered with buffer
- Electrophorese for 2 hours at 120 volts
- Reverse polarity of field and electrophorese for 1 minute at 120 volts

Electroelution Recovery

- Wipe off ends of tubing to remove any access buffer
- Carefully remove a clamp from one of the ends
- Transfer the TAE containing DNA in the tubing with a wide mouth P1000 pipet tip and add to a 50 ml Falcon tube; rinse the tubing twice with 0.5 ml $1 \times$ TAE

DNA concentration

- Add 15 ml DNA sample to an Amicon ultra centrifugal filter
- Spin at $4050 \times g$ for 20 min at 20°C
- Repeat the centrifugation when needed to concentrate the sample to ~ 0.2 ml
- Transfer the concentrated DNA to a microtube

- Rinse the membrane twice with 0.2 ml TE

6.3 Isopropanol precipitation

- Measure the sample volume
- Add 1/10 volume of 3 M sodium acetate (pH 5.3), and 1 volume of isopropanol
- Mix gently by inverting tube several times
- Place at -75°C for >30 min
- Pre-chill microcentrifuge to 4°C (this takes at least 15 min)
- Spin tubes at 4°C for 30 minutes at 13,500 rpm
- Withdraw the supernatant with a 1 ml pipet tip and discard (do not disrupt/loosen any pellet)
- Add 800 μl **cold** 80% ethanol (-20°C) to the tube, and invert gently three times
- Spin tube at 4°C for 10 minutes at 13,500 rpm
- Withdraw all ethanol with a pipet tip (do not disrupt/lose any pellet)
- Dry the pellet at room temperature until no liquid is visible (do not heat)
- Resuspend the pellet in ~ 20 μl of water
- Place at 50°C for 5 minutes and then 4°C overnight, to fully resuspend the DNA

Blunt End Repair

End-repair

- The sheared DNA inserts is end-repaired following the protocol of NEB Quick Blunting Kit
- Heat-inactivate the quick blunting enzyme mixture on a 70 °C heat block for 10 minutes (residual activity of T4 polynucleotide kinase may remains, leading to phosphorylation and self ligation of the cloning ready pJC8 in ligation reaction (section 3a))
- Cool down slowly to room temperature and place the sample on ice

DNA clean-up

Please note: extraction should be carried out in a fume hood. Waste should be disposed in a designated container.

- Add 400 µl TE and 500 µl phenol:chloroform:isoamyl alcohol (25:24:1) to the tube
- Invert the sample gently, and spin the tube for 5 min at 13,400 rpm
- Transfer the upper phase into a new tube
- Add 500 µl chloroform:isoamyl alcohol (24:1), mix the sample gently and then spin for 3 min at 13,400 rpm
- Repeat steps c-d once
- Add 0.1 vol of 3 M Na-acetate (pH 5.3), and 1 vol of isopropanol
- Place at -80 °C for 25 min, spin the mixture at 4 °C at 13,400 rpm for 30 min
- Remove supernatant using a 1 ml pipet tip and discard
- Add 1 ml cold (-20 °C) 80% ethanol, and revert the tube 4 times

- Spin the tubes at 4 °C and 13,500 rpm for 10 minutes
- Remove all liquid carefully (avoid touching any precipitate)
- Air-dry the pellet (do not heat or SpeedVac)
- Dissolve the DNA in 20 µl water, and combine resuspensions if necessary
- Estimate DNA concentration using λ DNA as a standard on 0.8% agarose gel
- Store at -20 °C until needed

Part 3. Ligation Reaction

The pJC8 and inserts are mixed in a molar ratio of 10:1 to minimize the formation of chimeric clones. The reaction was performed following the protocol for the NEB Ligation Kit

- a. Set up a 20 µl reaction according to kit instructions, mix and spin down the mixture briefly
- b. Incubate the tube at room temperature for 4 hours, or overnight at 16 °C
- c. Heat-inactivate the sample on the 70 °C heat block for 10 minutes
- d. Cool down the tube slowly to room temperature
- e. Store the ligation mixture at -20 °C until needed

Part 4. Packaging ligation reaction

E. coli host preparation

- Streak *E. coli* HB101 from stock at -75 °C on LB-Sm₁₀₀ agar plate
- Incubate overnight at 37 °C
- Inoculate one colony of *E. coli* HB101 in 3 ml of LB (**No Sm!**)
- Place the culture on shaker at 37 °C overnight at 220 rpm
- If needed, store the culture at 4 °C for up to 5 days
- Add 50 µl of the overnight culture to 10 ml of LB containing 10 mM MgSO₄ and 0.2% maltose (**No Sm**)
- Incubate the culture at 37 °C, 220 rpm until an OD₆₀₀ of **0.6-1.0** is reached
- Use or store at 4 °C for up to 72 hr

Packaging:

- Take 1 tube of Stratagene packaging extract (Gigapack III XL) from -80 °C freezer
- Add 1-4 µl of ligation mixture to the tube when the packaging extract begins to thaw
- Mix the sample **gently** with a pipet tip and spin down briefly (**no bubbles**)
- Incubate at room temperature for 2 hr
- Add 500 µl of phage dilution solution (see recipe) to the tube

- Add 25 μ l of chloroform to the tube
- Invert to mix, spin at 1000 rpm for 2min and infect HB101 (or store at 4°C until ready to plate).

Titering packaging reaction

- Mix 10 μ l of packaged DNA with 100 μ l of *E. coli* HB101 (section 4.1)
- Incubate the mixture at room temperature for 30 min
- Add 400 μ l of LB, and incubate at 37 °C for 60 min (invert tubes every 15 min)
- Pellet cells for 3 min at 13,400 rpm
- Resuspend and spread cells on a 150 mm \times 15 mm LB-Tc₂₀ plate
- Incubate overnight at 37 °C
- Count the number of clones on the selection plate

Large-scale packaging and plating

Based on the number of colonies obtained in the small-scale titering test, scale up ligation and packaging reactions.

- Titer the pooled packaging reactions in the same manner as the small test reaction
- Mix the packaging reaction with freshly cultured *E. coli* HB101 in a 1:10 ratio (v/v)
- Incubate at room temperature for 30 minutes

- Add appropriate volume of LB and incubate at 37 °C for 60 min (shake tubes every 15 min)
- Concentrate and plate approximate vol of infected cells on 150 mm x 15 mm LB-Tc₂₀ agar plate, to obtain ~2000 clones per plate
- Collect all clones in LB-Tc₁₅, and concentrate to a small vol (e.g. 80,000 clones in 50 ml)
- Add DMSO to a final concentration of 7%, aliquot 1 ml in labeled vials, and then save at -75 °C
- Congratulate yourself for successfully constructing a fantastic cosmid library

