# Bacteriophage λ Plant Genomic Library Construction

## I. Preparation of the insert DNA

Starting DNA should be of high molecular weigh (>50kb) and should be completely restriction digestible. Check the size and quality of DNA by running an aliquot of uncut DNA next to an *Eco*RI cut portion on a 0.4% agarose, 1X TEA gel overnight at 20 –30 V.

**Perform a partial digestion of the Genomic DNA.** (If using the λFixII vector kit from Stratagene in which the λ DNA has been digested with *Xho*I and partially filled in with dCTP and dTTP, then cut the DNA with *Mbo*I, *Sau*3A, *Bgl*II or *Bam*HI.)

### Pilot *Sau*3A Digestion

Prepare a DNA master mix cocktail consisting of:

| | Per Rxn | For 6 Rxns |
|---|---|---|
| DNA (~2.5µg) | 12.50µl**** | 75.0µl |
| 10X NEB *Sau*3A I Buffer | 1.5µl | 9.0µl |
| 10mg/ml BSA | 0.15µl | 0.9 |
| 10mg/ml RNase A | 0.15µl | 0.9µl |
| Mix gently | | |

(**** The Genomic DNAs used were approximately 2µg per 10µl or 0.2µg per µl.)

Dilute the *Sau*3A I restriction enzyme to 0.1U/µl (10X dilute as that suggested for the full scale partial digestions) in storage buffer.

| Stock | | Vol |
|---|---|---|
| *Sau*3A | (4U/µl) | 1.0µl |
| Buffer | (10X) | 4.0µl |
| dd$H_2O$ | | 35.0 |

**_Sau_3A I storage buffer**

10mM Tris ($_{pH\ 7.4}$)
50mM KCl
1mM DTT
0.1mM EDTA
200µg/ml BSA
50% glycerol

Suggested *Sau*3A I dilution series (dilutions are given in Units of *Sau*3A I per µg DNA in the final reaction mix):

| Diln: | 0.05U/µg | 0.10U/µg | 0.15U/µg | 0.20U/µg | 0.25U/µg | 0.30U/µg |
|---|---|---|---|---|---|---|
| Units: | (0.125U) | (0.25U) | (0.375U) | (0.50U) | (0.625U) | (0.75U) |
| Vol: | 1.25µl | 2.5µl | 3.75µl | 5.0µl | 6.25µl | 7.5µl |

Label 6 tubes according to the enzyme dilution

Add the appropriate volume of enzyme to each.

Aliquot 14.3µl DNA cocktail to each of the six tubes.

Mix gently.

Incubate at 37°C for 1.25h.

Add 4µl Loading Dye (the EDTA and SDS stop the reaction).

Load onto a 0.8% TEA agarose gel.

**5X Gel loading buffer with dye**
(Good for just about all applications)

| | 50ml | 5X Conc |
|---|---|---|
| 80% Glycerol | 47ml | 75% |
| Bromophenol Blue | 125mg | 0.25% |
| Xylene Cyanol | 125mg | 0.25% |
| 1M Tris (pH.7.4) | 0.5ml | 10mM |
| 5M NaCl | 0.1ml | 10mM |
| 0.5M EDTA | 1.0ml | 10mM |
| 10% SDS | 0.5ml | 0.1% |

The general rule of thumb is to use 1µL of gel loading buffer dye per 5µL of sample.

Run gel overnight for 500V-h (30-35V for ~14-16h, on a standard sized gel).

# Bacteriophage λ Plant Genomic Library Construction

## *Sau*3A Digestion

**Full-scale partial digestions**

Prepare a DNA master mix cocktail consisting of:

| | Per Rxn | Per 5 Rxns |
|---|---|---|
| DNA (~25µg) | 132.0µl | 660µl |
| 10X NEB *Sau*3A Buffer | 15.0µl | 75µl |
| 10mg/ml BSA | 1.5µl | 7.5µl |
| 10mg/ml RNase A | 1.5µl | 7.5µl |
| Mix gently | | |

Dilute the *Sau*3A restriction enzyme to 1.0U/µl in storage buffer.

| Stock | | Vol |
|---|---|---|
| *Sau*3A | (4U/µl) | 22.5µl |
| Buffer | (10X) | 9.0µl |
| dd$H_2O$ | | 58.5µl |

*Sau*3A dilution series based on empirical data from pilot digestions and previously constructed libraries in which 0.15U/ug to 0.20U/ug seemed to be quite optimal. The size and quality of the starting DNA material will play a role in what is the ideal enzyme concentration per µg of DNA.

| Diln: | 0.05U/µg | 0.10U/µg | 0.15U/µg | 0.20U/µg | 0.25U/µg |
|---|---|---|---|---|---|
| Units: | (1.25U) | (2.50U) | (3.75U) | (5.0U) | (6.25U) |
| Vol: | 1.25µl | 2.5µl | 3.75µl | 5.0µl | 6.25µl |

Label 5 tubes, one for each dilution.

Add the appropriate volume of enzyme to each.

Aliquot 150.0µl of the genomic DNA cocktail to each of the five tubes.

Mix gently.

Incubate at 37$^{o}$C for 1.25h.

Heat-denature the *Sau*3A enzyme at 65$^{o}$C for 20 minutes.

(Stopping Point; freeze the samples)

**B. Perform a partial end fill with dGTP, dATP and Klenow**

There should be approximately 25µg of *Sau*3A digested DNAs in 150µl. If the concentration of DNA is substantially less, reduce the amount of Klenow proportionally (see the cautions regarding the use of excess Klenow to DNA ratios below).

Add to that:

| | Concentration in Reaction | | |
|---|---|---|---|
| (1) 5µl 10mM dGTP | 333µM | | |
| (2) 5µl 10mM dATP | 333µM | | |
| (c) 15U Klenow | 15U/25µg | Klenow at 50U/µl | 5.0µl |
| (d) Mix gently | | 10X NEB Pol buffer | 50.0µl |
| | | $ddH_2O$ | 445.0µl |
| | | Aliquot per reaction: | 25µl |

(e) Incubate at RT for 15min

(f) Phenol-extract the sample (See Phenol Extraction and EtOH precipitation of DNA for complete details on phenol extraction and Ethanol Precipitation of DNA).

In this instance however one must be gentle so as to avoid shearing the DNA, which may result in fragments that are of the same size as that which are to be extracted from the gel but with non-ligatable ends. If fact it is probably wise if an aliquot; e.g., 10% is removed prior to phenol extraction and ethanol precipitation in order to assess if any damage has been done to the DNA. Then run this on another area of the same gel as the DNA you plan to cut out of the gel.

