Shotgun Library Construction

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Plasmid Lysate (this is the Qiagen Plasmid Maxi Kit Procedure with Eric's added notes)**:**

Day 1

1. Streak out the strain of interest onto an LB plate with the appropriate antibiotic selection (if Ampicillin use 100-150μg amp/ml). Incubate plate overnight at 37°C.

Day 2

2. Inoculate 250ml LB/antibiotic (if Ampicillin use 100-150μg amp/ml) with a single colony. Shake ~225-300RPM at 37°C overnight.

Day 3

Prepare DNA using Qiagen Plasmid Maxi Kit. Follow the Qiagen Maxi Kit Protocol for DNA preparation. Be sure to read the Qiagen Handbook because it provides additional comments on the "dos and don'ts" of the procedure.

3. Harvest the cells by pelleting them in 250ml bottles in the SLA1500 rotor at 6000 rpm for 10min at 4°C. Pour off the spent medium. Quick spin cells down to pull down the last bit of medium; remove this with an L1000 or Pasteur pipette.

Note: Most procedures call for centrifugation temperatures of 4°C. This means setting the chamber temperature to 4°C (by adjusting the blue dial) not 0°C. Biological and aqueous samples will freeze if centrifuged at 0°C because the rotor chamber will cool to temperatures below 0°C in order to maintain 4°C rotor temperatures. Note also that if the rotor is at room temperature, it can be quickly cooled to 4°C by centrifuging it at 4°C for 15min and 1000RPM. Be sure to set the over temperature knob (by adjusting the red dial) above ambient temperature otherwise the centrifuge will not operate.

4. Resuspend cells in 10.0ml Qiagen Buffer P1. Transfer the resuspended cells to an Oak Ridge tube.

5. Add 10.0 ml Qiagen Buffer P2. Mix gently but thoroughly by inverting 4-6 times. Incubate at room temperature for 5 minutes maximum.

6. Add 10.0 ml of Qiagen Buffer P3. Mix gently but thoroughly by inverting 4-6 times. Incubate on ice for 20 minutes (an hour or more seems to be fine too).

7. Centrifuge using the SS34 Rotor in the Sorval at 16,000RPM (~20,000 x g) for 30 minutes at 4°C. As soon as the centrifuge has stopped transfer the supernatant (which contains the plasmid DNA) to a 2nd clean and sterile Oak Ridge tube. Leave as much of the white precipitate behind as possible.

8. Centrifuge this 2nd tube using the SS34 Rotor in the Sorval at 16,000RPM (~20,000 x g) for 15 minutes at 4°C. Meanwhile prepare the Qiagen column (next step).

9. Equilibrate a Qiagen-tip 500 by applying 10ml of Qiagen Buffer QBT. Allow this buffer to drain completely from the column by gravity flow.

10. Apply the supernatant containing the plasmid DNA from step 8 to the equilibrated Qiagen column and allow it to enter the resin by gravity flow. Qiagen recommends adding the supernatant immediately; i.e., immediately after the centrifuge has stopped remove the oak ridge tube from the centrifuge and add the supernatant to the column.

11. Once the supernatant has completely passed through the resin, add 30ml of Qiagen Wash Buffer QC. Allow this to completely pass through the resin. Repeat the washing step with a second 30ml volume of Qiagen Wash Buffer QC.

12. Once the supernatant has completely passed through the resin, elute the DNA from the column by adding 15ml of Qiagen buffer QF. Collect this eluate in an Oak Ridge tube (or some other collection vessel) strategically placed below the column.

13. Precipitate the DNA by adding 11ml of isopropanol (2-propanol) to the collected eluate. Mix thoroughly but gently and then immediately centrifuge at 16,000RPM (~20,000 x g) for 30 minutes at 4°C. Carefully, so as to not dislodge the DNA pellet decant the supernatant off of the DNA pellet.

Note: Isopropanol-DNA pellets are often quite difficult to visualize. In addition, isopropanol-DNA pellets do not "stick" to the sides of polypropylene tubes (the Oak Ridge and microfuge tubes that we use in the lab) as do ethanol-DNA pellets. Therefore to avoid loosing the pellet, label the Oak Ridge tube with a piece of colored time-tape and then orient the this tube in the rotor such that the tape is facing the outside wall of the rotor. Then when decanting, keep one eye on the pellet to insure that you don't throw it out. Alternatively, decant the supernatant into a second, clean and sterile Oak ridge tube so you can recover the DNA pellet later if need be.

