

Video Article

Large Insert Environmental Genomic Library Production

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Abstract

The vast majority of microbes in nature currently remain inaccessible to traditional cultivation methods. Over the past decade, culture-independent environmental genomic (*i.e.* metagenomic) approaches have emerged, enabling researchers to bridge this cultivation gap by capturing the genetic content of indigenous microbial communities directly from the environment. To this end, genomic DNA libraries are constructed using standard albeit arduous laboratory cloning techniques. Here we describe the construction of a large insert environmental genomic fosmid library with DNA derived from the vertical depth continuum of a seasonally hypoxic fjord. This protocol is directly linked to a series of connected protocols including coastal marine water sampling [1], large volume filtration of microbial biomass [2] and a DNA extraction and purification protocol [3]. At the outset, high quality genomic DNA is end-repaired with the creation of 5'-phosphorylated blunt ends. End-repaired DNA is subjected to pulsed-field gel electrophoresis (PFGE) for size selection and gel extraction is performed to recover DNA fragments between 30 and 60 thousand base pairs (Kb) in length. Size selected DNA is purified away from the PFGE gel matrix and ligated to the phosphatase-treated blunt-end fosmid CopyControl vector pCC1 (EPICENTRE <http://www.epibio.com/item.asp?ID=385>). Linear concatemers of pCC1 and insert DNA are subsequently headfull packaged into phage particles by lambda terminase, with subsequent infection of phage-resistant *E. coli* cells. Successfully transduced clones are recovered on LB agar plates under antibiotic selection and archived in 384-well plate format using an automated colony picking robot (Qpix2, GENETIX). The current protocol draws from various sources including the CopyControl Fosmid Library Production Kit from EPICENTRE and the published works of multiple research groups [4-7]. Each step is presented with best practice in mind. Whenever possible we highlight subtleties in execution to improve overall quality and efficiency of library production. The whole process of fosmid library production and automated colony picking takes at least 7-10 days as there are many incubation steps included. However, there are several stopping points possible which are mentioned within the protocol.

Protocol

Fosmid library construction has been divided into four main steps and sub-divided into several parts (see Fig.1 for an overview).

Step I (see protocol "DNA extraction from 0.22 µM Sterivex filters" [3])**Part 1: Enzyme-catalyzed cell lysis****Part 2: Purification of environmental DNA by CsCl density gradient centrifugation and DNA recovery****Part 3: Quality control of extracted DNA by gel electrophoresis****Step II****Part 1: Enzymatic modification steps of recovered environmental DNA**

All reagents necessary for the end-repair step of the environmental DNA are included in the pCC1 Fosmid Library Production Kit from EPICENTRE. Ideally, your genomic DNA should be adjusted to 0.5 µg/µl.

1. Thaw all reagents on ice, combine the following and briefly centrifuge the tube to get all liquid to the bottom:
 - x µl sterile water
 - 8 µl 10X end-repair buffer
 - 8 µl 2.5 mM dNTP mix
 - 8 µl 10 mM ATP
 - up to 20 µg sheared insert DNA (~0.5 µg/µl)
 - 4 µl end-repair enzyme mix
 - 80 µl total reaction volume
2. Incubate at room temperature for a minimum time of 45 minutes up to 60 minutes and heat inactivate the enzyme mix at 70°C for 10 min (in the mean time, prepare a low-melting agarose gel for subsequent size-selection of end-repaired DNA, see below).

Part 2: Size-selection of genomic DNA by pulsed-field gel electrophoresis (PFGE)

1. Re-suspend 1.5 g of low melting agarose in 150 mL of 1.0X TAE running buffer and add a small magnetic stir bar. Cover the flask with microwavable plastic wrap and boil quickly in the microwave until agarose is completely dissolved. Stir solution until it is cold enough to pour into the gel tray (~60°C).
Note: do not include ethidium bromide or SYBR Gold either in the gel or in the running buffer.
2. Assemble the gel tray to cast your gel and choose a comb which will give you sufficient space to load the complete end-repair mix into one or two wells (80 µl plus appropriate amount of loading buffer).

