Screening lambda cDNA or genomic libraries

Media

Need LB plates (9 cm or 14 cm) to plate the library and do secondary screens. I typically screen about 500,000-1 million plaques for a primary cDNA screen. This fits on 11-22 14 cm plates. I do the secondary screens on 9 cm plates - you should pour about 20 of these so you will be prepared in you get a bunch of positives. After pouring the plates, let them age/dry at room temperature for 2-3 days. This is necessary to help the top agarose stick. You will also need LB top agarose containing 10 mM MgCl2 or MgSO4. I use 0.9% agarose ("low electroendo-osmosis" agarose is best, it is slightly low melting. If regular agarose is used, you may need to go down to 0.7% to get the top agarose to spread evenly before it sets.)

Note: many protocols call for using special "NZY" plates for plating lambda libraries. In practice, LB medium works fine.

Bacterial host strain

- For most lambda strains use RY1073 or C600 HflA or LE392 for lambda hosts.
Actually almost any e coli (like TG1) are ok for some lamda vectors, like lambda ZAP II. For lambda gt11 use the host Y1090. The Barstead mixed stage C. elegans cDNA library is in the original lamda ZAP vector, and requires a host carrying the supF mutation, such as Y1090.

Growing the cells:

grow up 40 ml of bacteria o/n in LB

spin 5 min, 5K

resuspend in 10 mM MgCl2 (1/2 original volume)

these "2X cells" can be kept in the fridge & used for ~1 month

Note: optionally, can add maltose to 0.2% to the LB medium used to grow the cells. Make a sterile filtered stock of 10% maltose to supplement the LB. Maltose induces higher expression of the lambda receptor. In practice, the screening will work fine even if you don't add maltose to the medium.

Titering the library
Libraries are stored at 4° C in a tightly sealed tube, with a drop of chloroform at the bottom (chloroform keeps bacteria from growing but won't harm the phage). For very long term storage, add 10% DMSO, freeze in liquid nitrogen, store at -70°. Titers will range from 10^6-10^11 phage per ml. Often a 1000-fold diluted stock of the original library is titered and stored for use. Further dilutions made to use for a particular experiment are discarded after the experiment is done - these more dilute phage solutions tend not to hold their titer.

Phage will be diluted in 1X phage buffer. Here is the recipe for 10X phage buffer:

10X phage buffer

100 mM Tris pH7.5

100 mM MgCl2

500 mM NaCl

Prepare some top agarose:

Melt a bottle of top agarose in the microwave. For 100 mm plates, put 3 mls in each of several 13X100 mm glass culture tubes in a 50-55° temp block. For 15 cm plates need 6.6 ml top agarose (use a bigger tube and a 50-55° water bath).

to titer (on 100 mm plates):

Put various dilutions of phage in 100 µl 1X phage buffer. Always do a no phage control! This will help you distinguish between dilutions that give you no phage (look like the no phage control) and dilutions that give complete lysis (too many phage so that no individual plaques can be distinguished).

To 100 µl diluted phage

add 100 µl 2x cells

incubate 37° for 20'

plate with 3 ml top agarose (on a 100 mm plate)

To plate, squirt the phage/cell mixture into a tube, vortex quickly, pour onto a 100 mm LB plate, quickly tilt the plate around to evenly coat the plate with the top agarose. Do not use LB plates straight out of the cold room - they will cause the top agarose to set before you can spread it evenly. Let the plates sit a few minutes for the top agarose to harden. Then turn the plates upside down and move to 37°.
grow 37° for 6-8 hours for cDNA library, ~10 hrs for a genomic library. This should be enough time to get nice clear ~1 mm plaques.

You want the library to be plated for screening at a density such that the plaques can grow to about 1 mm in diameter before they begin to just barely touch each other. For your first screen, get an experienced person to look at your titering plates to give you advice about what dilution to use. If you are going to titer on 10 cm plates and plate on 15 cm plates, you will have to multiply everything by 2.25 to adjust to the large plates.

**Plate out for screening**

use L plates that have been aged at room temp for 2-3 days

for top agarose (agar doesn't stick to the underlying plate as well)

want 15,000 plaques/9cm plate or 45,000 per/14 cm plate

grow until plaques begin to touch. cDNA phage grow fast (~6hrs).