14. Wash the DNA pellet by adding 5-10ml of 70% ethanol. Invert the tube to wash over the DNA pellet and then centrifuge at 16,000RPM (~20,000 x g) for 15 minutes at 4°C. Carefully decant the 70% ethanol off of the DNA pellet. After decanting the supernatant off of the DNA pellet, give the Oak Ridge tube a 30 second "quick spin." This will bring down all traces of ethanol; remove this with an L1000 pipette.

Note: Ethanol-DNA pellets in general tend to be more opaque whereas isopropanol-DNA pellets tend to be more translucent. Ethanol-DNA pellets also seem to stick better to the

sides of polypropylene tubes; nevertheless, pay attention such that the pellet is not discarded when decanting off the 70% ethanol.

15. Air-dry the pellet by leaving the tube open (uncapped but loosely covered with either a piece of aluminum foil or a kim wipe) on the bench for about 30 minutes (the Qiagen handbook recommends 5-10 minutes, which seems quite short; longer periods are okay too; i.e., overnight). Most importantly all traces of ethanol must be removed because it inhibits later enzymatic manipulation and also causes the DNA to "jump out of the well" when loaded onto an agarose gel.

16. Resuspend the DNA in a total volume of 600µL $T_{10}E_{0.1}$ (or ddH_2O). To insure maximal recovery add 400µl of $T_{10}E_{0.1}$ (or ddH_2O) to the Oak Ridge tube and incubate it in the 65°C water bath for ~10-30 minutes. Give the Oak Ridge tube a 30 second quick-spin to pull all liquid to the bottom of the tube and then transfer this to a microfuge tube. Add an additional 200µl of $T_{10}E_{0.1}$ (or ddH_2O) to the Oak Ridge tube and swirl this around rinsing the sides and then add this to the microfuge containing the first 400µl.

Note: For long-term storage, DNA is best resuspended in TE because it is more stable. However most sequencing facilities prefer that DNA be dissolved in ddH_2O and for short-term storage ddH_2O is perfectly fine. Over time (on the scale of months to years) however, DNA will undergo acid hydrolysis if resuspend in ddH_2O.

17. Determine the DNA concentration by spectrophotometric analysis at OD_{260}. (See Protocol: *L\Methods\SpecOperation* or ask somebody.)

The DNA concentration in µg/ml = (OD260) X 50 X (Dilution factor). Usually the yield of plasmid DNA recovered from a 250ml culture and a Qiagen-tip 500 column from these large insert plasmids ranges from about the very low end of 50µg/ml (30µg total DNA per 600µl) to ~600ug/ml (360µg total DNA per 600µl). To obtain an accurate reading on the spec requires that the OD_{260} reading be between 0.1 and 1.0. Thus diluting the DNA 20-fold (5µl of Plasmid DNA into 95µl of $T_{10}E_{0.1}$ (or ddH_2O)) will result in an OD_{260} reading within the optimal range. To confirm that both the DNA concentration and the construct are what you think they are it is best to digest 0.5µg of plasmid DNA with *Not*I, *Sal*I and/or *Xba*I restriction enzymes, depending on which enzymes were used to create the clone (or all three) and run these, and a known concentration of a molecular weight marker on a ~0.8% regular agarose gel overnight.

Preparative Restriction Digest:
(To obtain sufficient quantities of DNA for hydroshearing purposes)

	For 500µl Reaction Volume
Plasmid DNA	100µg (200µl if the DNA is 0.5µg/µl)
10X NEB Buffer	50µl (1/10 vol)
100X BSA	5µl (1/100 vol)
Enzyme	250U (e.g., if the enzyme is 20U/µl add 12.5µl)
ddH_2O	_____ µl (to a final volume of 500µl)
Incubate at 37°C	~1h
Enzyme	250U
Incubate at 37°C	~2-3h more to overnight

Optional: Heat Kill the Enzyme by incubating in the 65°C water bath for 20 minutes. (The loading dye contains SDS so any residual enzymatic activity will be inactivated when loaded onto the gel.)

