Appendix A9-1 Fosmid library construction protocol

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Fosmid Library Construction

1 Required Reagents and Equipment

1.1 For Isolation of High-Molecular-Weight Genomic DNA

1.1.1 For Sucrose Gradient Preparation

1. Sucrose (density gradient grade).
2. TE buffer (10m M Tris-HCl and 1 mM EDTA, pH 8.0).
3. Ultracentrifuge tubes (Beckman Ultra-Clear™ 25 × 89 mm).
4. Rack to hold tubes.
5. Silicon rubber stopper.
6. Bio-Rad peristaltic pump (with long blunt-end Calibrated Pipette [100 µl, Drummond scientific company] attached to pump tubing).

1.1.2 For Tissue Sample Digestion

1. Nuclei isolation buffer: No. 2 and No. 10 (KURABO, cat. NR-2025 and NR-10025).
2. Proteinase K solution (20 mg/mL).
3. 0.5 M EDTA (pH 8.0).
4. Liquid nitrogen.
5. Mortars, pestles.
6. 50 mL screw-cap tubes.
7. 55 °C incubator.

1.1.3 For Sucrose Density Gradient Centrifugation

1. Laboratory benchtop microfuge.
2. 1.5 mL sterile centrifuge tube.
3. Swing out compatible ultracentrifuge and rotor (Beckman SW28).
4. Weighing balance.

1.1.4 For Collection and Selection of Large DNA Fragments

1. Agarose, 50 × TAE buffer and ethidium bromide (EtBr) (5 mg/mL).
2. Bio-Rad Peristaltic pump (with long blunt-end Calibrated Pipette [100 µl, Drummond scientific company] attached to pump tubing).
3. Amicon Ultra-15 Centrifugal Filter Devices.
4. 96 well plate.

5. Laboratory benchtop microfuge.
6. CHEF Mapper system (Bio-Rad).
7. λHindIII marker, Lambda PFG marker and low-range PFG marker (New England Biolabs).
8. UV transilluminator.
9. Fluorometer.

1.2 For Fosmid Library Construction

1.2.1 For DNA Shearing

1. Syringe with a blunt-end needle.
2. Ultrafree-0.5 PBHK centrifugal filter unit (Millipore).
3. CHEF-Mapper system (Bio-Rad).
4. 1% Agarose and 10 × TBE.

1.2.2 For Size Selection of End-Repaired DNA

1. 1% LMP agarose gel in 0.5 × TBE buffer.
2. SYBR® Gold Nucleic Acid Gel Stain (Molecular Probes, Invitrogen).
3. Low-range PFG marker (New England Biolabs) and Fosmid Control DNA (Copycontrol™ HTP Fosmid Library Production Kit).
4. Safe imager™ blue-light Transilluminator (Invitrogen).

1.2.3 For Recovery of the Size-Fractionated DNA

1. GELase 50X Buffer and GELase enzyme preparation (Copycontrol™ HTP Fosmid Library Production Kit).
2. Razor blades and ruler.
3. Weighing balance.
4. Water bath or incubator.
5. Ultrafree-0.5 PBHK centrifugal filter unit (Millipore).

1.2.4 For DNA Ligation

1. DNA ligation kit ver.2.1 (Takara).
2. pCC2FOS™ Fosmid Vector (Copycontrol™ HTP Fosmid Library Production Kit).
3. PCR thermalcycler.

1.2.5 For Test Transformation

1. MaxPlax Lambda Packaging Extracts (Copycontrol™ HTP Fosmid Library Production Kit).
2. Phage Dilution Buffer (10 mM Tris-HCl [pH 8.3], 100 mM NaCl, 10 mM $MgCl_2$).
3. Chloroform.
4. EPI300-T1R Phage T1-resistant *E. coli* Plating strain (Copycontrol™ HTP Fosmid Library Production Kit).
5. LB broth containing 10 mM $MgSO_4$.

