
2016 Protocols

# Metagenomic Library Construction

Adapted from the Protocol Provide to us by
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**Purpose:** To construct a cosmid library with environmental DNA inserts of >30 kb.

**Procedure:**

**Preparation of Cloning Vector**

1. *E. coli* DH5α carrying pJC8 or pJC24 was streaked on LB-Tc plate and incubated overnight at 37 °C
2. 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
3. Cosmid was isolated using Plasmid Miniprep kit (Fermentas)
4. 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
5. 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 (*Pml*I), 10 units of Fast alkaline phosphotase and 1 × FastDigest Green buffer in a final volume of 500 µl
6. 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 µl *Eco*72I to the sample and incubate for 30 min
7. *Eco*72I- and SAP-treated pJC8 DNA was Loaded onto 0.8% TAE agarose gel and run at 5 V/cm for 1 h
8. 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
9. DNA was visualized under UV light and the position of pJC8 backbone (11 kb) was marked
10. The gel was reassembled and the region of gel containing 11 kb pJC8 was excised
11. DNA was isolated using Gel Extraction kit (Bio Basic Inc), and quantified using NanoDrop spectrophotomer
12. DNA (30 ng) was run on 0.8% gel to confirm the amount and size of isolated DNA compared with 1 kb DNA ladder (Fermentas)
13. In order to test the efficiency of dephosphorylation and ligation of the prepared pJC8, three reactions were carried out: reaction 1 contained 50 ng DNA in 1 × ligation buffer (20 µl). Reaction 2 consisted of 50 ng DNA and 5 Weiss units of T4 DNA ligase in 1 × ligation buffer (20 µl). Reaction 3 was composed of 50 ng DNA , 5 Weiss units of T4 DNA ligase and 10 unit of T4 polynucleotide kinase in 20 µl of 1 × ligation buffer. The reactions were incubated at room temperature for 2 hr or overnight at 16 °C
14. Five microliters of ligation reaction were transformed into 100 µl of DH5α competent cells by heat-shock
15. Following adding 1m l 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 *Eco*72I restriction and SAP dephosphorylation was calculated

**Genomic Isolation**

1. Grow 5 ml of bacterial cells (such as *Sinorhizobium meliloti* Rm1021) overnight in rich medium
2. Collect cells and wash once with 0.85% NaCl
3. Resuspend cells in 500 µl of TE, 25 µl of 25% SDS, 62.5 µl of 5 M NaCl and 12.5 µl of proteinase K (10 mg/ml)
4. Incubate at 65 $^{o}$C (15 min – overnight) to obtain clear lysate
5. Extract DNA twice with equal vol of phenol
6. Extract twice with equal vol of phenol:chloroform:isoamyl alchol (25:24:1)
7. Extract once with equal vol of chloroform:isoamyl alchol (24:1)
8. Precipitate DNA with ethanol
9. Remove RNA with RNase A

**Metagenomic DNA Isolation**

1. Weigh 5 g soil sample
2. Mix with 13.5 ml of extraction buffer (100 mM Tris-HCl (pH 8.0), 100 mM sodium EDTA (pH 8.0), 100 mM sodium phosphate (pH 8.0), 1.5 M NaCl, 1% cetyltrimethyl ammonium bromide) and 100 µl of proteinase K (10 mg/ml) in 50 ml a Falcon tube by shaking at 37 $^{o}$C and at 225 rpm for 30 min
3. Add 1.5 ml of 20% SDS, and incubate in a 65 $^{o}$C water bath for 2 h with gentle end-over-end inversions every 15 min
4. Collect supernatants after centrifugation at 6,000 × g for 10 min at room temperature and transfer to 50 ml centrifuge tubes
5. 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 $^{o}$C for 10 min, and centrifuging as before
6. Combine supernatants and extract DNA with chloroform isoamyl alcohol (24:1)
7. Precipitate DNA with 0.6 volume of isopropanol at room temperature for 1 h; or
8. Obtain crude nucleic acids by centrifugation at 16,000 × g for 20 min at room temperature, washed with cold 70% ethanol. Resuspend DNA in water to give a final volume of 500 µl

**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**

1. Pour 100 ml of 1% pulse field certified agarose (BIO-RAD, cat#162-0137) gel in 1 × TAE buffer
2. Load 50 ng *Xba*I-digested λ DNA (25 kb) and 50 ng λ DNA (48.5 kb) as DNA Markers
3. Load ~500 ng of metagenomic/genomic DNA

Run the gel with following parameters

| | |
|---|---|
| Agarose | 1% |
| Buffer setting) | 1 × TAE (1 × TBE in BIO-RAD CHEF MAPPER actual |
| Temperature | 14 $^{o}$C |
| Voltage | 5.5 V/cm |
| Pulse | 0.5-10 sec |
| Angle | 120$^{O}$ |
| Running time | 20 hr |

**Visualize DNA**

1. Remove the gel and transfer to a **clean** glass plate
2. Place the gel on the top of DR-88M Transilluminator (Interscience$^{TM}$)
3. Take a photo of gel above an amber screen
    a. A high-quality cosmid library will be generated if the majority of DNA migrates in the >25 kb range.