3. Pour 2.2 L of 1.0X TAE into the electrophoresis chamber and turn on the machine to circulate the running buffer and set the cooling device to 14°C. The pump should run at 70 rpm to ensure sufficient circulation of the running buffer and keep the buffer at 14°C.
4. Pour the melted agarose into the gel tray on a well leveled surface once it is cooled down and make sure you avoid bubbles. Ensure the melted agarose is even on the gel comb, otherwise remove the comb and put it back into the melted agarose. Once the gel is solidified, remove the comb and load a MidRange I PFG marker in the fourth well of the gel. Extrude agarose from the gel syringe and slice a small plug from the end with a scalpel. Place the plug at the front of the well and seal with molten agarose. Place the gel into the electrophoresis chamber.
 Note: handle your agarose gel very carefully as low melting gels brake easily. Check if the running buffer has cooled down to 14°C before you start loading your gel. If your wells look blocked by agarose, try the following next time: before you remove the comb from the gel, add 1-2 mL TAE-buffer around the comb; this will result in nicer wells.
5. In the first well load 5 µl of λ HindIII digest followed by 1 µl of the fosmid control size marker (EPICENTRE, 100 ng/µl). Add 10 µl of sterile water to the size marker in a microcentrifuge tube as well as an appropriate amount of loading buffer. The larger volume makes it easier to load the gel. Directly next to the fosmid marker load your end-repaired and heat-inactivated mix with an appropriate amount of loading buffer. Close the lid and program the settings.
 Note: we really recommend loading different amounts of other DNA ladders to roughly quantify the amount of DNA or to visualize the extent of shearing of your genomic DNA. Ideal size markers are λ DNA-HindIII Digest and MidRange I PFG Marker (both from NEB).
6. Program the settings: use Auto algorithm if your PFGE system is equipped with this. The program automatically calculates the setting you need to separate DNA of a given size range. Settings used are: DNA size range 2-250 Kb; calibration factor: 1; gradient: [6 V/cm]; run time: 12:00 hours; included angle: 120°; initial switching time: 0.1 sec; final switching time: 21.79 sec; ramping factor a = linear.
7. To visualize your DNA, pour 250 mL of 1.0X TAE buffer into a container and add 25 µl of 10.000X SYBR Gold; mix gently before you place your gel into the solution. Stain your gel for 1 h in the dark as SYBR Gold is light sensitive.
 Note: handle your agarose gel very carefully as low melting gels brake easily.

Part 3: DNA-recovery by gel extraction and ligation of recovered DNA to the fosmid cloning vector

All reagents for this part are included in the EPICENTRE fosmid-library production kit.

