REPAIRING SHEARED DNA

90 µl sheared DNA, estimate quantity
16 µl 10mM dNTPs
16 µl T4 10X buffer
1.6 µl 100X BSA
7 µl T4 Polymerase
29.4 µl dH2O
160 µl TOTAL VOLUME

If you use a larger or smaller volume of sheared DNA, adjust the water volume. Incubate at Room Temperature for 15 minutes. Add 5 µl Klenow (5U/µl), mix carefully and incubate at RT for another 15 minutes. Add 35 µl dH2O and proceed with phenol extraction/EtOH precipitation. Wash with 70% EtOH.

Your final pellet should be dissolved in 10 µl TE.

This entire volume of DNA needs to be run on a 0.7% agarose gel. Take a photo of the gel and carefully size-fractionate between 1-3 kb. Minimize the amount of time your DNA is exposed to the UV light from the transilluminator. UV light damages DNA.

LIGATIONS

Total ligation volume is 15 µl.
Constants:
1.0 µl pBlue Script vector (We use pBluescriptII SK+, cut with EcoRV/CIAP) (Stored in -20C freezer)
1.5 µl 10X Buffer
0.75 µl ATP
1.0 µl T4 DNA ligase

At least 2 ligations per phage DNA fragments preparation should be done. Examples are: 1.0 and 2.0 µl, or 0.5 and 1.0 µl, or 2.0 and 4.0 µl. The remaining volume should be filled with dH2O. Check with Jen/Alexis for suggestions about DNA fragments volume.
Mix ligations carefully and incubate at RT for 1 hour.
Add 15 µl water and heat kill at 65ºC for 15 minutes.

The ligations can be transformed directly. If you aren't going to transform them right away, the ligations may be frozen in the -20C freezer. Make sure everything is labeled: contents, date, ligation number, initials, phage name.

TRANSFORMATIONS

You will be using E.coli XL1-Blue F’ competent cells for transformations. The stock in the Hatfull Lab was made by students and when the stock gets low, students need to replace them. This is not Molly's job, so DO NOT ask her to make cells for you. When it is time to make more competent cells, 2 or 3 students need to set up a time with Jen and Alexis and they will help you. The whole procedure takes a couple of days, so ample warning time is necessary.

Each frozen tube of cells is good for 5 transformations. The cells shouldn't be re-frozen once they are thawed.
Set everything up on ice. Label the electroporation cuvettes and set them on ice as well.

1. Put 1.0 µl of each ligation into a cuvette. You will also use 1.0 µl of pBlue (uncut, stored in cold box). Keep on ice.
2. Once the competent cells are thawed (use a fresh aliquot for your phage library), aliquot 40 µl of cells into a clean cuvette for each transformation. Each ligation gets its own transformation. You will also need 40 µl for a pBlue control and 40 µl for the ‘no DNA’ control. Make sure you label the transformation cuvettes.
3. Transfer the cell/ligation mix into the electroporation cuvettes. Be careful not to introduce any air bubbles into your sample. You may need to flick the cuvette several times to get any bubbles out.
4. Keep the cuvettes with sample on ice while you get the recovery tubes ready.
5. You will need a sterile culture tube for every ligation plus 1 tube for no DNA and 1 tube for the pBlue control. Aseptically add 1ml SOC broth to every culture tube. Make sure you label everything.
6. Have Jen or Alexis show you how to use the electroporator. (We use the one in the Lawrence Lab)
7. Recover your samples for 1 hour on a shaker in the 37ºC room. After plating, the remaining cells, may be stored in the cold box for a couple of days.
8. They should be plated on S-GAL Amp/Tet plates. (Amp=100 mg/ml, Tet= 12.5 mg/ml)
9. Plate 100 µl cells/plate. Also plate 100 µl of No DNA cells. For pBlue, Plate 100 µl LB and Place 1 µl cells in puddle to spread.