Ethanol-precipitate the DNA:
(See: *L\Methods\EthanolPrecipitationOfDNA* for additional technical information on ethanol precipitation of DNA)

Add 50µl of 3M NaOAc (pH ~5.5)
Add 1.0ml 95% Ethanol
Invert to mix
Spin ~2 minutes at RT
Decant off and discard the supernatant
Add 1.0ml 70% Ethanol to wash the DNA pellet
Spin ~2 minutes at RT
Decant off and discard the supernatant
Dry the DNA in the speed vac ~10 minutes
Resuspend in 80µl of $T_{10}E_{0.1}$
Add 20µl of loading dye (be sure that the TE and loading dye is free from any DNA contamination)

Load the DNA sample onto a 0.8% LOW MELTING POINT, 1X TEA gel. Run gel at low volts (~25 to 35V) overnight (20 to 15h), which ends up being ~500V-h.

Notes: The reason for the 500µl restriction digestion volume (instead of 1000µl) is that this leaves room in the microfuge tube to add ethanol and perform the precipitation in a single tube. It should be possible to load the 100µl sample volume (80µl of DNA plus 20µl of loading dye) in aliquots of 50µl into each of 2 adjacent wells using the larger **Idea Scientific** gel apparatuses, the 12 well metal combs and 150ml of gel volume. (Volumes with other gels will require that you do the empirical determinations.) This gel

apparatus also allows for buffer exchange between the two reservoirs; TEA does not have as high a buffering capacity as TBE and so the buffers in separated reservoirs can become polarized leading to the possibility of the gel melting. Low melting point agarose is required because this is the only type of agarose that β-agarase is capable of digesting. However it is also very expensive so it should only be used when β-agarase digestion is required. TEA (aka TAE but made up slightly differently) is the preferred buffer of choice over TBE because TBE can inhibit a variety of downstream enzymatic manipulations of DNA. The overnight run at low volts helps to insure good separation between the desired band to be cut out of the gel and any uncut plasmid that might potentially be carried along. Note also that if you are concerned that the digestion did not work it might be a good idea to run a small percentage of the digestion first (e.g., 0.5µl, which should contain 0.5µg of DNA); however this probably is not essential.

Band-isolation of the insert from low meting point agarose using β-agarase:

Note: Ideally the DNA should not be exposed to short wave μv because this will cause nicking of the DNA. Rather, obtain a hand held μv lamp with long wavelength μv and excise the band from the gel. Probably the best strategy for this is to place the gel onto the μv box (after you have wiped it down with ethanol and are sure it is clean) and cut out the region of the gel containing the band(s) of interest. Once all bands are dissected out, turn on the μv box's lamp and inspect the gel further.

Using a hand held long wavelength μv lamp, determine the region of the gel harboring DNA fragments of interest. The vector should run at 3.2kb. The inserts should be in the upper part of the gel. Note that any uncut DNA will also migrate in the upper part of the gel. Ideally you want to minimize the amount of uncut DNA taken along with the insert of interest. Ask if you are unsure.

Wipe a new razorblade with ethanol. Using this razorblade cut out the upper DNA band.

Transfer the agarose fragment to an appropriate sized container (microfuge tube if volume < 750µl or a 15ml conical tube if volume >750µl.

Quick spin the tube containing the band isolated fragments in agarose in order to pull the agarose down to the bottom of the tube. Approximate the volume of agarose by weighing the tube (a 1.7ml Sarstedt microfuge tube weighs ~0.9g).

Agarose Digestion:

Prior to β-Agarase digestion, the agarose fragment should be equilibrated to 1X β-Agarase buffer. This is outlined below:

Add >2 volumes of 1X β-Agarase buffer (relative to the estimated volume of the gel fragment isolated) to the dissected gel fragment.

Incubate on ice for ~30 min.

Quick spin, and remove the excess buffer.

Repeat with a second volume of β-Agarase buffer.

Incubate on ice for ~30 min.

Quick spin, and remove the excess buffer.

Melt the agarose by incubation at 65^{o}C to 70^{o}C for ~10 minutes. Intermittently gently mix the tube to insure complete agarose melting.