(g) Extract once with an equal volume of phenol (See Phenol Extraction and EtOH precipitation of DNA for complete details on phenol extraction and Ethanol Precipitation of DNA). Note also that this step may be unnecessary as a single phenol extraction may be sufficient.

(h) EtOH-precipitate the sample (See Phenol Extraction and EtOH precipitation of DNA for complete details on phenol extraction and Ethanol Precipitation of DNA).

(i) Resuspend in 25-40ul TE
(j) Add 5µl Loading Dye

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**Notes, recommendations and cautions from NEB about END FILLING with Klenow:**

**To inactivate the polymerase, NEB recommends phenol/chloroform extraction followed by alcohol precipitation of the DNA. Alternatively, a heat-inactivation can be effectively performed at 75°C for 20 minutes, but EDTA should be added to a final concentration of 10mM beforehand, in order to prevent the exonucleic activity of the polymerase from destroying the blunt ends.**

**Caution: too much enzyme, temperature higher than 25C, or reaction time longer than 15 minutes can result in 'overdoing' the reaction. This is the most common source of dissatisfactory result and should be avoided.**

**It is important that the deoxynucleotides are added to the reaction before adding the Klenow enzyme.**

**Regarding the CAUTION in the reaction conditions shown on page 81 of the 2000/01 NEB catalog:**

**NEB has empirically determined that the quantity of Klenow recommended in the protocol is optimal for producing the highest percentage of products with the desired blunt ends (which is not exactly what we are doing, because we are only performing a partial fill in). The goal of this Klenow reaction is to bring the rates of polymerization and exonucleic chew-back into equilibrium. An excess ratio of Klenow to DNA substrate**

**causes the exonucleic activity of Klenow to predominate. NEB has also determined that reaction temperatures higher than 25°C will yield a lower percentage of the desired blunt ends. Higher temperatures tend to unwind the ends of the DNA helix. The unwound helix appears to Klenow as single stranded DNA and, therefore, will be chewed back beyond the blunt end at temperatures higher than 25°C.**

**C. Band isolation of the insert using low meting point agarose and β-agarase.**

Load DNA onto a 0.8% LOW MELTING POINT Agarose/1X TEA gel along with a λ *Hin*dIII MW standard on an outside lane (or both outside lanes). Use a gel box in which there is buffer exchange between the two reservoirs otherwise the buffers become polarized and the gel will melt. TEA is the recommended buffer of choice when isolating fragments from gels but because it does not a high buffering capacity the buffers in separated reservoirs can become polarized. LMP Agarose is very expensive so use it sparingly.

Run gel overnight at ~30V (~500V-h)

**Note: Ideally the DNA should not be exposed to short wave *uv* because this will cause nicking of the DNA. Rather obtain a hand held *uv* lamp with <u>long wavelength</u> *uv* and excise the 9 to 23kb region of gel and then photograph the gel after you have removed the desired bands as outlined below:**

Using a hand held long wavelength *uv* lamp, determine the region of the gel harboring DNA fragments 9 to 23kb in size; i.e., between the 9.6 and 23kb λ bands.

Wipe a new razorblade with ethanol. Using this razorblade cut out the DNA fragments from each lane 9 to 23kb in size.

Transfer these fragments to an appropriate sized container (microfuge tube if volume < 750µl or a 15ml conical tube if volume >750µl. Pool all samples from the different lanes that are the correct size.

Photograph the gel after band dissection for a record of the DNA smear isolated.

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 and/or weight the tube (a 1.7ml Sarstedt microfuge tube weighs ~0.9g).

**<u>Agarose Digestion</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°C to 70°C for ~10 minutes. Intermittently gently mix the tube to insure complete agarose melting.

Transfer to the 42°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 1h 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 ~750µl 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.

**Isopropanol precipitate the DNA:**

Add 1/10 volume of 3M NaOAc pH6
Add 0.7X volume isopropanol
Spin for 2min at RT at max RPM in a microfuge
Decant off the supernatant.
Wash the DNA pellet by adding 1ml 70% EtOH, and invert several times.
Spin RT 2min.
Decant off the EtOH washate.
Quick spin the tube to pull all remaining ethanol down to the bottom of the tube.
Remove this ethanol with a pipette (without disturbing the DNA pellet).
Air dry the DNA pellet ~15-30 minutes on the bench top (cover it with foil or a kim wipe)
(Alternatively minimally dry the DNA pellet in speed vacuum; i.e., 5-7 min.)
Resuspend each DNA pellet in a volume of $T_{10}E_{0.1}$ that when all samples are combined will total approximately 500µl; i.e., if there are 3 microfuge tube all containing the same DNA, resuspend each in ~167µl.
Incubate at 65°C with intermittent gentle finger vortexing to insure complete resuspension of the DNAs.
Combine all resuspended DNAs for phenol extraction.

**Phenol extract the band isolated DNA:**

(The purpose of the above Isopropyl/EtOH precipitation prior to the following phenol extraction procedure is to concentrate the DNA into a volume suitable for phenol extraction in a microfuge tube. Butanol concentration of the DNA (See Sambrook) seems to cause shearing and is not recommended.) **(See Phenol Extraction and EtOH precipitation of DNA for complete details on phenol extraction and Ethanol Precipitation of DNA.)**

**Note: In all subsequent steps be gentle with the DNA; i.e., gentle finger vortex only.**

Add 400µl of TE (pH~7-8) saturated phenol to the 400µl of DNA.
Flick the tube multiple times (but be gentle) to insure complete emulsification between the aqueous and organic layers.
Spin for 2min at RT at max RPM in a microfuge.
Pull the upper aqueous layer to a new microfuge tube. Avoid the interphase.
Add 200µl TE saturated phenol and 200µl chloroform/Isoamyl alcohol.
Flick the tube multiple times as before.
Spin for 2min at RT at max RPM in a microfuge.

Pull the upper aqueous layer to a new microfuge tube.
Add 400µl of chloroform/Isoamyl alcohol.
Flick the tube multiple times as before.
Spin for 2min at RT at max RPM in a microfuge.
Pull the upper aqueous layer to a new microfuge tube.
Add 1/10 volume of NaOAc.
Add 2X volume 95% EtOH.
Spin for 2min at RT at max RPM in a microfuge.
Decant off the supernatant.
Wash the DNA pellet by adding 1ml 70% EtOH, and invert several times.
Spin RT 2min.
Decant off the EtOH washate. Blot the edge of the tube dry with a kim-wipe.
Dry pellet in speed vacuum 5-10min.
Resuspend the DNA pellet in 25µL $T_{10}E_{0.1}$.
Run an aliquot on a 0.75% gel to insure recovery, confirm size of bands extracted and estimate DNA concentration (confirm DNA concentration by spec analysis).
DNA should be about 1µg per 2.5µl.
This DNA will now serve as the insert for ligation to the λ arms.