6. LB plates containing 12.5 μg/mL chloramphenicol.
7. Water bath or incubator.
8. Thermostat shaker and 37 °C incubator.
9. Sterile culture tubes (50 mL).

1.2.6 For Insert Size Estimation

1.2.6.1 For Plasmid DNA Isolation

1. LB broth with 12.5 μg/mL chloramphenicol.
2. Thermostat shaker.
3. 10 mL culture tube (5 multi-tube).
4. Automatic plasmid isolation system (KURABO, PI-100).

1.2.6.2 For Fosmid Insert Size Analysis

1. *Not*I and *Eco*R I (Takara).
2. Low Range PFG molecular weight marker (New England Biolabs).
3. 1% Agarose, 0.5 TBE buffer, and EtBr (5 mg/mL).
4. 37 °C water bath or incubator or PCR thermalcycler.
5. CHEF Mapper system (Bio-Rad).
6. UV transilluminator.

1.2.7 For Storage of the Fosmid Library

1. Freezing media: LB media with 7.5% glycerol, autoclave, and then add 12.5 μg/mL chloramphenicol.
2. EPI300-T1R Phage T1-resistant *E. coli* Plating strain (Copycontrol^{TM} HTP Fosmid Library Production Kit).
3. LB broth containing 10 mM $MgSO_4$.
4. LB plates containing 12.5 μg/mL chloramphenicol.
5. Sterile culture tubes (50 mL).
6. Plastic pipette (50 mL).
7. Thermostat shaker and 37 °C incubator.
8. Cryogenic vials (2 mL).

2 Methods

2.1 Isolation of High-Molecular-Weight Genomic DNA

2.1.1 Making 20–40% Sucrose Density Gradients

1. Prepare two sucrose solutions, one containing 20% (w/v) sucrose and another containing 40% (w/v) sucrose in a TE buffer of 20 mM Tris-HCl (pH 8.0), and 5 mM EDTA (pH 8.0). TE buffer should be autoclaved at 125 °C for 25 minutes.
2. A Beckman ultracentrifuge tube (38 mL) is held upright in a tube rack. Layer the dense sucrose solution (40%) (16 mL) to the bottom of a centrifuge tube.

3. A long blunt-end Calibrated Pipette (100 μL) attached to pump tubing is submerged into the 20% sucrose solution, holding another end of the pump tubing slightly over the height of the 40% sucrose reservoirs and against the side of tube wall. Overlay carefully same amount of a lighter sucrose solution (20%) using peristaltic pump onto the dense sucrose (40%) solution. Do not allow air bubbles to pump into the tube. When the gradients are completed, stop the peristaltic pump and carefully remove the pump tubing from the ultracentrifuge tube. Don't disturb the interface at this point.
4. Cover the tube with a silicon rubber stopper.
5. Gently lay down the tube rack on its side, and allow to diffuse for about 2–3 hours.
6. Slowly straighten the tube racket.
7. Place the above sucrose gradient in the −80 °C freezer until be used.

2.1.2 Tissue Sample Digestion

1. Snap-freeze 0.5–1 g of freshly excised tissue in liquid nitrogen and then grind it to powder using a mortar and pestle prechilled with liquid nitrogen.
2. Allow the liquid nitrogen to evaporate, and transfer the powered tissue quickly to a 50 mL screw-cap centrifuge tube containing 5 mL extraction buffer (No. 2 solution, 2 mL; 0.5M EDTA [pH 8.0] 500 μL; proteinase K solution (200 μg/mL in final concentration) and No. 10 solution, 2.5 mL).
3. Allow the powder to spread over the surface of the lysis buffer, and then shake the suspension quickly and vigorously by hand.
4. When all the material is in solution, incubate the digest for at least 3 hours at 55 °C, shake gently.
5. Allow the digest to cool to room temperature, transfer it to 1.5–2 mL centrifuge tubes and pellet the precipitated protein/SDS complex by centrifuge at 15,000 rpm for 3 minutes at 25 °C in a micro-centrifuge.
6. Transfer carefully the supernatant to the top of sucrose solution with a large bore pipette.

