

## Genomic Library Construction – Lab Exercises

**Day 1:** Groups 1,3,5 should start by setting up the gel and the nebulization mixtures, then go to the Perl introduction. You will continue with nebulization and running gel afterward. Groups 2,4,6 should pour the gel, run the nebulization calibration series, load and start their gels, and then go to the Perl introduction. The gels will take about 45-60 minutes to run.

### Fragmenting the DNA

Prepare a 1% agarose gel in 1X TAE. To prepare the gel, weigh out 0.4 g agarose, pour it into an Erlenmeyer flask, add 40 ml 1X TAE and microwave to dissolve (2-3 min at power level 6). After the agarose is dissolved, add 2  $\mu$ l 10 mg/ml ethidium bromide, let the flask cool until it can be handled comfortably, then pour the gel. Groups 2,4,6 can pour the gel in the cold room to save time.

Caution! Ethidium bromide is a mutagen. Use care and wear gloves when handling solutions containing it. Dispose of all EtBr contaminated tips, etc. in hazardous waste.

### Shearing of DNA by nebulization:

You will need two nebulizers, one for a calibration series and one to prepare the insert DNA. Open nebulizers, affix thick tubing section to the inside center of the lid.

Keep both nebulizers on ice.

#### *1. Calibration series:*

In the cup of one clinical nebulizer, and on ice, mix:

- 200  $\mu$ l fosmid DNA (about 25  $\mu$ g)
- 250  $\mu$ l 50% sterile glycerol
- 50  $\mu$ l 3M NaOAc

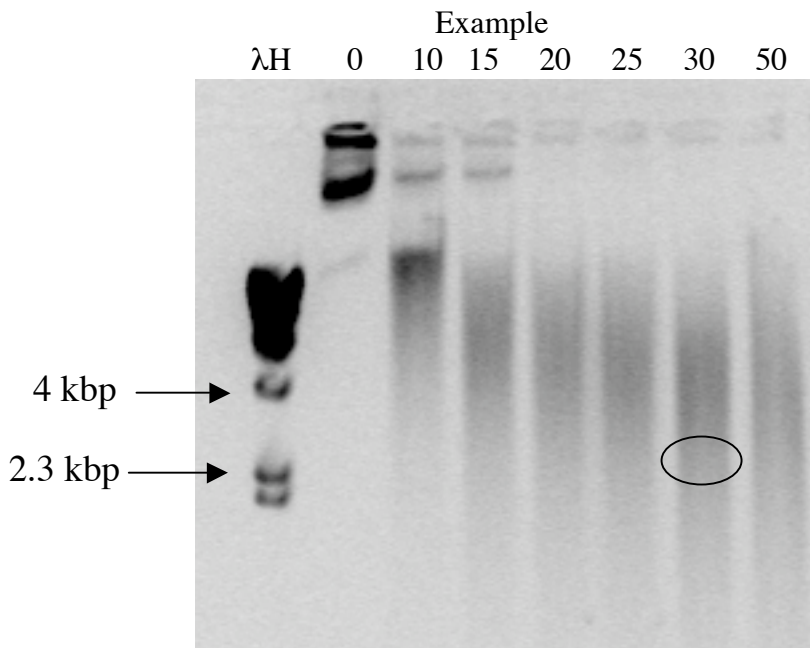
Remove a 15- $\mu$ L aliquot and save on ice (no-shear control). Label 7 additional sample tubes.

Nebulize the DNA with a stream of N<sub>2</sub> adjusted to 10 psi for 10 seconds, keeping the nebulizer on ice. Remove 15  $\mu$ l and save on ice. Adjust the N<sub>2</sub> tank regulator to 15 psi. Nebulize for 10 sec; remove 15  $\mu$ L. Repeat, adjusting the gas output to 20 psi, and then 25, 30, 40, and 50 psi. Remember to remove 15- $\mu$ l aliquots after each treatment. To each sample add 3  $\mu$ L of 6x gel loading solution. Keep all samples on ice. You will have 8 samples altogether. Discard the nebulizer and the remainder of the sample.

Hint: set your timer to count up and observe seconds, don't try to reset to 10 each time.

Load your samples (all 18  $\mu\text{l}$  or as much as you can fit in the wells) on the prepared gel. Make sure you also load 5  $\mu\text{l}$  DNA size markers on both sides of the gel. Run the gel at 100 V in TAE. MW markers are in tubes labeled " $\lambda$ -HindIII" and "1 kb ladder". Heat the  $\lambda$ -HindIII markers at 50°C while you are loading the other samples.

When the bromophenol blue has migrated half-way down the gel, turn off the power, remove gel from gel box and visualize the DNA under UV light. Wear gloves. Note the range of generated fragments for each of the applied pressures. Determine the optimum pressure for shearing into 2-3 kbp range fragments.