## II. Ligation of the insert DNA to λ Phage DNA

Thaw the λ DNA on ice. There is 10µl at 1µg/µl of λ DNA provided by Stratagene. (Since it is probably best not to freeze-thaw the λ vector DNA multiple times, it might be best to aliquot it into convenient volumes for use later.)

| | |
|---|---|
| $ddH_2O$ | |
| λ FIX II *Xho*I predigested DNA | 1.0µl (1µg) |
| Insert DNA (~1µg per 2.5µl)** | 2.5µl (~1µg) |
| 10X NEB Ligase Buffer | 0.5µl |
| NEB T4 DNA Ligase | 1.0µl |

**Control Ligation (which indicates the Klenow reaction worked preventing the insert from self-ligation):**

| | |
|---|---|
| $ddH_2O$ | 3.5µl |
| Insert DNA | 1.0µl |
| 10X NEB Ligase Buffer | 0.5µl |
| Pull for Pre Ligation gel | 2.0µl |
| NEB T4 DNA Ligase (400U/µl) | 1.0µl |

Incubate ligations overnight at 16°C. The Stratagene protocol recommends incubating at 4°C overnight. I don't know if the 4°C temperature is chosen because it results in more efficient ligation between the λ vector and the insert. I have always used 16°C (and oftentimes RT) and besides, the NEB quality control is a 30 minute ligation at 16°C using λ *Hae*III digested DNA test.

Store the ligated reaction mix at –20°C.

**Stratagene recommends a 1:1 molar ratio of vector cohesive termini to insert cohesive termini. This will depend upon the size of the insert DNA. If all fragments are 20kb then they recommend 0.4µg of insert to 1.0µg of λ FIX II. If you are unsure about vector to insert molar ratios, set up multiple ligations to the λ arms using different concentrations of insert DNA. Then package each ligation independently.

## III. Packaging

Remove the appropriate number of packaging extracts from the –80°C. **(Stratagene recommends 1.0 to 4.0µl of ligated DNA per packaging reaction. Since the ligations were in 5.0µl, package ½ or 2.5µl; the unused portion serves as a backup that should be stored at –20°C that can be retrieved later if necessary.)**

Quickly thaw the packaging extract by holding the tube between your fingers until the contents of the tube just begins to thaw.

**Immediately** add 2.5 µl of ligated DNA to the packaging extract.

**Gently** stir the contents of the tube with a pipette tip. Gentle pipetting is okay too.
**However, DO NOT introduce air bubbles!**

Quick spin the tube for 3-5 seconds to insure that all the contents of the tube are at the bottom.

Incubate at RT (22°C) for 2 hours. Do not exceed 2 hours. Maximal packaging efficiency occurs between 90 minutes and 2 hours. After about 2 hours the efficiency begins to drop and can drop dramatically after extended incubations.

Add 500µl of SM buffer to the packaged DNA.

Add 20µl of chloroform and **GENTLY** mix the contents of the tube.

Spin the tube briefly to sediment the debris.

The supernatant containing the phage is ready for titering. Store at 4°C for up to 1 month.

## IV. Titering the Library

### A. Growth & Preparation of the Host Strains

Streak out E. coli strain XL1-Blue MRA (P2) onto an NZY plate (LB will work too). Invert the plate and incubate overnight at 37°C. **(When performing secondary/tertiary screens or plate lysates use XL1-Blue MRA.)**

Pick a single colony of XL1-Blue MRA (P2) into 5ml of NZY supplemented with maltose (to 0.2% w/v) and grow at 30°C overnight with shaking at ~225 rpm (or at 37°C for about 4-6 hours or until $OD_{600}$ = 1.0).

Spin the cells down and decant the spent medium off of the cell pellet.

Resuspend the cell pellet in sterile 10mM $MgSO_4$ using about ½ the volume of culture medium. Determine the $OD_{600}$ of the resuspended pellet. Dilute the cells to $OD_{600}$ = 0.5.

**B. Preparation of the λ Bacteriophage**

Meanwhile prepare a serial dilution of the packaged λ bacteriophage in SM buffer. Stratagene states that 5 X $10^5$ to 1 X $10^7$ recombinant plaques should form. After SM addition there should be ~0.5 ml. So, this roughly translates to 1 X $10^6$ to 2 X$10^7$pfu/ml or about 1000 to 20,000pfu/µl. Prepare dilutions accordingly in order to attempt to flank that number in the dilutions plated.)

| | Dilutions | | | |
|---|---|---|---|---|
| | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ |
| λ Phage | 2.5µl (packaging reaction) | 2.5µl ($10^{-1}$) | 100µl ($10^{-2}$) | 100µl ($10^{-3}$) |
| SM | 22.5µl | 22.5µl | 22.5 | 22.5µl |

**Note:**

When preparing serial dilutions of bacteriophage, it is usually much more accurate to dilute 10µl into 100 to create a $10^{-1}$ dilution or 10µl into 1000µl to create a $10^{-2}$ dilution rather than to dilute 1 into 10µl or 1µl into 100µl because of pipetting error. This is particularly critical for the first dilution. (Using 2.5µl of a valuable packing reaction is a compromise.)

Add 200µl of XL1-Blue MRA (P2) cells at $OD_{600}$ = 0.5 to individual sterile culture tubes (the number of bacteriophage dilutions you plan to plate) in a test tube rack.

Add 10µl of each serial dilution of λ bacteriophage to the culture tubes containing the XL1-Blue MRF′ cells.

Place the test tube rack into a 37°C water bath for 15 minutes to allow the λ phage to attach to the cells.

Meanwhile melt NZY Top Agarose in the microwave and allow it to cool to ~48/50°C.

At the conclusion of the 15-minute incubation, add ~3 ml of the NZY top agarose to the test tube containing the λ phage & E.coli. Remove the tube from the rack (which is still sitting in the water bath) and give it a quick flick of your wrist mixing the contents and immediately pour it onto an NZY plate. (I usually do three plates at one by pipetting 10ml of the NZY top agarose from the bottle, dispensing ~3.3 to one tube, ~3.3 to second tube and then another ~3.3 to a third tube. I then pour the plates starting with the first tube I put the NZY into.)

Set the plate onto a level surface.

Allow the top agarose to cool (1~5 minutes).

Invert the plate and incubate overnight at 37°C.

Count the number of plaques.

Determine the titer; i.e., pfu/ml using the formula:

$$\frac{(\text{Number of plaques (pfu)} \times \text{Dilution Factor}) \times 1000\text{ul/ml}}{\text{Volume plated (µl)}}$$

Where the volume plated (in µl) refers to the volume of the λ bacteriophage solution added to the cells.