1. In the mean time prepare a water bath at 70°C and one at 45°C for the gel digestion step.
2. Take your stained gel together with an empty tared microcentrifuge tube and a sterile scalpel to the blue light transilluminator. Visualize your end-repaired genomic DNA and the fosmid control size marker. Excise a gel slice with a scalpel that migrated with and slightly above the position of the fosmid control size marker (excise a gel slice which is 5-7 mm wide) and transfer the slice to the tared microcentrifuge tube. Run a MidRange I PFG Marker on your gel as this helps you to not cut the gel too high.
 Note: lay plastic wrap on the blue light transilluminator before you put your gel on it to prevent cross-contamination and wear the viewing glasses before turning on the blue light. Do not excise gel slices where the genomic DNA is smaller than the fosmid control size marker as this will result in unwanted chimeric clones.
 You can cut and store gel slices containing lower or higher molecular weight DNA for constructing whole genome shotgun or BAC libraries, respectively.
 Stop 1: the recovered gel slice(s) can be stored at -20°C for up to one year
3. Weigh the tared tubes and calculate the weight of the gel slice(s). 1 mg of agarose will yield approximately 1 µl of molten agarose.
4. Melt the low melting agarose containing your genomic DNA in a water bath at 70°C for 10-15 minutes (we do not add gelase buffer). After your gel slice is completely melted, transfer the tube quickly to 45°C.
 Note: do not vortex your sample to help dissolving as this might shear your DNA.
5. Add 1 U (1µl) of GELase Enzyme Preparation per 100 µl of melted agarose. Incubate at 45°C for 1 hour and then add more GELase (2-4 µl) and incubate for an additional hour. Heat inactivate the GELase Enzyme Preparation at 70°C for 10 minutes.
 Place the tube on ice for 10 min and centrifuge the tube at maximum speed (13,000 rpm) for 15 min to pellet any insoluble particles. Carefully remove the upper supernatant and transfer it to a 15 mL Falcon tube and add 3 mL of sterile water to the tube.
 Note: avoid pipetting any pelleted agarose. As we add more GELase than included in the kit, we order extra GELase Enzyme Preparation separately.
6. Transfer the solution to a Amicon Ultra-4 Centrifugal Filter Unit with Ultracel-10 membrane and spin the tube at 4,000 g for 6-8 minutes and discard the flow through. Centrifuge until the amount of solution on the filter is reduced to approximately 100 ~ 500 µl. Add another 3 mL of sterile water to the empty Falcon tube from above and transfer the solution to the Amicon tube and repeat the centrifugation step. Wash a third time with 3 mL of water and discard flow through again. Reduce the final volume to 50 – 100 µl.
 Note: we use a swinging bucket rotor for the centrifugation step; simply place the Amicon centrifugal filter unit in to a 50 mL Falcon tube to hold it in place during centrifugation. Amicon filter safely retains 50 µl of solution on the filter even after extended centrifugation.
7. Transfer the remaining DNA solution from the Amicon tube into a pre-washed Microcon YM-50 Centrifugal Filter Unit and rinse the Amicon tube with an additional 50 µl of sterile water to recover all DNA. Centrifuge the Microcon YM-50 Centrifugal Filter Unit in a microcentrifuge at 10,000 g until the filter is still slightly covered with liquid. Check every minute if only a small amount of liquid is left on the filter. Turn the filter upside down and recover your DNA solution by a second centrifugation step at 1,000 g for 3 min in a fresh microcentrifuge tube. The resulting volume should not exceed 10-15 µl in total, otherwise your DNA might be too diluted in the ligation step.
 Note: be careful not to spin your filter device to complete dryness. If your membrane is dry, then add 10-15 µl water onto the membrane, agitate gently for 30 seconds and recover your DNA as described above.
8. Quantify your resulting DNA solution by NanoDrop or PicoGreen assay.
9. Recovered end-repaired DNA is now ready to be ligated to the pCC1-fosmid cloning vector. Thaw the following solution on ice and make sure the vector to insert molar ratio is 10:1.
 Note: 0.5 µg pCC1-fosmid vector ~ 0.09 pmoles vector.
 0.25 µg of ~40 Kb insert DNA ~ 0.009 pmoles insert DNA
 Increasing the amount of DNA used in the ligation step might be helpful if you should obtain only a few fosmid clones after plating infected *E. coli* cells on selection plates.
 Add the reagents in the order below and briefly centrifuge the tube to get all liquid to the bottom, tap the tube and spin again:
 - x µl sterile water
 - 1 µl 10X Fast-Link ligation buffer
 - 1 µl 10 mM ATP
 - 1 µl pCC1-fosmid vector (0.5 µg/µl)
 - x µl concentrated insert DNA (0.25 µg)
 - 1 µl Fast-Link DNA ligase
 - 10 µl total reaction volume

10. Incubate at room temperature for 2 hours and heat inactivate the enzyme for 10 minutes at 70°C.
 Stop II: store ligation mixture at -20°C or proceed to the phage packaging step.
 Note: A single ligation reaction will produce 10^3 - 10^6 clones depending on the quality of the environmental DNA. Based on the estimated number of clones that is required for particular coverage, you can scale up the ligation reaction. If the ligation is scaled up, then the amount of phage packaging extract should be scaled up accordingly as described below.