Transfer the tube(s) to the 42^{o}C water bath.

Add 1U of β-Agarase per 200µl of gel.

Incubate at 42°C for 1h.

To insure maximal agarose digestion, add a second 1U of β-Agarase per 200µl of gel about 30-60min into the incubation and incubate another 30-60 minutes.)

Heat-kill the β-agarase by incubating the sample at 65°C for 20 minutes.

If the sample was in a 15 ml conical tube, transfer ~500µl aliquots (or a reasonable volume that partitions the sample) to separate microfuge tubes.

Spin for 15 minutes, 4°C, max RPM in a microfuge to pellet undigested agarose carbohydrates.

Transfer the DNA-containing supernatant to a new microfuge tube.

Ethanol-precipitate*, 70% wash and dry the DNA as before.

Resuspend the DNA in 400µl of $T_{10}E_{0.1}$ (or is ddH_2O better??). Run 8µl of the recovered DNA on a gel to determine recovery. Afterwards, aliquot two 100µl volumes into separate tubes and hydroshear each separately. (Save 200µl of the band isolated fragment in case you need to repeat the hydroshearing process.)

Notes: The β-agarose buffer supplied by NEB (when diluted to 1X) is 10mM Bis Tris-HCl (pH6.5), 1mM Na_2EDTA. Eric routinely uses sterile Tris pH 6.5, 1mM EDTA and incubates at RT (instead of on ice at 4°C). Also note that one advantage of using the larger conical tubes is that 5ml of 1X β-agarase buffer can be added to a gel slice, which should help bring about faster equilibration in the 1X β-agarose buffer. *If the volume of DNA in the microfuge tube is 500µl or less, ethanol precipitation will work fine, if the volume is much greater then >500µl, then isopropanol precipitation is the preferred alcohol for the first precipitation step.

DNA Hydroshearing:
(The Hydroshear apparatus is located at the MCIC in Selby Hall.)

The objective of the Hydroshearing process is to generate fragments in the 1.0 to 2.5kb range, which has been empirically determined for our 9-20kb bacteriophage λ plasmid subclones. To do this, program the Hydroshear machine such that:

Speed code: **3**
Cycle: **20**
Volume: **100µl**

Prepare:
ddH_2O, 0.2N HCl, 0.2N NaOH

Blunting the hydrosheared DNA fragments:

Pool the two duplicate hydrosheared fractions

DNA recovered after Hydroshearing	~200µl
dNTPs (10mM stock that is 2.5mmol each)	20µl
10X T4 Polymerase Buffer	25µl
T4 DNA Polymerase*	20U (~7µl)
H_2O (if needed to bring volume to final volume to 250µl)	__µl

Incubate at 12°C for 20 minutes (lower temperatures favor end-filling while the higher temperatures favor 3'→ 5' Exo activity).

Ethanol-precipitate, 70% wash and dry the DNA after the **Blunting Step** is completed.

Resuspend the DNA in 40µl of $T_{10}E_{0.1}$

Add 10µl of loading dye (again be sure that the TE and loading dye are free from any DNA contamination)

Load the DNA sample onto a 1.0% LOW MELTING POINT, 1X TEA gel. (The larger fragments are best recovered using 0.8% LMP agarose whereas 1% is probably better for the smaller hydrosheared fragments. Run the gel such that good resolution is obtained in the 4.0 to 0.5kb range (25-35V for 20-14hours). The low melt agarose is required for β-agarase enzymatic digestion of the agarose for band isolations. If the Millipore kit will be used, then regular agarose is better.

Notes: After hydroshearing there are several possible and obtainable DNA ends. Those with 5' overhangs, those with 3' overhangs or a combination thereof. Only those with 5' overhangs are capable of being filled in, those with 3' overhangs require the Exo activity of Klenow. Therefore it is essential to use the Klenow* enzyme that retains the 3'→ 5' Exo activity (NEB cat# M0210). Klenow is also a very active enzyme and can create problems when used in excess; i.e., more is not better. Alternatively T4 DNA polymerase can also be used. T4 DNA polymerase has a very active 3'→ 5' Exo activity and for this reason these reactions are best carried out at 12°C. Regardless of which enzyme is used, follow the recommended procedures for either Klenow or T4 Pol, which can be found in either the NEB catalog or Sambrook. Although the hydrosheared fragments should be in the targeted size range (1.0 to 2.5kb), there will be many quite small fragments present in this population of fragments. Small fragments have a much higher success rate when it comes to ligation to the vector. Therefore they must be eliminated by an additional size selection through gel isolation step.