**Notes:** Several steps greatly aid the ease and success of poring good phage onto plates:

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(1) Position the 37°C and 48°C water baths right next to each other with enough room in front of them for you to work.

(2) Use NZY plates that have been prewarmed to 37°C

(3) Use sterile glass pipettes that have been prewarmed to ~50°C.

## V. Amplifying (Plating) the Library

Generally the primary library is amplified to generate a secondary library. However if you time things well, you can also screen the primary library at the same time that you amplify it. After plating and recovery of the amplified library, we have maintained the primary library plates at 4°C for several years and are still able to recover phage. Other microbes may eventually overtake the phage on the plate so if probes and other things are not ready to go, it would be better to just amplify the library and screen the amplified library. An idealized timetable for screening and amplifying is presented below:

Day 1 Plate out the entire packaged library as it was being amplified.

Day 2 Perform plaque lifts on the plated library, bake nylon membrane filters

Day 3 Pre-prehybridization and Prehybridization

Day 4 Hybridization

Day 5 Washes

Day 6 Develop autoradiograms

Day 7 Core plaques of interest

Overlay SM buffer onto remainder of phage on plate. Process as if these were from a normal amplification procedure. Store at +4°C until completion of the 2nd ary screen.

Day 8 Plate eluted phage from cored plaques (this is the secondary screen).

Day 9 Perform plaque lifts on the 2nd ary screen.

Day 10 2nd ary screen pre-prehybridization and prehybridization.

Day 11 2nd ary screen hybridization.

Day 12 2nd ary screen washes.

Day 13 Develop 2nd ary screen autoradiograms.

Day 14 Core 2nd ary screen plaques of interest

Combine phage eluate from all of the original **PRIMARY** screen's cored plaques. Process these combined eluates as for amplification. Add this eluate to the remainder of amplified library. Add DMSO. Aliquot the amplified library into convenient volumes and freeze these at –80°C for long-term storage.

# Bacteriophage λ Plant Genomic Library Construction

**Primary screen and amplification**

**Growth & Preparation of the Host Strains**

Streak out E. coli strain XL1-Blue MRA (P2) (and XL1-Blue MRA) onto an NZY plate (LB will work too). Invert the plate and incubate overnight at 37°C.

Pick a single colony of XL1-Blue MRA (P2) into 5ml of NZY supplemented with maltose (to 0.2% w/v). NZY contains Mg, which is required for phage adhesion to the maltose binding protein of E. coli and maltose induces expression of this protein. Grow at 30°C overnight with shaking at ~225 rpm (or at 37°C for about 4-6 hours or until $OD_{600}$ = 1.0).

Spin the cells down (use the clinical centrifuge on setting # 5 for 5 to 10 minutes) and decant the spent medium off of the cell pellet.

Resuspend the cell pellet in sterile 10mM $MgSO_4$ using about ½ the volume of culture medium. Determine the $OD_{600}$ of the resuspended pellet. Dilute the cells to $OD_{600}$ = 0.5.

**Preparation of the λ Bacteriophage**

Presumably the titer of the original library has already been performed. From this number determine how many µl of packaged library will yield 5 x $10^4$ pfu (50,000). This is the number of phage that can be plated onto one large (137mm) petri dish plate.

Hopefully the titer of the primary library is around $10^6$. This means that 20 large plates will be required to plate the entire library.

Add 600µl of XL1-Blue MRA (P2) cells at $OD_{600}$ = 0.5 in sterile 10mM $MgSO_4$ to individual sterile culture tubes (the number of plates you plan to plate) in a test tube rack.

Add 50,000 pfu λ bacteriophage to each culture tube containing the XL1-Blue MRA (P2) cells.

Place the test tube rack into a 37°C water bath for 15 minutes to allow the λ phage to attach to the cells.

Meanwhile melt NZY Top Agarose in the microwave and allow it to cool to ~48/50°C.

At the conclusion of the 15-minute incubation, add ~6.5-7.5 ml of the NZY top agarose to a test tube containing the λ phage & E. coli. Remove the tube from the rack (which is still sitting in the water bath) and give it a quick flick of your wrist mixing the contents and immediately pour it onto an NZY plate.
**Note:** I usually do three plates at one by pipetting 25ml of the NZY top agarose from the bottle, dispensing ~7.5ml NZY top agarose to one tube, ~7.5ml to second tube and then another ~7.5ml to a third tube. I then pour the plates starting with the first tube I put the NZY into.)

Pour onto freshly prepared NZY plates (**use only freshly prepared plates for amplification**).

Allow the top agarose to cool (1~5 minutes).

Invert the plate and incubate 6-8 hours at 37°C. Ideally lysis should proceed only long enough such that each individual phage plaque just starts to come into contact with the neighboring plaques.

**Plaque lifts**

Label each plate with a designation; i.e., #1, #2… #n.

Chill the plates at 4°C for ~2h (this helps keep the layer of top agarose from peeling off of the plate's agar layer when the filter membranes are peeled off).

Meanwhile, in pencil, gingerly label a series of 137mM Hybond N filters with the same designation as that which the plates are labeled. This can be performed on the bench top; although sterile conditions are generally a priority, absolute sterile conditions are not critical to the success of the screen. However, wear gloves at all times when handling these filter membranes because oils from your hands will contaminate the Hybond N inhibiting the transfer of DNA to the filter membrane.

After the plates have cooled, lay the appropriately labeled membrane onto the corresponding plate. (I have the pencil side of the membrane facing the top-agarose surface. Just be consistent throughout.) To do this, fold the membrane (without creasing it) such that the folded part is centered over the plate. Touch the membrane to the plate and let adhesion pull the membrane down onto the plate. This is performed the same way as if you were laying a membrane onto a gel for Southern Blotting. It takes 10 to 30 seconds for the filter membrane to pull itself down onto the plate and to lay completely flat with no air bubbles under the filter disc. Once you touch the plate with the filter, don't lift it and then lay it back down because DNA is instantly transferred, just go with what you've got; these filter membranes cost several dollars each and things don't need to be absolutely perfect!

Once the filter is laid onto the plate move on to the next plate. Continue until the entire series of membranes has been laid onto the plates.

Fit a one or 5 ml syringe with a small diameter needle; e.g., 25G.

Draw ~1ml of permanent India ink into the syringe.

Asymmetrically mark the membrane and plate poking 4 to 5 holes through the membrane into the agar with a syringe needle. Leave just a small hole with a speck of ink around it.

Repeat this process for the entire series of plates.

Using a pair of forceps, grasp one end of the filter and with one fell swoop, lift the filter off of the plate. Set it onto a piece of Whatman filter paper. Do this for the entire series of filters.

Allow these filters to dry ~15 minutes to 1hour. (It does not seem to matter if the filters dry to the point of curling.)