Step III

Part 1: Phage-packaging of vector-insert ligation product

1. Two or three days before intended phage packaging streak out provided EPI300-T1^R plating strain on a plain LB plate and incubate overnight at 37°C to obtain single colonies. This plate can be sealed and stored at 4°C for future use.
2. The day before the packaging reaction inoculate 50 mL of LB broth + 10 mM MgSO₄ (add 500 µl 1M MgSO₄) with one single colony of the plate generated in step 1. Shake at 225 rpm and 37°C overnight.
3. The day of the packaging reactions inoculate fresh 50 mL LB broth + 10 mM MgSO₄ with 5 mL of the overnight culture prepared in step 2. Grow at 37°C to an OD₆₀₀ of 0.8 to 1.0 and do not exceed OD₆₀₀ of 1.0! Dilute your sample before measuring on the spectrophotometer so that you get an accurate result. Store cells on ice or at 4°C until further used.
 Note: Stop III: culture may be stored at 4°C for up to 72 hr if necessary; however using a fresh grown up culture is highly recommended.
4. Thaw, on ice, the MaxPlax Lambda Packaging Extracts, 1 tube for every ligation reaction carried out previously. Thawing will take 10-15 minutes. Meanwhile, place 1.5 mL tube on ice to be pre-chilled.
 Note: do not leave thawed phage on the ice too long. Otherwise phage infection efficiency will drop.
5. Once thawed **immediately** transfer 25 µl of each packaging extract to a second pre-chilled 1.5 mL microfuge tube and place on ice. Return remaining packaging extract to -80°C.
 Note: do not store the packaging extract with dry ice or any other CO₂ source.
6. Add 10 µl of the ligation reaction to each 25 µl of the thawed extracts on ice. Mix by pipetting the solution several times, avoid introducing air bubbles. Briefly centrifuge the tubes to get all liquid to the bottom of the tube and incubate the packaging reactions at 30°C for 90 min. After 80 min of incubation thaw remaining packaging extract on ice.
 Note: use a water bath rather than heating block for the 30°C incubation step.
7. After 90 min packaging reaction is complete add the remaining 25 µl of packaging extract to each reaction tube. Incubate reactions for additional 90 min at 30°C. After 90 min incubation add 100 µl of the prepared Phage Dilution Buffer in each packaging tube and mix gently.
 Note: if your efficiency should be low at the end, your phage extracts might be too old or have been exposed to dry ice or any other CO₂ source. In our experience, phage packaging at room temperature (2 times 90 min) instead of 30°C followed by a final additional incubation for 2 hours at 30°C increased the amount of resulting clones.
8. Add 5 µl of chloroform to each tube and mix gently resulting in a total volume of 165 µl in the tube. A viscous precipitate may form after chloroform addition; this will not interfere with library production. However avoid this precipitate as well as the organic chloroform phase when pipetting the phage particles.
 Stop IV: at this point the packaged phage particles may be stored for several days at 4°C.
 Note: for the best phage infection efficiency, use freshly packaged phage.

Part 2: Infection of the library host *E. coli*

1. Add packaged phage to EPI300-T1^R host cells (OD₆₀₀=0.8-1.0) in the ratio of 400 µl of EPI300-T1^R cells for every 10 µl of phage particles. To avoid pipetting any chloroform we use 125 µl of the packaged phage particles and 5 mL of prepared EPI300-T1^R host cells. Mix gently and then incubate at 37°C for 30 min.
 Note: be careful not to transfer chloroform to the host cells when you remove the phage particle from the phage packaging tube. If you are unsure, take less of the packaged phage particles.

Part 3: Plating and titering

1. Add 5-7 sterilized glass beads on four small LB + 12.5 µg/mL chloramphenicol petri dishes.
2. Spread two times 50 µl and two times 10 µl of the phage infected EPI300-T1^R cells on four separate plates. To ensure even spreading, add some LB broth (~50 µl) to the plates with the 10 µl of phage infected cells. Shake the plate horizontally to evenly spread out the cells on the plate. Let the plate dry for 15 min and then remove the beads by inverting the plate.
 Note: these plates are used to determine the number of transformants and to calculate the appropriate dilutions necessary in the colony picking step to avoid too high colony density.
3. Place the plates upside down in a 37°C incubator for 16 to 24 hours up to 48 hours until colonies form. Check plates after 24 hours to prevent fast growing clones of growing into neighbor colonies.
4. Meanwhile, harvest the residual cells left-over from the 5 mL infection by centrifugation at 3,500 g for 10 minutes at 4°C. Discard the supernatant. Add 1 mL LB/ 20% glycerol to the tube. Re-suspend the cell pellet and aliquot it to 100 µl each in 2 mL cryovial tubes. Freeze immediately and store at -80°C for further use in the colony picking step.
5. Next day, count the number of clones on the plates and determine the titer. Make sure to include the correct dilution factor to calculate the total amount of fosmid-clones.
 Note: this procedure generates between 10,000 and 50,000 fosmid-clones in total; however depending on the quality and purity of the extracted environmental DNA, a range between 3,000 up to 80,000 fosmid clones is observed.

Step IV

Part 1: Agar- and well plates preparation

1. Pour media into plates as follows; include 12.5 µg chloramphenicol per mL LB:
 - 100X15mm petri dish: 25mL LB agar
 - 150X15mm petri dish: 50mL LB agar
 - 245X245mm square bioassay plate: 250mL LB agar

Note: it is important to pour the media on a well leveled surface to make depth of agar evenly across the plate. We recommend using 245X245 mm plates for fosmid libraries so you do not have to handle many plates.