Charon 4 (genomic library vector) grows slowly (~10 hours).

put plates in fridge for >1 hr

**plaque lifts**

-make sure plates are chilled to 4° C.

-many people take more than one lift from each plate (you can take up to about 7 serial lifts). This allows you to probe with multiple probes simultaneously. Also, this can help with a problem that occurs if you have poor technique with hybridization - this often leads to random background spots that can be confused with positive plaques. By probing two sets of lifts with the same probe, genuine positives can be recognized since they occur on both lifts. I have reproducibly good technique and therefore only make and use one set of lifts.

- lay on nitrocellulose filters (numbered with pencil)

bend filter at middle and touch to plate-then let the wetted area spread to edges of filter. For 2nd and subsequent lifts, let the filter sit an extra 30 sec on the plate to soak up stuff before you lift it off.

- Mark filter and plate with assymetrical holes - puncture in 4 places with a 25 g needle filled with india ink. On second and subsequent lifts, just use the needle without ink and poke holes where the plate is marked.
- Using flat (e.g "millipore") forceps, gently lift the filter off the plate, making sure the top agarose stays on the plate.

- After the lifts seal the plates with parafilm and store at 4°. It's best to keep things clean during the lifts so that fungus doesn't end up growing on your plates.

Alkaline denaturation

- pull off filter, lay DNA side up in 1.5 M NaCl, 0.5 M NaOH 30-60 sec

- transfer to 1.5 M NaCl, 0.5 M Tris pH 8 for 5'

- optional: rinse in 2X SSPE to get salt off the filters

- lay on 3MM filter paper to dry

- bake in a vacuum oven at 80° for 2 hours

Heat denaturation - this is a newer and more convenient alternative to the alkaline denaturation method

- after lifting the filters, let dry DNA side up for a few minutes.

- interleave the filters with pieces of Whatman paper, and put the stack on an upside down lasagna dish in the autoclave, with a few extra whatmann sheets at the top and bottom.

- Autoclave 5 minutes. Start timing the 5 minutes when the chamber temp. is up to 100° C; turn the autoclave cycle off (fast exhaust) when 5 minutes is up.

- Bake the filters in a vacuum oven at 80° for 2 hrs. Filters may come out with an odd smell.

Hybridization to lamda plaque lifts

Probing the filters; a few hours to make the probe and prehyb, overnight to incubate blot and probe, a few hours to wash the next day, 1-2 days to expose the autorad.

1. Labelling the probe-obviously, follow all the normal radioactivity safety precautions

- If you store it frozen, take the alpha 32P-dATP out of the freezer to thaw.

- Put 50 ng DNA to be labelled in a total volume of 18.9 µl H2O. For labelling fragments from a restriction digest: I prefer not to do the labelling in the low melt agarose, since the
agarose somewhat inhibits the labelling reaction and the specific activity of the resulting probe is only marginally adequate. It is easy to purify the fragment either using agarase, or by adsorption to glass as in the commercial Qiex kit, and this will increase the efficiency of labelling considerably.

- Boil the DNA to denature it for a few minutes. Briefly spin the tube and put on ice to cool. Add 6.6 µl 5X OLB, 5 µl hot dATP (50 µCi), and 2 µl Klenow.

- Allow the labelling reaction to proceed at room temp 30 min to overnight. The reaction appears to go to completion by 45 min.

- Some people use the entire reaction in their hybridization. However, anyone intelligent will run a spin column at this point and measure the amount of counts incorporated, since this is the main factor determining the strength of signal on the autorad. Add about 20 µl of 50 mM EDTA to stop the reaction and run a spin column as usual. Expect about 50% of the counts to be incorporated for an average labelling.

2. Prehybridization of the filters- can do this while the probe labels.

Wet the filters in a tray of 6X SSPE. Make some prehybridization buffer. Put the filters in a stack in an appropriate hybridization container and add the buffer (just enough to fully cover the stack of filters), and incubate at 65° for a couple hours or longer. The best containers to use for this are pyrex "crystallization dishes". These are round dishes with flat bottoms that come in several diameters - use one just bigger than your filters. Put some saran wrap over the top and use a rubber band to seal the saran wrap on the top. In our lab we have a hybridization oven in which a rocking platform can be used. Just set the dish rocking in the oven.