Meanwhile cut three circular pieces of Whatman chromatography filter paper such that they will neatly fit inside a large, #3, 1.4L round Rubbermaid container.

Lay these pieces of Whatman chromatography filter paper inside each plastic Rubbermaid container.

Label the 1st denaturation, the 2nd neutralization, and the 3rd, 2X SSC.

Place a small volume of each of the respective solutions into the appropriately labeled container; just enough to wet the filter paper.

Lay the first filter (e.g., filter #1), DNA side up (the side of the filter that was facing the λ phage & top-agarose) onto the piece of Whatman chromatography filter paper wetted with the **DENATURATION** solution. The ideal conditions are such that the DNA side of the Hybond N filter should float in the DENATURATION solution above the Whatman chromatography filter paper and that the DENATURATION solution should not submerge or flow over the Hybond N filter.

Incubate 2- 5 minutes with occasional gentle shakings.

At the conclusion of the denaturation step, grasp the filter with a pair of forceps and lift it out of the solution. Remove the excess DENATURATION solution by running the non-DNA side over the edge of the plastic Rubbermaid container allowing the drips of DENATURATION solution to fall back into the container.

Transfer this filter to the **NEUTRALIZATION** solution using the same technique used to lay the filter into the denaturation solution.

Incubate 2 - 5 minutes in the NEUTRALIZATION solution with occasional gentle shakings.

Transfer this filter to the **2X SSC** and incubate 2 - 5 minutes again with occasional gentle shakings.

At the conclusion of the 2X SSC incubation, remove the filter from the 2X SSC and place it onto the piece of Whatman filter paper.

Process each membrane in this stepwise fashion; i.e., Denaturation 2-5 minutes, Neutralization 2-5 minutes, and 2X SSC 2-5 minutes. After the filter is removed from the solution discard the solution and replace it with fresh solution (you can use the same piece of Whatman chromatography filter paper for all filters and changes of solutions) before placing the next filter onto the Whatman chromatography filter paper. It is really important to completely denature and neutralize the DNA on each filter. Therefore periodically dump out the old solutions and add fresh solutions.

Once all filter membranes have been processed compile them into a stack inserting a kim-wipe or piece of paper toweling between each filter and then wrap them in Aluminum foil. Bake this package at 80$^{o}$C for 3 hours under vacuum. Once baked these filters are quite stable and can be stored at RT (indefinitely?). However the phage on the plates is not so stable thus the filters should be hybridized as soon as possible.

**Note for High Throughput Processing; process multiple filters at once.** To do this peel the filters off of the plates and then lay multiple filters onto a single piece of nylon mesh cut to the dimensions of a large container. Place the nylon mesh first into the denaturation solution. After incubation for 2-5 minutes, pickup the entire mesh, allow the denaturation solution to drain, and then lay the mesh into the neutralization solution. Continue in this manner until all filters are have been run through all buffers.

# Bacteriophage λ Plant Genomic Library Construction

## Preparation of Probe

**Background:**

The Lambda (λ) ZAP II vector has internal T3 and T7 primer sites. This is because the plasmid vector pBluescript SK- exists as a resident component of the λ vector. Plasmid pBSSK- therefore also has T3 and T7 sites, whereas the pGEM plasmid series have the T7 and SP6 primer sites. The T3, T7 and SP6 sites are the binding sites for the RNA polymerases from bacteriophage T3, T7 and SP6 respectively, which can come in very handy for other types of experiments (e.g., *in vitro* RNA synthesis and/or *in vitro* transcription translation reactions) but if not removed from the probe may lead to background hybridization between the probe and all λ clones. On the other hand the pUC plasmid series do not have any of these bacteriophage specific promoter binding sites. (Note however that all plasmids harboring the *lacZ* gene encoding β-galactosidase have the M13Forward and M13Reverse sites because these primers are in the *lacZ* gene itself.)

**Objective:**

To remove the common sequences between that which is used as the probe and the λ vector that is being screened.

**Strategy:**

Each clone will require a slightly different strategy depending on the vector within which it exists. One very straightforward technique is to digest the plasmid construct with the restriction enzyme *Pvu*II and then band isolate the fragment containing the insert. *Pvu*II cuts the *lazZ* gene twice. These two sites flank the polylinker (one *Pvu*II site is about 65 bases proximal to one end of the polylinker and the other *Pvu*II site is about 150 bases proximal to the other end of the polylinker). If there is a *Pvu*II site in the cloned insert of interest, this strategy may not work (it will depend how big the insert is). Alternatively and/or if you don't have enough plasmid DNA to band isolate an insert, then PCR amplification of the plasmid insert using the M13F and M13R primers is a very quick way to generate insert DNA. If the cloned insert is in a vector like pGEM, then the T7 site can be removed using an enzyme that is between the T7 site and the T overhang cloning site; e.g., *Sph*I. (Again be sure that the enzyme does not cut the insert.) Then when labeling, one can use the SP6 primer to prime the reaction because it will not cross hybridize with the λ vector DNA. Alternatively if the cloned insert is in a vector that has both T3 and T7 sites, such as the pBluescript vectors, then remove one of the primer sites (again by restriction digestion using an enzyme between the primer site and the cloned insert, which does not cut the cloned insert), band isolate this fragment and then label with a gene specific primer.

# Bacteriophage λ Plant Genomic Library Construction

## I. PCR amplification of Plasmid Inserts:

### Procedure:

### A. General Procedure

1. Turn on Robocycler to warm it up.
2. Thaw all necessary components (on ice)
3. Label PCR tubes.
4. Aliquot complimentary primers to respective PCR tubes. Place on ice.
5. If performing multiple reactions, prepare the necessary volume of reaction cocktail. Do this on ice.
6. Add reaction cocktail to tubes with complimentary primers.
7. Place in Robocycler and Start PCR process.

### B. Reaction Conditions:

| | Vol to use | Final Rxn [] |
|---|---|---|
| DNA (5ng/µl) | 5.0µl | (25ng) |
| 10X Reaction Buffer | 5.0µl | (1X) |
| dNTP mix | 1.0µl | |
| $ddH_2O$ (to final vol of 50µl) | 33.0µl | |
| *Taq* Polymerase | 1.0µl | 2.5U |
| DMSO | 2.5µl | 5% |
| (Required for G/C rich clones; i.e., barley) | | |
| Primer/Oligo #1 (M13F = EJS39) | 1.25µl | 125ng |
| Primer/Oligo #2 (M13R = EJS40) | 1.25µl | 125ng |

(No need to overlay sample with mineral oil when using the Robocycler fitted with the Hot Top)

### C. Suggested PCR Conditions (Program #16 on Robocycler):

| Program | Temp | Time | # Cycles |
|---|---|---|---|
| 1 | 95°C | 2 min | 1 |
| 2 | 95°C | 1min | 30 |
| | 55°C | 1 min | |
| | 72°C | 1 min | |
| 3 | 72°C | 5 min | 1 |

**II. Removal of T7 and or T3 sequences from Insert.**

Several options exist (see background, objective and strategy discussion above):

**A. The ole standby: phenol extraction and EtOH precipitation**

(1) Phenol, Phenol/Sevag & Sevag extract the PCR product (to remove all residual Taq).
(2) Restriction digestion this PCR product with an enzyme that eliminates the T7 site leaving the SP6 intact.
(3) Band-isolate the appropriate sized fragment (this insures removal of all primers and dNTPs)
(4) Confirm and estimate percent recovery on another gel.
(5) Label this fragment.