Size-Selection of Hydrosheared DNA using Electrophoresis:

Note: As with the band-isolation of the large inserts, the DNA should not be exposed to short wave μv because this will cause nicking of the DNA. Rather, obtain a hand held μv

lamp with **Long Wavelength** μv and excise the desired region from the gel. (The hand held μv lamp belonging to Clay Sneller's lab seems to be a little better than our hand held μv lamp for detecting this size class of DNA. However it has a sliding screen that lets through either long wave or short wave μv, so beware; i.e., be sure to us long wave μv. Again probably the best strategy for this is to place the gel onto the μv box (after you have wiped it down with ethanol and are sure it is clean) and cut out the region of the gel containing the hydrosheared sizes of interest. If there are multiple lanes containing different hydrosheared fragments, it might be easiest to load two lanes containing markers towards the gel's outside lanes and simply slice the entire region above (>2.0kb) and below (<1.0kb) the desired fragment sizes and separate the above and lower portions from the desired region. Then simply trim to size the lane with the DNA. Once all bands are dissected out, it might be a good idea to reconstruct the gel (minus the dissected regions), turn on the μv box's lamp and photograph the gel for future reference. If the hydrosheared DNA is not exactly the right size, then isolate the next best size class that exists. Realize the most important thing is to eliminate the small stuff that ligates much more efficiently than the larger stuff. Note also that for band isolation either a strategy using β-agarase digestion of the agarose or the Millipore gel Extraction kit can be employed. However the β-agarase digestion will probably result in greater yields because of the relatively large size of the gel region being dissected.

<u>β-Agarase Digestion of the Agarose</u>:

Prior to β-Agarase digestion, the agarose fragment should be equilibrated to 1X β-Agarase buffer. This is outlined below:

Add >2 volumes of 1X β-Agarase buffer (relative to the estimated volume of the gel fragment isolated) to the dissected gel fragment.

Incubate on ice for ~30 min.

Quick spin, and remove the excess buffer.

Repeat with a second volume of β-Agarase buffer.

Incubate on ice for ~30 min.

Quick spin, and remove the excess buffer.

Melt the agarose by incubation at 65^{o}C to 70^{o}C for ~10 minutes. Intermittently gently mix the tube to insure complete agarose melting.

Transfer the tube(s) to the 42^{o}C water bath.

Add 1U of β-Agarase per 200µl of gel.

Incubate at 42^{o}C for 1h.

To insure maximal agarose digestion, add a second 1U of β-Agarase per 200µl of gel about 30-60min into the incubation and incubate another 30-60 minutes.)

Heat-kill the β-agarase by incubating the sample at 65^{o}C for 20 minutes.

If the sample was in a 15 ml conical tube, transfer ~750µl aliquots (or a reasonable volume that partitions the sample) to separate microfuge tubes.

Spin for 15 minutes, 4^{o}C, max RPM in a microfuge to pellet undigested agarose carbohydrates.

Transfer the DNA-containing supernatant to a new microfuge tube.

Ethanol-precipitate, 70% wash and dry the DNA.

Resuspend the DNA in 10µl of $T_{10}E_{0.1}$

Note: If the volume of DNA in the microfuge tube is 500µl or less, ethanol precipitation will work fine, if the volume is much greater then >500µl, then isopropanol precipitation is the preferred alcohol for the first precipitation step.

Montage DNA extraction Kit:

Note: This is simply a 10min spin followed by an ethanol-precipitation. The recovered yields will probably be lower than those obtained by β-agarase digestion and several Montage DNA gel extraction devices will be required because the maximum recommended gel slice is 100µl. Use of this kit is not recommended for quantitative recovery of the large inserts in the previous band isolation step.

Ethanol-precipitate, 70% wash and dry the DNA after the 10minute spin.