3. Hybridization: pour the prehyb sol'n out. Add hybridization solution (just enough to cover the filters). Denature the probe DNA by A) heating to 100° C for 5-10 min, or B) add NaOH to 0.2 M, incubate 5' room temp (the SSPE will buffer this out when you add it to the hyb solution). Add the probe to the blot/hyb. solution, mix well, and incubate overnight at 65° on a rocker in the hybridizatio oven.

4. Posthybridization: Make 1 liter of post hyb. wash solution, and heat in a water bath (or microwave) to 65°. Pour out the hyb solution into a 50 ml plastic disposable centrifuge tube if it is to be reused (probe is good for a couple of weeks, and must be boiled 5 min and chilled on ice before reuse. You should certainly save the probe from your primary screen, you can reuse it for the secondary screen). Rinse the filters (in the dish) briefly with some wash solution. Remove the filters to a tupperware dish. Add a generous amount (a few hundred mls) of wash solution. Wash the filters 3X30' in the 65° hybridizatio oven. At this point the geiger counter should not (or just barely) detect counts on the filters.
Dab the filters on 3MM paper to get rid of excess liquid, wrap the moist filters in Saran wrap, tape to an old autorad, apply some strips of phosphorescent markers next to them (Stratagene), and take an exposure with an intensifying screen at -80°. Try overnight - if your technique was good this will be sufficient.

**Solutions:**

**5X OLB**: (oligonucleotide labelling buffer)

- 250 mM Tris pH 8.0 250 µl 1M
- 25 mM MgCl2 25 µl 1M
- 5 mM β-mercaptoethanol 0.35 µl 14.4 M stock
- 2 mM dCTP 20 µl 100 mM stock
- 2 mM dGTP 20 µl 100 mM stock
- 2 mM dTTP 20 µl 100 mM stock
- 1 M HEPES pH 6.6 500 µl 2 M (2.38 g + 4ml H20, adjust to 6.6 w/ NaOH, adjust vol to 5 ml)
- 1 mg/ml oligonucleotides (random primers: Pharmacia # 27-2166-01)
  
  H20 to 1 ml

**20X SSPE:**

- 175.3 g NaCl, 27.6 g NaH2PO4.H20, 7.4 g EDTA: dissolve in ~800 ml H20, adjust to pH 7.4 w/ ~6.5 ml 10 N NaOH. Adjust volume to 1 liter.

**Prehybridization solution**: note that some leave out the Denhardt's

- 30 ml 20X SSPE
- 1 ml boiled 10 mg/ml salmon sperm DNA (store frozen in 1 ml aliquots)
- 10 ml 50X Denhardt's solutions (see below)
- 5 ml 10% SDS (add some H20 first to prevent this from ppting)
  
  H20 to 100 mls
**Hybridization solution:** Same as prehyb sol'n above except without the Denhardt's

**50X Denhardt's solution:**

5 g Ficoll

5 g polyvinylpyrrolidone

5 g BSA (pentax fraction V)

500 ml H2O

Mix overnight. Then filter thru a disposable filter unit and freeze in 10 ml aliquots. Alternatively, add EDTA to 5 mM and store at 4°.

**Posthybridization wash solution:** 0.2X SSPE, 0.5 % SDS

Recipe: 10 ml 20X SSPE, 940 ml H2O, 50 ml 10% SDS

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**Secondary screening of lambda clones**

- line up the plate with the autoradiogram to locate positive plaques

- scoop up the top agar around the hit with a pasteur pipette, an area about the size of the capitol letter O.

- put in 1 ml 1X phage buffer at 4° for 5-6 hours

- dilute a small amount of this 100 fold, and add 2.5 µl of the diluted phage to 100 µl 1x phage buffer, and 100 µl 2X cells, incubate 37° for 20', and plate on a 9 cm plate with 3 ml top agar. Want to get a few hundred plaques

8/16/93 - with a lambda zap library I needed to dilute 10 fold more than as above for 2°, i.e. 1 to 1000, then use 2.5 µl.