**B. Kits:**

(1) Microcon-PCR (removes primers up to 137 bases and dNTPs but not Taq polymerase)
(2) Micropure-EZ Enzyme (removes Taq)

**Probe Labeling**

(See Oligonucleotide labeling protocol for additional details and stock solutions)

Probe for Library Screening

| | |
|---|---|
| (1) DNA (50-100ng gel-isolated fragments) | 10.0µl |
| (2) $dH_2O$ | to 100.0 |
| (3) Primer (125ng/µl) | 3.0µl* |
| (4) Boil | 10min |
| (5) Chill on ice | |
| | Per Rxn |
| (6) Soln A | 4µl |
| (7) HEPES | 4µl |
| (8) dA | 2µl |
| (9) dG | 2µl |
| (10) dT | 2µl |
| (11) BSA | 4µl |
| (12) $^{32}$P-dCTP | 10µl |
| (13) $ddH_2O$ | 35.0 |
| (14) Klenow | 2l |
| (15) 37°C | ~1h |
| (16) STOP | 100µL |

Hybridization:

Transfer one membrane to a small volume of 2X SSC (in a round Rubbermaid dish). (Optional: massage off all agarose from the filter membrane.) Transfer the filter to second round Rubbermaid dish and incubate with shaking at RT for about 15 minutes. Process each filter in this fashion.

Begin preparing the pre-pre-hyb, pre-hyb and hyb solutions; you will need approximately ~0.2 ml/cm$^2$, ~0.1 ml/cm$^2$, and ~0.05 ml/cm$^2$ of membrane surface area respectively. See the table.

**Note**: Church buffer (Church, G.M. and Gilbert, W. (1974) Genomic Sequencing. Proc. Natl. Acad Sci. USA. 81: 1991-1995) may be used in place of the following hybridization solutions.

Hybridization solutions
(100 x 137mm filters)

| Stock Solutions | Pre-Pre-Hyb (~15,000 cm$^2$ total) | Pre-Hyb (400ml) (4ml/filter; 0.026ml/cm$^2$) | Hyb (200ml) (2ml/filter; 0.013ml/cm$^2$) |
|---|---|---|---|
| Formamide | - | 200.0 ml (50%) | 100 ml (50%) |
| SSC (25X) | 20.0 ml (1X) | 80.0 ml (5X) | 40.0 ml (5X) |
| Na-PB pH 6.8 (2M) | - | 10.0 ml (50mM) | 2.0 ml (20mM) |
| Denhardt's (100X) | - | 20.0 ml (5X) | 2.0 ml (1X) |
| SDS (10%) | 25.0 ml (0.5%) | 8.0 ml (0.2%) | 2.0 ml (0.1%) |
| Dextran sulfate (50%) | - | - | 40.0 ml (10%) |
| $dH_2O$ | 455 ml | 82.0 ml | 14.0 |

Add the pre-pre hyb solution and incubate the membrane with shaking at ~60$^o$C for ~1h. Do this in the same round sealable Rubbermaid container.

Discard the pre-pre hyb solution.

Add the pre-hyb solution to the round sealable Rubbermaid container containing the membranes.

Incubate at 42$^o$C with shaking for 4h to overnight.

Discard the pre-hyb solution into an appropriate waste container. Formamide is targeted to the reproductive organs and is very toxic. Alternatively put the pre-hyb solution in a bottle, store it at –20$^o$C and then reuse it for the secondary and tertiary library screenings.

Boil the probe for 2- 10 minutes and quick chill on ice.

Add the boiled probe to a small aliquot of hyb solution and mix well. Then add the bulk of the hyb solution to the round sealable Rubbermaid container containing the membranes. Then add the probe in the hyb solution to the Rubbermaid container containing the membranes.

Incubate at 42°C with shaking overnight.

Discard the hyb solution into an appropriate $^{32}P$ radioactive waste container.

Low Stringency Washes: Wash the membrane 3 or 4 times at RT for 15 – 30 minutes each wash until no more counts come off in wash buffer. Discard the wash solution into an appropriate $^{32}P$ radioactive waste container.

High Stringency Washes: Wash the membrane 3 or 4 times at 55°C for 1-2 hour each wash until no counts come off in wash buffer. (Discard the wash solution into an appropriate $^{32}P$ radioactive waste container.)

Wash solutions

| Stock solution [direct from shelf] | Low (1000 ml) | High (250 ml) |
|---|---|---|
| SSC (20X) | 100 ml (2X) | 10 ml (0.2X) |
| SLS (10%) | 5 ml [0.125 g] (0.05%) | 5 ml [0.125 g] (0.05%) |
| Na-PPi (5%) | 4 ml [0.05g] (0.02%) | 2 ml [0.025g] (0.01%) |
| $dH_2O$ | 891 ml | 983 ml |

Notes: The above wash volumes are approximated for 32 filters prehybridized in one container and then partitioned between two Rubbermaid containers for hybridizations with two different probes. Adjust your volumes appropriately.

Lay the membranes on a piece of filter paper and/or kim-wipe to blot away the excess moisture.

Prepare a "mount" for the membranes by wrapping a thin sheet of boxboard (e.g., the ones that come in the film packages are perfect) with Saran wrap. Tape it down on the backside. Saran wrap is critical here. Other brands of plastic wrap cause high backgrounds in autoradiograms.

Lay the membrane on top of the Saran wrapped mount.

Cover this with another piece of Saran Wrap.

Insert a glow in the dark ink marked filter paper to help orientate the autoradiogram after it is developed. (Note put the glow-in-the-dark, ink-marked filter papers in the dark room for 5 min to reduce their fluorescence capacity.)

Place into film cassette.

For 1° library screening use an intensifying screen and place the film cassette into the –80°C for 2-3d. For 2° and 3° library screening, the intensifying screen is less critical. When using an intensifying screen the film and membrane need to go into the –80°C. This combination increases signal intensity ~3X above that without the screen.)