Resuspend the DNA in 10µl of $T_{10}E_{0.1}$

Vector Preparation:

This is an expensive reaction and requires some skill. If you only need a small amount of vector, ask another person in the lab if you can get some vector DNA from them. If you need a lot of vector then take your time and do this right because cut, dephosphorylated vector will store indefinitely at –20°C in TE and is an invaluable resource to have. The importance of starting with the highest quality plasmid DNA for cloning purposes cannot be overemphasized. The use of $CsCl_2$ isopycnic centrifugation produces the best quality DNA for vector preparation; Qiagen columns are second best but are much quicker and require less skill.

Set up the restriction digest for the plasmid vector.

In this case it is preferable to cut a fairly large quantity of DNA (25-30µg) in a larger and convenient volume; e.g., 150-200µl. Since it is very important to cut the DNA to completion, use an excess of restriction enzyme; e.g., to cut 25µg of DNA use 250 units of enzyme; add 125U at the beginning of the digestion period and another 125U around half way through the digestion period. Often however the use of excess restriction enzyme can lead to other problems. Therefore it is best to review the properties unique to each restriction enzyme; i.e., read the blurb provided in the NEB catalog.

	150µl Reaction Volume
ddH_2O	__µl (to bring final volume to 150µl)
Plasmid DNA	__µl (25µg)
10X NEB Buffer	15µl (1/10 vol)
100X BSA	1.5µl (1/100 vol)
Restriction Enzyme	12.5µl (125U if Enzyme is 10U/µl) DNA
Incubate at appropriate temp	~1h
Add a second aliquot of restriction enzyme	12.5µl (125U if Enzyme is 10U/µl) DNA
Add CIP	__µl (use 0.5U/µg vector DNA whether 5' overhangs or blunt ended fragments).
Incubate at appropriate temp	~2-3h

Phenol Extract the DNA following the protocol "Phenol Extraction and Ethanol Precipitation of DNA" (See: *L\MethodsPhenolExtraction*)

Resuspend the DNA in a volume that will allow you to load the sample into one or two lanes on a gel.

Add 2µl of Loading Dye Buffer per 10µl of DNA sample. (Store the sample at –20°C if you are not going to load onto a gel right away.)

Load onto a 1% Agarose (Regular agarose), TEA gel and electrophorese for 2-4 hours at ~50V.

With the agarose gel still sitting on the gel casting tray and using the handheld μv lamp and a razor blade, dissect just the linearized plasmid DNA band away from all other contaminating plasmid fractions (open circular and supercoiled). Be careful not to slice up the gel-casting tray. Discard everything from the gel-casting tray except the desired band.

Set the gel slice up on the casting tray as it was prior to dissection and pour a 1.0% LOW MELTING POINT, 1X TEA gel around this band.

Electrophorese the plasmid DNA band out of the regular agarose and into the LMP agarose.

Dissect the plasmid band from the LMP agarose and recover the DNA using β-agarase digestion (same procedures apply here as above for β-agarase digestion of agarose).

Ethanol precipitate the DNA by adding 1/10 volume 3M NaOAc pH ~5 and 2.5 volume EtOH. Spin, wash and dry plasmid.

Resuspend the plasmid DNA in 600µl and perform a phenol-extraction. NEB states that any residual digested agarose carried along with the DNA should not interfere with ligations. (Eric does this extra phenol extraction step, however it may be optional; but realize that Eric obtains 95% of his clones with inserts)

Ethanol precipitate the DNA by adding 1/10 volume 3M NaOAc pH ~5 and 2.5 volume EtOH. Spin, wash and dry plasmid.

Resuspend the DNA in a convenient volume (i.e. 0.2 µg/µl)

Run an aliquot (~0.5µg) on a gel to confirm complete digestion and recovery of the plasmid DNA after the phenol extraction and EtOH precipitation.