- take plaque lifts as before, and probe with the same hyb mix used in the primary screen (boiled for 10 min to denature before use).

- pick well isolated positive plaques from the secondary plates into 1 ml 1X phage buffer.
Lambda ZAP cDNA popout

follow a modification of the instructions from Stratagene. At the last step, they tell you to plate too much of the final mix - try 1/100th of their recommendation in order to get nicely isolated colonies.

1. Core a well isolated plaque from the secondary or tertiary plate and transfer to a microfuge tube containing 500 µl 1X phage buffer. Vortex to release the phage.

2. In a 50 ml conical tube mix
   a) 100 µl 2X cells
   b) 200 µl of your phage stock
   c) 10 µl of the M13 helper phage R408 (106-1011 pfu/ml)

   Incubate mixture at 37° for 15 minutes

3. Add 5 ml LB medium and incubate 4-6 hours at 37° in a roller.

4. Heat the tube at 70° for 20 minutes, then spin 5 minutes at 4000 RPM.

5. Decant the supernatant into a sterile tube. This contains the Bluescript plasmid packaged at an f1 phage particle. It can be stored at 4°.

6. To plate the rescued plasmid, combine the following in two 15 ml tubes:
   a) 1 µl of the phage stock from step 5 above and 100 µl 2X cells
   b) 1/100 µl of the phage stock from step 5 above and 100 µl 2X cells

   Incubate at 37° for 15 minutes.

7. Plate 1 µl from each tube on LB/Amp plates and incubate overnight at 37°.

   Colonies appearing on the plate contain the pBluescript plasmid with the cloned DNA insert.

The remainder of the protocol is for genomic and other nonpopoutable phage:

Preparation of plate lysates
High titer phage stocks can be prepared either from liquid culture, or more reproducibly, from plate lysates. For plate lysates, need to use NZCYM agarose (not agar) plates and top agarose (not agar) since agar contains inhibitors of restriction enzymes that will contaminate the prep.

- mix ~20 µl of phage from the plaque purified stock with 80 µl 1X phage buffer and 100 µl 2X cells, incubate 37° for 20 min, and plate on 90 mm plates as usual. May want to scale up and do several plates per phage.

- incubate the plates 8-12 hours. Want the lawn to be almost confluently lyzes (mealy looking plates are the best).

- overlay each plate with 3 ml 1X phage buffer. Place on a rotating platform at room temp for 1-2 hours. Phage diffuse up into the buffer

- pour the supernatant off into a 15 ml centrifuge tube (don't take the sup. off with a pipette - you'll just end up sucking off lots of coli and agarose along with the phage).

- spin 5 min ~5000 rpm, and keep the cleared sup. Add a drop or chloroform if you want to keep the phage stock a long time.

**Quick analytical lambda DNA prep**

This is the quickest and most reliable lambda DNA prep I've found. It was handed down through Hogness lab lore. Its problem is that it exposes the DNA to DEPC, which is potentially mutagenic, so DNA prepared this way should only be used for restriction mapping or preparing hybridization probes; a nonmutagenic prep should be used if the DNA is to be subcloned for further analysis.

- to 0.4 ml high titer phage stock (supernatant from a plate lysate) add 4 µl 1 mg/ml DNAs.e I. Incube 30 min at room temp. (Digests colo genomic DNA).

- spin 10 min in microfuge, keep sup.

- add 40 µl 0.5 M Tris pH 7.4, 0.2 M EDTA. Add 5 µl diethylpyrocarbonate (DEPC), and vortex (not all the DEPC will go into solution).

- incubate 5 min at 70°, then 10 min on ice.

- microfuge 10 min, collect sup (the DEPC and heat denatures and ppts. phage proteins, releasing the lambda DNA)

- add 145 µl 7.5 M NH4OAc, and 545 µl isopropanol with 0.1% DEPC, incubate 5 min room temp, spin 10 min room temp., discard sup.
- resuspend in 200 µl TE, add 75 µl 7.5 M NH4OAc, 650 µl EtOH, -20° for 10 min, spin 10 min at 4°, wash pellet with ice cold 70% EtOH, resuspend in 50 µl TE.

- use 5 µl for a restriction digest in a total volume of 25 µl. RNAse before loading on a gel.