**Shearing DNA (if necessary)**

1. Transfer metagenomic/genomic DNA with a wide mouth pipette tip to a well-labeled microtube and bring the final volume to 100 µl with TE (DNA, 0.2 µg/µl). If required, concentrate large volumes with isopropanol precipitation or dry DNA down to 100 µl.
2. Shear the DNA manually using a GC syringe
    a. Wash the syringe before and after shearing each sample with 0.2 M HCl (5 times), 0.2 M NaOH (5 times, **freshly** made), and TE buffer (5 times)
    b. # of passes through the syringe = 1-5 cycles (depending on the sizes of sheared DNA checked by PFGE)
3. Collect the sample in a clean tube and place it on ice immediately.

**Size Fractionation**

1. Turn on pulse field gel apparatus (BIO-RAD, CHEF MAPPER), and add 1 × TAE Buffer(~2.6 L)
2. 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
3. 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)
4. Add loading dye solution to the sheared DNA
5. Mix and spin down the sample briefly
6. 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)
7. Load 50 ng of λ DNA and *Xba*I-cut λ DNA into other small wells (leave a small well between each sample and markers).
8. Run the gel overnight with the following parameters:

| | |
|---|---|
| Agarose | 1.0% |
| Buffer | 1 × TAE (1 × TBE in actual setting) |
| Temperature | 14 °C |
| Voltage | 5.5 V/cm |
| Pulse | initial 1.0 – final 6.0 sec |
| Run Time | 20 hrs |
| Angle | $120^0$ |

**Visualize gel and cut out DNA**

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

**Electroelution Preparation**

Prepare Dialysis Tubing

1. Cut dialysis tubing (12,000 MWCO, Sigma D-9652) in approximately 15 cm pieces (diameter 12 mm, one per gel slice)
2. Immerse the tubing into 1 L of 2% sodium bicarbonate and 1 mM EDTA in a 2 L glass beaker
3. Boil for 10 min
4. Rinse 3 times with $ddH_2O$
5. Boil in $ddH_2O$ for 10 min
6. Decant $ddH_2O$
7. Submerge the tubing in 20% ethanol and 1mM EDTA, and store at 4 °C

**Electroelution**

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

**Electroelution Recovery**

1. Wipe off ends of tubing to remove any access buffer
2. Carefully remove a clamp from one of the ends
3. 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 × TAE

**DNA Concentration**

1. Add 15 ml DNA sample to an Amicon ultra centrifugal concentrater filter (30,000 MWCO, Millipore)
2. Spin at 4050 × g for 20 min at 20 °C
3. Repeat the centrifugation when needed to concentrate the sample to ~0.2 ml
4. Transfer the concentrated DNA to a microtube
5. Rinse the membrane twice with 0.2 ml TE

**Isopropanol Precipitation**

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

**Blunt End Repair**

The sheared DNA inserts is end-repaired following the protocol of End-It DNA End-Repair kit (Epicentre Biotechnologies)

a. Reaction setup

| | |
|---|---|
| Electroeluted DNA | 20 µl |
| 10 × Buffer | 10 µl |
| 2.5 mM dNTPs | 10 µl |
| 10 mM ATP | 10 µl |
| ER enzyme mix (2µl /5ug DNA) | 2µl |
| $H_2O$ | 48 µl |
| --- | --- |
| Total volume | 100 µl |

b. Cap the tube, mix gently, and spin down (no vortexing)

c. Incubate at room temperature for 60 minutes

d. Heat-inactivate the end-repair enzyme mixture on a 70 °C heat block for 10 minutes (residual activity of T4 polynucleotide kinase may remain, leading to phosphorylation and self-ligation of the cloning ready pJC8 in ligation reaction (section 3a))

e. 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.