## 2. Preparative sample:

In the second nebulizer, combine

- 50  $\mu\text{l}$  fosmid DNA (about 50  $\mu\text{g}$ )
- 150  $\mu\text{l}$   $\text{H}_2\text{O}$
- 250  $\mu\text{l}$  50% sterile glycerol
- 50  $\mu\text{l}$  3M NaOAc

Shear the DNA for the optimum pressure and time. Transfer the DNA to a microfuge tube and measure the volume remaining. Precipitate by adding 2 vol of cold ethanol. Leave at -20 during dinner period.

**Day 1, evening:**

*BAL31 Nuclease treatment*

Recover the DNA by centrifugation for 10 minutes, rinse with 80% cold ethanol, and drain and dry the pellet at room temperature.

Resuspend the DNA in 49  $\mu$ l TE.

Add 50  $\mu$ l 2x BAL31 buffer

1  $\mu$ l BAL31 Nuclease (1 unit, from NEB, a mix of "fast" and "slow")

Incubate 5 min at 30°C.

Clean up the reaction using Qiaquick PCR purification kit from Qiagen. Add 5 vol buffer (~500  $\mu$ l) PB immediately after the 5 min incubation. Add the sample to the filter assembly and spin 1 min. Discard flow-through. Add 750  $\mu$ l buffer PE; spin 1 min; discard flow-through. Spin again 1 min to dry the membrane. Elute DNA into a fresh tube with 40  $\mu$ l EB applied right on top of the unit's membrane, without touching it; spin 1 min.

Store at -20 overnight.

## Day 2:

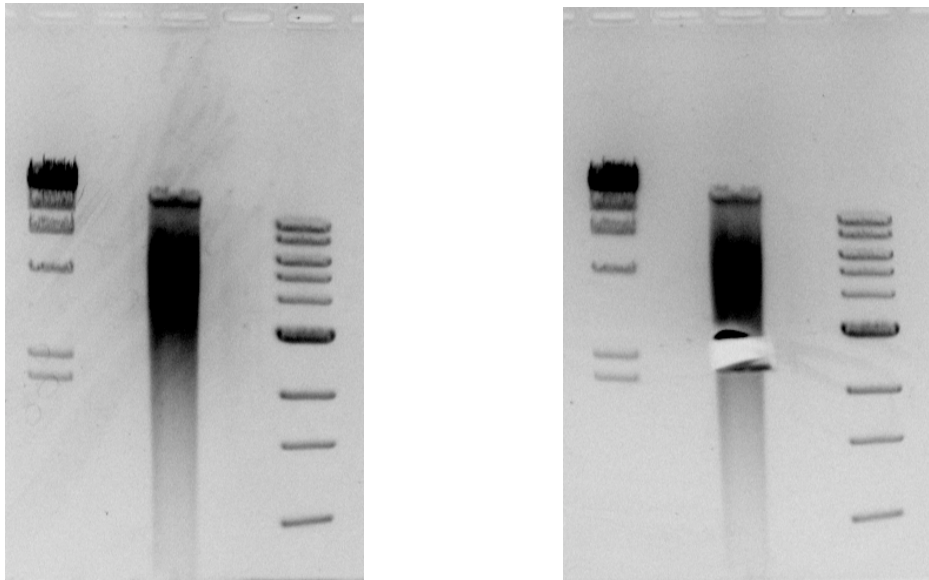
### Size fractionation of insert fragments

Add 7  $\mu$ l gel loading solution to the Bal31-treated DNA.

Run out the fragments on a 1% agarose gel at about 8 V/cm (preferably in a cold room) until the bromophenol blue has migrated halfway down the length of the gel. Make sure that you have molecular weight markers on both sides of the experimental lane.

### **[While gel is running, you will be starting cDNA library construction]**

Visualize the DNA using the ultraviolet light transilluminator in the darkroom. Using a clean razor blade, excise the region containing DNA fragments in the 2-3 kbp size. Work quickly to avoid excessive nicking of the DNA. Place excised chunks in pre-weighed microfuge tubes and weigh again to determine approximate weight of the agarose plug. Excise the regions of the gel containing the desired size fragments.



Extract DNA using Qiaquick Gel Extraction. Add 3 vol buffer QG and melt the agarose by incubating at 50°C for 5 min. Add the solution to the filter assembly and spin 1 min. Discard flow-through. Add 750  $\mu$ l buffer PE; spin 1 min; discard flow-through. Spin again 1 min to dry the membrane. Elute DNA into a fresh tube with 44  $\mu$ l EB applied right on top of the unit's membrane, without touching it; spin 1 min.