# Bacteriophage λ Plant Genomic Library Construction

## Preparation of λ DNA from λFixII Vector

This procedure is designed to prepare DNA from one or two 147mm plates. Once the PEG precipitated phage pellet is recovered, there are two options for DNA preparation. Option 1 is to prepare DNA using microfuge tubes, phenol extraction and multiple precipitation steps. This is useful when handling numerous samples and works extremely well for plate lysate yields recovered from a single plate. The DNA is suitable for restriction digestion and subcloning purposes. The second option is to use anion exchange chromatography and the components of the Qiagen Lambda DNA Isolation Kits. As such, the procedure closely parallels the information provided in the Qiagen Lambda Handbook (read the Qiagen Lambda Handbook).

Note that prior to initiating the following you will need to determine the titer of the λ bacteriophage stock.

**A. <u>Plate Lysates</u>**

Pick an overnight of XL1Blue MRA into the appropriate volume of NZY Maltose and grow to $OD_{600}$ = ~1.0. Ideally the E. coli should have been recently streaked out onto an NZY plate; i.e., it should be less than one-week old.

Spin the culture down and resuspend in ½ the original culture volume of sterile 10mM $MgSO_4$.

Determine the $OD_{600}$ of the resuspended culture and then bring it to $OD_{600}$ = 0.6. A good starting point is to dilute 250µl of the resuspended culture into 10mM $MgSO_4$ and determine the $OD_{600}$ of this (using 10mM $MgSO_4$ as the blank). Then bring the remainder of the resuspended culture to $OD_{600}$ = 0.6. 600µl of cells at $OD_{600}$ is required for each large 147mm plate. Use these resuspended cells the same day; i.e., use them fresh.

Aliquot 600µl of these cells into two separate (prelabeled) standard 100 X 125mM culture tubes sitting in a test tube rack. (Option 1 works well for one plate, Option 2; i.e., the Qiagen protocol is optimized to obtain DNA from two large plates.)

Aliquot 125,000 pfu of λ bacteriophage to each tube of E.coli cells. Generally the standard volume is 100µl but if your phage titer is 5 x $10^5$ pfu/ml, then add 500µl. However don't dilute the phage too much otherwise the kinetics of the infection process may be inadvertently altered.

Place the rack containing the tubes into the 37°C water bath for 15-20 minutes. (The λ bacteriophage will adhere to the E. coli cells while sitting at room temperature but λ DNA is not introduced into cells until incubation at 37°C.)

Meanwhile melt NZY Top Agarose in the microwave and allow it to cool to ~48/50°C.

At the conclusion of the 15-minute incubation, add ~6.5ml of the NZY top agarose to the test tube containing the λ phage & E. coli. Remove the tube from the rack and give it a quick flick of your wrist mixing the contents and immediately pour it onto an NZY plate.

Pick up the plate and tilt it around to evenly disburse the top agarose mixture.

Allow the top agarose to cool (~5 minutes).

Invert the plate and incubate at 37°C for ~9 hours.

Add 10ml of SM to each plate and incubate at RT for ~4 hours with gentle shaking (or overnight at 4°C).

Transfer the SM, which now contains the phage particles to an Oak Ridge tube.

Add 1ml of SM to each plate to recover as much of the remaining phage as possible.

Add chloroform to final volume of 2% (~500µl per 2 large plates of lysate).

Swirl to thoroughly mix the chloroform with the SM. The chloroform will lyse and kill residual E. coli cells.

Remove the chloroform and as much cell debris as possible with an L1000 and then centrifuge at >10,000 x g for 10 minutes.

Decant the supernatant into a clean Oak Ridge tube.

**B. DNA Isolation Procedure** (Read the Qiagen λ Handbook; volumes of Qiagen Buffers may need to be adjusted. The corresponding steps in the Qiagen λ Handbook are in **bold** above each series of steps in the Stockinger Lab protocol.)

**Qiagen Step 2:**

Add 40µl of **L1** (containing RNase A and DNase I) to the 20ml volume of phage supernatant. (The RNase A and DNase I will digest residual E. coli RNA and DNA that exists outside of the phage particle.)

Incubate at 37$^{o}$C for 30 minutes.

**Qiagen Step 3:**

Add 4 ml of ice-cold **L2**. Mix very gently. Incubate on ice for 60 minutes (longer; i.e., O/N is okay). (Buffer L2 contains PEG and NaCl, which cause the phage particles to precipitate.)

**Qiagen Step 4:**

Centrifuge at >10,000 x g for 10 minutes.

Discard the supernatant by aspirating it off.

Quick spin the tube to pull all remaining liquid towards the bottom of the tube. Remove all traces of liquid by aspiration (which removes the RNase A and DNase I).

**At this point in the procedure there are two options: Option 1 or Option 2. Follow the respective protocol depending on your needs.**

**Option 1. DNA preparation using microfuge tubes, phenol extraction and multiple precipitation steps**

Resuspend the pellet in 350µl of **L3**. (It is safe to add **L3** and leave this to incubate on the phage pellet overnight at 4°C.) Resuspend the pellet completely by pipetting it up and down.

Transfer the resuspended phage to a microfuge tube.

**From the next step on through the completion of the DNA isolation, BE VERY GENTLE in all mixing steps. Once the phage particle is broken open, the λ DNA is very prone to shearing because it is linear and quite large.**

Add 350µl of **L4**. Gently invert the tube and mix the contents. Incubate at 70°C for 20 minutes. (Buffer L4 is 4% SDS; this disrupts and solubilizes the proteins of the λ phage particle, which in turn liberates the phage DNA inside.)

At the conclusion of the 70°C incubation, cool the tube by placing on ice for ~5min.

Add 350µl of **L5**. Gently invert the tube 4-6 times to mix the contents. (Buffer L5 is 3M K-acetate pH 5.5. It forms a precipitate with the proteins and SDS.)

Centrifuge at 4°C for 30 minutes at maximum g in a microfuge.

Gently pull off the supernatant into a fresh tube using a P1000.

Add 60ul 5M NaCl.

Add 750µl 2-propanol (isopropanol).

Gently invert to mix.

Spin 2-5 min in a microfuge tube.

Decant off the supernatant.

Wash the DNA pellet with 70% ethanol.

Quick spin the tube to pull down all residual liquid.

Remove the residual liquid with a pipette.

Add 600ul $T_{10}E_{0.1}$ pH 7.9.

Incubate at 60-68°C for 5-10 min to resuspend the DNA.

Add 400µl TE equilibrated phenol (either freshly thawed from the –20°C or stored less than one month at +4°C).

Gently flick the tube with your finger and/or gently invert back and forth until you have achieved complete emulsification of the inorganic and organic phases.