1. Add 400 µl TE and 500 µl phenol:chloroform:isoamyl alcohol (25:24:1) to the tube
2. Invert the sample gently, and spin the tube for 5 min at 13,400 rpm
3. Transfer the upper phase into a new tube
4. Add 500 µl chloroform:isoamyl alcohol (24:1), mix the sample gently and then spin for 3 min at 13,400 rpm
5. Repeat steps c-d once
6. Add 0.1 vol of 3 M Na-acetate (pH 5.3), and 1 vol of isopropanol
7. Place at -75 $^{o}$C for >30 min, spin the mixture at 4 $^{o}$C at 13,400 rpm for 30 min
8. Remove supernatant using a 1 ml pipet tip and discard
9. Add 1 ml cold (-20 $^{o}$C) 80% ethanol, and revert the tube 4 times
10. Spin the tubes at 4 $^{o}$C and 13,500 rpm for 10 minutes
11. Remove all liquid carefully (avoid touching any precipitate)
12. Air-dry the pellet (do not heat or SpeedVac)

13. Dissolve the DNA in 20 µl water, and combine resuspensions if necessary
14. Estimate DNA concentration using λ DNA as a standard on 0.8% agarose gel
15. Store at -20 °C until needed

**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 Fast-link ligation kit (Epicentre Biotechnologies)

1. Reaction setup

| | | |
|---|---|---|
| Insert DNA (0.25 µg) | 2.9 µl | |
| pJC8 vector (0.75 µg) | 3.5 µl | |
| 10 × ligation buffer | | 2.0 µl |
| 10 mM ATP* | 1.0 µl | |
| T4 DNA ligase | 2.0 µl | |
| Water | 8.6 µl | |
| --- | --- | --- |
| Total volume | 20 µl | |

* Use 0.5 mM ATP as high concentration of ATP inhibits blunt ligation

2. Mix and spin down the mixture briefly
3. Incubate the tube at room temperature for 4 hours, or overnight at 16 °C)
4. Heat-inactivate the sample on the 70 °C heat block for 10 minutes
5. Cool down the tube slowly to room temperature
6. Store the ligation mixture at -20 °C until needed

## Packaging Ligation Reaction

***E. coli* host preparation**

a. Streak *E. coli* HB101 from stock at -75 °C on LB-$Sm_{100}$ agar plate
b. Incubate overnight at 37 °C
**c.** Inoculate one colony of *E. coli* HB101 in 3 ml of LB (**No Sm**!)
d. Place the culture on shaker at 37 °C overnight at 220 rpm
e. If needed, store the culture at 4 °C for up to 5 days
f. Add 50 µl of the overnight culture to 10 ml of LB containing 10 mM $MgSO_4$ and 0.2% maltose (**No Sm**)
g. Incubate the culture at 37 °C, 220 rpm until an $OD_{600}$ of **0.8-1.0** is reached
h. Use or store at 4 °C for up to 72 hr

**Packaging**

1. Take 1 tube of Stratagene packaging extract (Gigapack III XL) from -75 °C freezer
    a. Stratagene phage dilution solution (1000 ml)

| | |
|---|---|
| NaCl | 5.8 g |
| $MgSO_4 \cdot 7H_2O$ | 2.0 g |
| 1 M Tris-HCl (pH 7.5) | 50.0 ml |
| 2% (w/v) gelatin | 5.0 ml |
| $H_2O$ | up to 1000 ml |

    Autoclave
2. Add 4 µl of ligation mixture to the tube when the packaging extract begins to thaw
3. Mix the sample **gently** with a pipet tip and spin down briefly (**no bubbles**)
4. Incubate at room temperature for 2 hr
5. Add 500 µl of phage dilution solution (see recipe) to the tube
6. Add 25 µl of chloroform to the tube
7. Invert to mix, spin at 1000 rmp for 2min and infect HB101 (or store at 4°C until ready to plate.

**Titering Packaging Reaction**

a. Mix 10 µl of packaged DNA with 100 µl of *E. coli* HB101 (section 4.1)
b. Incubate the mixture at room temperature for 30 min
c. Add 400 µl of LB, and incubate at 37 °C for 60 min (invert tubes every 15 min)
d. Pellet cells for 3 min at 13,400 rpm
e. Resuspend and spread cells on a 150 mm × 15 mm LB-$Tc_{20}$ plate
f. Incubate overnight at 37 °C
g. 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.

a. Titer the pooled packaging reactions in the same manner as the small test reaction
b. Mix the packaging reaction with freshly cultured *E. coli* HB101 in a 1:10 ratio (v/v)
c. Incubate at room temperature for 30 minutes
d. Add appropriate volume of LB and incubate at 37 °C for 60 min (shake tubes every 15 min)
e. Concentrate and plate approximate vol of infected cells on 150 mm x 15 mm LB-$Tc_{20}$ agar plate, to obtain ~2000 clones per plate
f. Collect all clones in LB-$Tc_{15}$, and concentrate to a small vol (e.g. 80,000 clones in 50 ml)

g. Add DMSO to a final concentration of 7%, aliquot 1 ml in labeled vials, and then save at -75 °C