2.1.3 Sucrose Density Gradient Centrifugation

1. Having applied the sample to the top of the gradient, the tube should be handled and loaded into the rotor very carefully. Begin centrifuging as soon as possible.
2. The ultracentrifuge tubes was centrifuged in a swinging bucket SW28 type rotor (Beckman) at 25,000 rpm for 16–20 hours at 20 °C with an acceleration profile of 7 and deceleration profile of 0.
3. After running, the tube should be removed immediately from the rotor, taking great care not to disturb the layer of sucrose.

2.1.4 Collection and Selection of Large Size DNA Fragments

1. The tube should be held steady and upright in a tube stand.
2. Carefully insert the pump tubing (the end with long blunt-end Calibrated Pipette) into ultracentrifuge tube containing a sucrose gradient, ensuring the Calibrated Pipette rests at the bottom of the tube.
3. Start the pump at 2 mL/min, fractions of equal volume are then collected in 96 well plate from the bottom of the tube. A

total of 48 × 0.6 mL fractions are appropriate. The fractions can now be stored at 4 °C.

4. Analyze the fractions on 1% agarose gel to resolve the large size DNA fragments.
5. Combine the above large size DNA fragments into Amicon Ultra-15 Centrifugal Filter Devices and centrifuge at 25 °C, 500 g for 20 minutes to concentrate DNA. Meantime, add TE buffer two times and centrifuge at the same conditions in order to change sucrose solution into TE buffer. The retentive volume should be about 500–1,000 μL. Determine the DNA concentration using fluorometer.
6. Test the actual size of the DNA by first running a small amount of it by Pulse Field Gel Electrophoresis

Cast 1% agarose gel of the appropriate concentration in 0.5 × TBE buffer. Allow the gel to harden for 1 hour at room temperature.

Use a bubble level to ensure that the CHEF apparatus is completely flat on the laboratory bench.

Place the agarose gel in the CHEF apparatus, add 3 L 0.5 × TBE buffer to just cover the gel, and cool the buffer to 14 °C.

Load each sample into the center wells of the agarose gel. Load the lambda ladder PGF marker and low-range PFG marker into the wells on the two sides of the gel and then seal the wells with the 1% agarose reserved at 65°C.

Run the gel with CHEF apparatus (separation range 10–100 kbp) at 14C in 0.5 × TBE buffer for 20 hours.

After electrophoresis, Stain the gel with EtBr and take a photograph under UV light.

2.2 Library Construction

Fosmid library was construction following the protocol of Copycontrol™ HTP Fosmid Library Production Kit with slight modifications.

2.2.1 Preparation

Prior to beginning the Fosmid Library Production procedure, streak out the EPI300-T1R cells on an LB plate. Do not include any antibiotic in the medium. Grow the cells at 37 °C overnight and then seal and store the plate at 4 °C.

2.2.2 Shearing the Insert DNA

1. Randomly shear the DNA by passing it through a syringe with a 21G needle**.**
2. Aspirate and expel the DNA from the needle 20–50 times.
3. Test the extent of shearing of the DNA by running a small amount of it by with CHEF apparatus (separation range 10K bp to 100 kbp) at 14 °C in 0.5 × TBE buffer for 20 hours.
4. After electrophoresis, Stain the gel with EtBr and take a photograph under UV light.

2.2.3 End-Repair of the Insert DNA

1. Thaw and thoroughly mix all of the reagents listed below before dispensing. Combine the following on ice:

x μL sterile water

8 μL 10 × End-Repair Buffer

8 μL l 2.5 mM dNTP Mix

8 μL 10 mM ATP

up to 20 μg sheared insert DNA

4 μL End-Repair Enzyme Mix

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80 μL Total reaction volume

2. Incubate at room temperature for 45 minutes.
3. Add gel loading buffer and incubate at 70 °C for 10 minutes to inactivate the End-Repair Enzyme Mix.