### Repairing the fragments

To recovered fragments, add:

- 5  $\mu$ l 10x T4 DNA polymerase buffer
- 1  $\mu$ l 20 mM each dNTPs
- 1  $\mu$ l (5 units) T4 DNA polymerase
- 1  $\mu$ l (5 units) Klenow

Incubate 30 minutes at room temperature

Clean up repaired fragments using Qiaquick PCR Purification. Add 5 vol buffer (~250  $\mu$ l) PB. Add the solution to the filter assembly and spin 1 min. Discard flow-through. Add 750  $\mu$ l buffer PE; spin 1 min; discard flow-through. Spin again 1 min to dry the membrane. Elute DNA into a fresh tube with 25  $\mu$ l EB applied right on top of the unit's membrane, without touching it; spin 1 min.

### Setting up ligation reactions

In your ice bucket, find tube labeled "pUC19"; this contains plasmid pUC19, cut with *Sma*I and dephosphorylated (for details, see "Plasmid Library Construction" handout).

Prepare 2 microfuge tubes:

In each, and on ice, mix:

- 3  $\mu$ l water
- 4  $\mu$ l pUC19 *Sma*I/CIP (200 ng)
- 2  $\mu$ l 10x T4 DNA ligase buffer
- 1  $\mu$ L T4 DNA ligase

Add 10  $\mu$ l repaired DNA fragments to the first tube. Add 10  $\mu$ l water to the second tube; it will be the "vector only" control.

Mix contents by carefully pipetting up and down a few times.

Incubate overnight at 16°C.

**Day 3: (Fit this in during incubation steps of cDNA protocol.)**

Transfer 15  $\mu$ l from each ligation into a fresh tube and save the remainder on ice.

Pour a 1% agarose gel in TAE (+ ethidium bromide).

Add 3  $\mu$ l of gel loading solution to the 15  $\mu$ l ligation reactions. Load on the prepared gel; also load DNA size markers (5  $\mu$ l).

Run out at 80 V in TAE. When the bromophenol blue has migrated 3/4<sup>th</sup> down the gel, remove gel from gel box and take a photograph.

Use 0.5-1  $\mu$ l of the ligation reactions to transform electrocompetent *E. coli*. Set the voltage to 1800V and use a 1mm cuvette. After delivering the pulse, resuspend the cells in 1 ml of LB broth. Incubate for 30 min at 37°C with very mild agitation. Plate out 25, 50, and 100  $\mu$ l aliquots on LB plates supplemented with 100  $\mu$ g/ml ampicillin, Xgal, and IPTG. Incubate the plates overnight at 37°C.

**Day 4: Pick colonies into deep well blocks for overnight growth and sequencing template preparation.**

Combine Superbroth and ampicillin to 150  $\mu$ g/ml.

Use large-volume 8 channel pipetter to dispense 1.2 ml medium to each well of a 96 deep well block.

Use toothpicks to pick well isolated colonies, leaving toothpick in the well to mark location. Pick as many of the white colonies as possible, but include blue colonies as well.

When finished, remove all the toothpicks and cover the block with an Airpore membrane.

Incubate in shaker at 37°C overnight. They will be centrifuged and stored for you.

## Library Sequencing – Lab Exercises

You will need:

96 well PCR plate for the cycle sequencing reactions

96 well plate containing plasmid templates prepared from your picked clones

Shallow trough to contain your master mix

0.5-10 ul multichannel pipette and two boxes of tips

Reaction components: DMSO, 5X buffer (400mM Tris-HCl, pH9, 10mM MgCl<sub>2</sub>), BDT, water, M13F primer

Make up master mix:

200 ul water

40 ul primer

40 ul 5X buffer

50 ul BDT (0.5 ul/reaction, “1/16<sup>th</sup>” chemistry)

10 ul DMSO

Pipette this into the trough and use the multichannel pipetter to dispense 3 ul to each well of the PCR plate. Tap gently to slide the droplets to the bottom of the well.

With fresh tips, and using fresh tips for each column, transfer 3 ul of template to the corresponding well of the PCR plate. Cover the plate with a rubber mat and give it a quick spin in the tabletop centrifuge to bring all the liquid to the bottom.

Cycle the reactions using either the PE9700 or MJ thermal cyclers using the BigDye60 program.

### **Sequencing Reaction Cleanup:**

Spin plate briefly.

Add 30 $\mu$ l 75% Isopropanol, seal with Costar foil, invert a few times to mix, shake sample to bottoms of wells.

Incubate at room temp for 15 minutes.

Centrifuge at 2800 RCF for 30 minutes.

Remove foil, invert on paper towel, "swirl" inverted plate and paper towel in circular motion on benchtop until most of the IPA wicks out.

**Do not bang/slap/slam/tap inverted plate or pellet could be lost.**

Add 50 $\mu$ l 70% IPA, seal with foil.

Spin at 2000 RCF for 10 minutes.

Remove foil and invert onto fresh paper towels.

With plate inverted on paper towel, spin at 200 RCF for 1 minute.

Air dry for 20 minutes.

Resuspend in 10 $\mu$ l HiDi Formamide.

Spin plate briefly.

Seal with Costar foil. Store in freezer or run on 3730XL immediately.