TEST COLONIES

1. Aseptically aliquot 2 ml LB/Amp into several test tubes. Pick a white colony with a pipet tip and inject directly into the tube.
2. Test 8-12 white colonies per ligation.
3. Grow the cultures overnight (about 16 hours) in the 37ºC room on a shaker.
4. The next day, take 1.5 ml from each tube and put it into a clean eppendorf tube.
5. Spin tubes for 30-60 seconds and pipet off the supernatant.
6. Follow the protocol in the Qiagen mini-prep kits.
RESTRICTION DIGESTS

Once the DNA from your test colonies has been mini-prepped, restriction digests need to be set up. We typically use EcoRI and HindIII.

MASTER MIX
1X Restriction Digests
1.0 µl 10X React 2 Buffer
7.0 µl dH2O
always add enzymes last
0.5 µl EcoRI*
0.5 µl L HindIII*

*Volumes of enzymes are given in this protocol, however enzymes are sold in units. A unit of enzyme is the amount of enzyme necessary to digest 1 µg of DNA/hour. You will need enough enzyme to digest the sample. Check with Jen and Alexis for accurate conversions.

Each tube will have 9 µl of your master mix. Add 1.0 µl of DNA to each tube. Digest for 3 hours at 37°C. A thermocycler can be used, or you may put your samples in the water bath or warm room.

Once the digest is complete, run 10 µl of each digested DNA sample on a gel with a 1KB ladder. Show the picture to Jen or Alexis.

If you cannot run your digests on a gel that same day, place them in the cold box or -20 ºC freezer.

PICKING BLOCKS

You will need large quantities of LB broth for picking blocks. Make 500 ml at a time. This isn't Molly's job either; she will give you the recipe, but it is your job to make and autoclave it. Once the LB is cool, add ampicillin (100 mg/ml).

Obtain blocks from Jen or Alexis. If they need to be sterilized, one of them will tell you how to do it. Aseptically aliquot 1ml LB/amp per sample well. Pick white colonies with a pipet tip and inject one colony directly into each well. Cover the block with Qiagen Airpore tape and shake at 37°C for 24 hours. There are special rectangular clamps on all the shakers for this purpose. The next day, spin your blocks down (5 minutes at 1500 G) Use the centrifuge in the Genome Center. Dump liquid and tips into the sink, then put the tips into the trash. Cover the blocks with 3M tape and store them in the -20°C freezer in the Genome Center. Make sure everything is labeled properly. Name, Date, Sample Name, etc.

USING THE BIOROBOT

The preparation of DNA templates for the sequencing reactions is accomplished using the BioRobot 96, an efficient way to get quality high out-put sequencing reactions.
To use the biorobot

1. Schedule a time with Jen and Alexis well ahead of the time (at least one day in advance) you will need to use it. The time will be confirmed and written on the Genome Center board.

2. You must use the Biorobot under Jen and Alexis’ direction.

General outline of what occurs:

1. Spin down the blocks of cultures. The blocks of cultures need to grow 20-24 hours and then the cells are harvested in our centrifuge for 5 minutes at 1500 RPM.
2. Decant the liquid into the sinkh with paper towels. If you are not going to prep the DNA right away, put a piece of freezer tape over the block, put the lid back on and store in our -20 °C freezer. If you are going to prep the DNA right away, it can go on the robot.
3. The computer that runs the robot goes through the protocol, but it will be reviewed with you.
4. Once the DNA templates are recovered, you will set up sequencing reactions.

SEQUENCING REACTIONS

MASTER MIX 1X
4.5 µl dH2O
1.0 µl Big Dye
1.5 µl ABI 5X Buffer
0.016 µl primer (SK or KS)

Make one master mix for forward and another for reverse reactions. (100X/each usually works)
This master mix should be kept on ice. Distribute 7 µl of master mix to all 96 wells. You will need two 96 wells to plate for each block.
Aliquot 3 µl DNA into plates. Jen/Alexis will show you how to do this. Seal the plates.
The cycling takes 2.5 hours.

CLEAN UP REACTIONS
Again, see Jen and Alexis for specific direction. The following is the manufacturer’s protocol for cleaning up reactions.