2.2.4 Size Selection of the End-Repaired DNA

1. Prepare a 1% LMP agarose gel in 0.5 × TBE buffer.
2. Load the end repaired insert DNA into the center wells of the agarose gel. Load low range PFG marker into the wells on the two sides of the gel, seal the wells with the 1% agarose reserved at 65 °C. And then load 500 ng of Fosmid Control DNA into the wells on the sides of low range PFG marker wells.
3. PFG electrophoresis was performed with CHEF apparatus (separation range 10–100 kbp)
4. Following electrophoresis, stain the gel with SYBR® Gold stain.

Incubate the gel in a polypropylene container including 1 × staining solution for 10–40 minutes at 4 °C freezer. Place the gel on Safe Imager™ blue-light transilluminator and visualize the DNA.

5. Excise gel slice containing sample DNA that migrated with and slightly above (higher MW) the position of the 36 Kb Fosmid Control DNA markers by a ruler and new blade razors.
6. Transfer the slice to a tared sterile 2-mL tube.

2.2.5 Recovery of the Size-Fractionated DNA

1. Weigh the 2 mL tubes to determine the weight of the gel slice(s). Assume 1 mg of solidified agarose will yield 1 μL of molten agarose upon melting.
2. Warm the GELase 50 × Buffer to 45 °C. Melt the LMP agarose by incubating the tube at 70 °C for 10–15 minutes. Quickly transfer the tube to 45 °C.
3. Add the appropriate volume of warmed GELase 50 × Buffer to 1 × final concentration. Carefully add 0.5 U (0.5 μL) of GELase Enzyme Preparation to the tube for each 100 μL of melted agarose.
4. Keep the melted agarose solution at 45 °C and gently mix the solution. Incubate the solution at 45 °C for at least 1 hour.
5. Transfer the reaction to 70 °C for 10 minutes to inactivate the GELase enzyme.
6. Transfer the above reaction solution (step 5) into a ultrafree-0.5 PBHK centrifugal filter unit, and then centrifuge at 25 °C, 500 g for 20 minutes to concentrate DNA. Meantime, add TE buffer two times and centrifuge at the same conditions in order to change GEL buffer into TE buffer. The final retentate volume is about 9–10 μL.
7. Determine the DNA concentration by fluorometer.

2.2.6 Ligation Reaction

1. Using Takara ligation kit ver.2.1.

Combine the following reagents in the order listed and mix thoroughly after each addition.

10 μL solution I

1 μL CopyControl pCC2FOS Vector (0.5 μg/μL)

9 µL concentrated insert DNA (0.25 µg of 40 Kb DNA)

20 µL Total reaction volume

2. Mix contents gently and briefly centrifuge the tube to get all liquid to the bottom.
3. Incubate overnight at 16 °C.

2.2.7 Packaging the Fosmid Clones

1. The day before the Lambda Packaging reaction, inoculate 10 mL of LB broth + 10 mM $MgSO_4$ with a single colony of EPI300-T1R cells and shake overnight at 37 °C.
2. After ligation reaction, immediately transfer 10 µL ligation into fresh 1.5 mL centrifuge tube and keep in –80 °C, save the remainder on ice.
3. Thaw on ice, one tube of the MaxPlax Lambda Packaging Extracts for every 10 µL ligation reaction performed.
4. When thawed, immediately transfer 25 µL (one-half) of each packaging extract to a second 1.5 mL microfuge tube and place on ice. Return the remaining 25 µL of the MaxPlax Packaging Extract on to –80 °C freezer for using in step 8.
5. Add 10 µL of the ligation reaction to each 25 µL of the thawed extracts being held on ice.
6. Mix by pipetting the solutions several times. Avoid the introduction of air bubbles. Briefly centrifuge the tubes to get all liquid to the bottom.
7. Incubate the packaging reactions at 30 °C for 90 minutes.
8. After the 90-minute packaging reaction is complete, add the remaining 25 µL of MaxPlax Lambda Packaging Extract to each tube.
9. Incubate the reactions for an additional 90 minutes at 30 °C.
10. At the end of the second 90-minute incubation, add Phage Dilution Buffer to 1 mL final volume in each tube and mix gently. Add 25 µL of chloroform to each. Mix gently and store at 4 °C.