2. Thaw your glycerol stock containing the prepared fosmid clones on ice. Spread the amount appropriate for the plate you want to use based on your calculated titer (preparing the glycerol stock additionally concentrates your cells ~ 5 fold). Incubation time for cell growth is 24 hours at 37°C.
Stop V: when colonies formed, store sealed agar plates at 4°C for up to one month until they are used in the colony picking step.
Note: colonies need to be spread out evenly, distant enough from each other and should grow approximately 1 mm in diameter. Plating cells by using glass beads can generate a good separation between colonies. On a 100X15mm Petri dish, usually 150~250 colonies are a good density for highest robot picking efficiency and for 22 x 22 cm, no more than 2,000 colonies should be obtained.
3. On the days of colony picking, prepare 96 or 384 well plates filled with LB broth supplemented with 12.5 µg chloramphenicol per mL broth as well as 20% glycerol for library storage at -80°C. Well plates are filled with the automated QFill3 plate filling system as described below.
4. Autoclave 2 sets of glass bottles (500 mL), lids and silicone tubing. Do not autoclave manifold, including needle assembly and mounting flange. Assemble QFill3 near a Bunsen burner flame or within a hood to create and keep a sterile environment.
Note: make sure that the silicone tubing is inserted into the pinch valve by pressing the pinch valve activator and sliding the tubing fully into the valve. (This works like a brake for dispensing medium to the next column).
5. Sterilize dispensing needles by passing through ~50 mL of 1% bleach, autoclaved dH₂O and 80% ethanol, one at a time and place a tip box lid on the platform to collect waste solutions. After sterilization, change to the other set consisting of bottle, lid and silicon tubing.
6. Fill bottle with ~300 mL medium. Run enough medium through to get rid of any remaining ethanol from the cleaning step. Once the tubing is cleaned, load a 96/384 well plate on the platform. Press "start" to fill up your plate; each well should be filled with 200 µl of prepared LB broth. To clean dispensing needles, run ~ 50 mL of 80% ethanol through.
Note: make sure your well plate is labeled before filling.

Part 2: colony picking robot setup

1. We are using the clone picking robot QPix2 according to the user's manual. Users should be familiar handling their robot and how to set up everything to guarantee highest picking efficiency.
2. To create a sterile environment around the clone picking area, turn on the UV light for 30 minutes. While the UV light is on, prepare 500 mL of 1% bleach, 500 mL of sterile dH₂O and 500 mL 80% ethanol. When the UV light shuts off fill up the trays as labeled. Always pour the solutions into the correctly labeled solution trays. From back to front fill in 1% bleach, then autoclaved dH₂O and 80% ethanol in the front tray. Make sure they are full. Picking needles cannot be washed properly without sufficient washing solution.
Note: monitor 80% ethanol over time. It will evaporate and needs to be topped up.
3. After setting up the parameters for optimal picking, place the prepared agar plates with fosmid clones and well plates between the posts in the Q-Pix working area. Make sure that you have taken all the plate covers off!
4. Plates should be tight against front right corner, so it can not move during picking. Then close the front panel.
Note: This is extremely important!
5. Once all parameters are set the picking procedure can begin. When all picking is completed rinse the brushes and trays with deionized water and set on the bench to dry. Clean up any spills that may have occurred, and wipe down the inside of the Q-Pix with 80% ethanol.

Part 3: Overnight incubation and library storage

1. Ensure that all glycerol stock plates are labeled, and place them in the 37°C incubator for 24 hours. Leave a note on the incubator that contains the date, number of plates incubated, time of incubation, time for removal and your initials.
2. For long time storage, put the grown overnight incubations into a -80°C freezer.

Representative Results:

The presented protocol describes the procedure how to generate an environmental fosmid library to capture the genetic content of a microbial community in a given habitat. This protocol should create a fosmid library which is representative to the genetic content of the sampled environment and should enable the reader to modify and optimize critical steps if the amount of fosmid clones obtained at the end of the whole procedure is too low.

Discussion

A procedure is described how to most efficiently generate a large-insert fosmid library with genomic DNA derived from a coastal water sample. Up-stream genomic DNA extraction is described in a separate protocol [3].

As the fosmid-library production is a multistep-process, plan at least two to three weeks in time for the whole procedure including all four presented steps. The extraction of genomic DNA is the most crucial step and all down-stream steps rely on the quality and quantity of extracted genomic DNA; so consider spending enough time for this step and work carefully. Quality and quantity control of your extracted DNA is very important. It can happen that the number of fosmid clones is low especially if this is the first library you are going to make. As the fosmid library construction by itself is straight forward, failure is mainly caused by insufficient quality and/or quantity of your extracted and purified genomic DNA. Consider to re-extract genomic DNA and pay special attention to the steps involved in purification and DNA-recovery if your library construction was not successful.

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