Notes: Since CIP can be added directly to a restriction digest (the recommended NEB buffer is NEB buffer #3, however NEB indicates that buffers #2 and # 4 work equally well), it is easiest to just add the CIP at the halfway point in the digestion period when the second aliquot of enzyme is added. At the conclusion of the incubation it is absolutely essential to remove all traces of CIP from the reaction. The surest way to do this is to phenol extract the sample prior to ethanol precipitation and electrophoresis. One could phenol extract after the band isolation step but since an EtOH precipitation step is required to bring the DNA down to a volume that can be loaded on a gel, the extra time spent with the phenol extraction serves as added insurance that all traces of CIP are eliminated. Some procedures also recommend adding 1/10 volume 0.5M EGTA and incubating the CIP reaction at 65^{o}C for 45 min (which inactivates the enzyme) prior to phenol extraction (which denatures the protein) however this step is probably not necessary. **NEVER** use old phenol. If the phenol has been at 4^{o}C more than 3 months toss it out and prepare fresh. (Phenol is generally buffered against TE and can be stored

indefinitely at –20^{o}C and aliquots pulled out of the freezer for regular daily use.) The reason for electrophoresis of the restriction-digested plasmid DNA through regular agarose the first time is that there is much better resolution between the various plasmid DNA forms including: closed circular plasmid DNA (nicked), linearized plasmid DNA and supercoiled (completely undigested) in regular agarose over LMP agarose. For a complete description of this strategy and its methodology see the section GEL PURIFICATION OF THE *Eco*RI DIGESTED pT7T3-Pac DNA in the CSHL press book *Genome Analysis, A laboratory manual, Volume 2, Detecting genes*, p92-92. This vector DNA can be stored for many years at –20^{o}C; simply pull it out of the freezer and use it in a ligation.

Ligation:

Insert DNA	2.5µl
Blunt Cut Vector (e.g. *Hinc*II or *Sma*I), ΔP, GI	1.0µl (20ng/µl)
10X NEB ligation buffer	0.5µl
T4 Ligase	1.0µl

RT 4h or 16^{o}C O/N

Competent Cells:

Use commercially available XL1-Blue ultracompetent cells (Stratagene, San Diego CA) for the highest transformation efficiency.

Transformation:

Transform 1-2µl of the ligation mix into commercially available XL1-Blue ultracompetent cells (Stratagene, San Diego CA).

(See appropriate protocol; i.e., for transforming $CaCl_2$ competent cells see: *L\Methods\CaCl$_2$Transformation* or for electroporation see: *L\Methods\Electroporation*.

Determination of the Shotgun Library Quality:

Determine the insert size and % of clones harboring inserts by either mini prep or PCR. A high quality library should have inserts of the expected size in ~90% of the clones.

Plasmid Mini Preps:

1. Isolate mini prep DNA from 12-24 while colonies. (See: *L\Methods\PlasmidMinis*.)

2. Digest plasmid DNA using *Eco*RI and *Hind*III. Although NEB recommends 10X NEB buffer for *Eco*RI, I think NEB 10X buffer #2 is a better choice. Be sure to include RNase in the reaction.

3. Electrophorese restriction digests and determine insert size and % of clones harboring inserts. A high quality library should have inserts of the expected size in ~90% of the clones.

PCR screening:

1. Add 20µl of ddH_2O to the appropriate number of 0.2ml PCR tubes; label these. Prepare a grid on an LB_{AMP}/X-Gal/IPTG plate to match the labeling of the 0.2ml PCR tubes.

2. Using sterile technique and a sterile toothpick, touch a single white colony with the toothpick. Touch the toothpick first to the labeled position on the template grid plate and then transfer the toothpick to the 0.2ml PCR tube. Place the plate in the 37^{o}C incubator overnight

3. Place the labeled PCR tubes into the Robocycler; activate Robocycler Program #81, which will boil the samples for the appropriate length of time. (These tubes are probably very expensive; can we use tubes that will work in either the Tetrad or a heat block?

4. Set up the PCR reaction:

(a) Transfer the boiled sample to a second PCR tube	5µl
(b) Add 10X Taq buffer	5µl
(c) Add M13Forward Primer (E81) (1pmol/µl)	10µl
(d) Add M13Reverse Primer (E110) (1pmol/µl)	10µl
(e) Add dNTPs (10mM stock; 2.5mmol each dNTP	4µl
(f) Add ddH_2O to bring final volume up to 50µl	15µl
(f) Taq Polymerase	1µl
(g) Run Robocycler Program #82.	

5. Electrophorese a 5µl portion of the PCR product to determine insert size and % of clones harboring inserts. A high quality library should have inserts of the expected size in ~90% of the clones.