2.2.8 Titering the Packaged Fosmid Clones

Before plating the library, titering the phage particles (packaged fosmid clones).

1. Add 1 µL of above phage particle dilution to 100 µL of the prepared EPI300-T1R host cells and incubate each for 20 minutes at 37 °C.
2. Spread the infected EPI300-T1R cells on an LB plate + 12.5 µg/mL chloramphenicol and incubate at 37 °C for 20 hours to select for the Fosmid clones.
3. Count colonies and calculate the titer of the packaged phage particles.

2.2.9 Insert Size Estimation

2.2.9.1 Plasmid DNA Isolation

1. Randomly pick colonies with sterilized toothpicks and inoculate each into 2 mLl of LB containing 12.5 µg/mL chloramphenicol in a sterile 5 multi-tube culture tube, then add 2 µL CopyControl Induction Solution. Grow the cells at 37 °C overnight with vigorous shaking.

2. The recombinant plasmid DNA was isolated by the automated method (Automatic plasmid isolation system [KURABO, PI-100]).
3. Resuspend the DNA pellets in 90 μL of TE buffer, pH 8.0.
4. Determine the DNA concentration using spectrophotometer.

2.2.9.2 Size Determination of Inserts

1. About 800 ng of fosmid DNA was digested with *Not*I and *Eco*RI, respectively.

The general reaction mixture are as follows:

Not I 1 μL

10 × H Buffer 2 μL

0.1%BSA 2 μL

Substrate DNA 800 ng

Sterilized distilled water up to 20 μL

*Eco*R I 1 μL

10 × H Buffer 2 μL

Substrate DNA 800 ng

Sterilized distilled water up to 20 μL

2. Spin the reaction mixture briefly and incubate at 37 °C for 3–4 hours.
3. Transfer 10 μL reaction solution, dispense 2 μL of 6 × DNA loading buffer into each tube. Spin the samples briefly.
4. Prepare a CHEF agarose gel by pouring 150 mL of 1% agarose in 0.5 × TBE buffer at about 50 °C into a gel casting tray.
5. Load digested DNA. Use Lambda DNA PFG marker and low range PFG marker as the size marker.
6. Run the gel with CHEF apparatus (separation range 10–100 kbp)
7. Stain the gel with EtBr. Take a photograph of the gel and analyze the insert sizes.

2.2.10 Long-term Stockage of the Fosmid Library

If the test colonies meet the requirement for average insert size and empty vector rate, transform all ligated DNA into EPI300-T1R cells. Based on the titer of the packaged clones and the estimated number of clones required, calculate the volume of the packaged clones that will be needed to prepare the Fosmid library.

1. Prepare an adequate supply of LB plates with 12.5 μg/mL chloramphenicol.
2. Based on the titer of the phage particles, dilute the phage particles with Phage Dilution Buffer to obtain the desired number of clones and clone density on the plate.
3. Mix the diluted phage particles with EPI300-T1R cells in the ratio of 100 μL of cells for every 10 μL of diluted phage particles.
4. Adsorb at 37 °C for 20 minutes.

5. Spread the infected bacteria on an LB plate +12.5 μg/mL chloramphenicol and incubate at 37 °C overnight.
6. Next morning, add 2 mL of freezing media (LB broth + 12.5 μg/mL chloramphenicol) to each plate. Suspend and scrape the colonies off the agar and transfer the suspension into a 2 mL cryogenic vial.
7. Add glycerol (final concentration of 7.5%) to each cryogenic vial, and then store transformation glycerol mixture immediately at –80 °C.