Spin the microfuge tubes in a microfuge for 2-5 minutes at RT.

Remove the upper aqueous layer to a new microfuge tube.

Add 400µl of Sevag (chloroform/isoamyl alcohol; 24:1).

Gently flick the tube with your finger and/or gently invert back and forth several times.

Remove the upper aqueous layer to a new microfuge tube.

Add 60µl NaOAc.

Add 1ml 95% ethanol

Gently invert to mix

Spin 2-5 min in a microfuge tube

Decant off the supernatant

Add 1ml of 70% ethanol

Gently invert to mix

Spin 2-5 min in a microfuge tube

Decant off the supernatant

Quick spin the tube to pull down the residual liquid

Remove the residual liquid with a pipette

Allow the DNA to air-dry 1h to O/N at RT by leaving the microfuge tube open on the bench top. Cover the microfuge tube with a piece of foil so that dust and other debris do not fall into the tube.

Resuspend the DNA in 150ul $T_{10}E_{0.1}$ pH 7.9

Store at –20$^{o}$C. Use 10-15µl per restriction digest and ~50µl for subcloning purposes

**Option 2. DNA preparation using Oak Ridge Tubes, anion exchange chromatography, and components of the Qiagen Lambda DNA Isolation Kits.**

**Qiagen Step 5:**

Resuspend the pellet in 3ml of **L3**. (It is safe to add the Qiagen Buffer **L3** and leave this to incubate on the phage pellet overnight at 4°C.) Resuspend the pellet completely before proceeding by pipetting it up and down. (Buffer L3 is 100mM NaCl, 100mM Tris-HCl pH 7.5 and 25mM EDTA.)

**From the next step on through the completion of the DNA isolation BE VERY GENTLE in all mixing steps. Once the phage particle is broken open the λ DNA is very prone to shearing because it is linear and quite large.**

**Qiagen Step 6:**

Add 3 ml of buffer of Qiagen Buffer **L4**. Gently invert the tube and mix the contents. Incubate at 70°C for 20 minutes. (Buffer L4 is 4% SDS; this disrupts and solubilizes the proteins of the λ phage particle, which liberates the phage DNA inside.)

Place the tube into ice.

**Qiagen Step 7:**

Add 3ml of Qiagen Buffer **L5**. Gently invert the tube 4-6 times to mix the contents. (Buffer L5 is 3M K-acetate pH 5.5, which forms a precipitate with the proteins.)

Centrifuge at 4°C for 30 minutes at >15,000 x g.

Gently decant the supernatant into a fresh tube.

**Qiagen Step 8:**

Centrifuge a second time at 4°C for 10 minutes at >15,000 x g to obtain a particle-free cleared lysate.

**Qiagen Step 9:**

Meanwhile, as the lysates are spinning equilibrate a Qiagen-tip 100 by applying 4 ml of Qiagen Buffer QBT to the column. Allow it to drain completely.

**Qiagen Step 10:**

After the second centrifugation, directly apply the supernatant to the equilibrated Qiagen-tip 100 column. Allow the buffer to enter the column by gravity flow and allow it to drain completely.

**Qiagen Step 11:**

Wash the Qiagen-tip 100 column with 10 ml of Qiagen Buffer QC. Allow the buffer to enter the column by gravity flow and allow it to drain completely.

**Qiagen Step 12:**

Elute the DNA with 5ml of Qiagen Buffer QF into a clean (and sterile) oak ridge tube.

**Qiagen Step 13:**

Precipitate the DNA by adding 3.5ml of RT isopropanol to the eluted DNA. Mix by gentle inversion

Centrifuge at >15,000 x g for 30 minutes at 4°C.

Carefully decant the supernatant. The DNA pellet will be very difficult to see.

**Qiagen Step 14:**

Wash the pellet with 10 ml of 70% Ethanol.

Centrifuge at >15,000 x g for 10 minutes at 4°C.

Carefully decant the supernatant.

Quick spin the tube to pull all residual traces of ethanol down to the bottom of the tube. Remove these with the L1000µl pipette.

**Qiagen Step 15:**

Air-dry the pellet for 5-10 minutes. Do not vacuum dry this DNA because this may result in over drying of the DNA, which then makes it very difficult to dissolve.

Dissolve the DNA in a suitable volume of $T_{10}E_{0.1\ (pH\ 7.9)}$. Incubate the tube containing the DNA at 65-70°C to aid in the DNA dissolution. Suggested volume to dissolve DNA: Two plate lysates at optimum phage lysis and using the Qiagen buffer volumes add 120µl of $T_{10}E_{0.1\ (pH\ 7.9)}$ to the oak ridge tube, incubate at 65°C for a while, quick spin these tubes. Remove the 120µl to a microfuge tube. Add another 180µl of $T_{10}E_{0.1\ (pH\ 7.9)}$ to the oak ridge tube to rinse any residual DNA and then pool these two fractions to yield a total volume of ~300µl. This method yielded sufficient DNA that restriction digestion of 10-15µl was easily visualized on EtBr stained agarose gels.

## Fingerprinting Genomic Clones using Restriction Digestion and Hybridization

### Restriction Enzyme Digestions

| | Per reaction |
|---|---|
| DNA | 15.0µl |
| 10X Enzyme Buffer | 2.0ul |
| BSA | 0.2µl |
| Enzyme | 0.5µl |
| 37°C | 1-4h |
| Loading Dye | 4.0µl |
| 65-70°C | 5-10 min |

Load the reaction product on a 1% agarose, 1X TEA gel, run 30-35 V for 14-16h. Blot the gel. Hybridize with probes of interest.

Restriction-digest the clones with a panel of enzymes. Digestion with *Not*I, *Sal*I and *Xba*I should drop out the insert, which will resolve distinct from the λ arms. The λ left arm will run at 20kb; the λ right arm will run at 9kb. Use of other enzymes such as *Eco*RI and *Hin*dIII will include portions of the λ arms in the junction fragments. Heating the reaction to 65°C-70°C prior to loading on a gel denatures the 12 bp COS sites.

# Bacteriophage λ Plant Genomic Library Construction

## Subcloning λ Clones

**Digestions**

| | |
|---|---|
| λ DNA | 51µl |
| 10X NEB buffer 3 | 6.0µl |
| BSA | 0.6µl |
| RNase | 0.3µl |
| *Not*I | 1.0µl |

37°C

65°C (Heat Inactivation) 20 minutes

Ethanol-precipitate the DNA:

Add 1/10 volume of NaOAc
Add 2X volumes ethanol
Spin for 2min at RT at max RPM in a microfuge
Decant off the supernatant.
Wash the DNA pellet by adding 1ml 70% EtOH, and invert several times.
Spin RT 2min.
Decant off the EtOH washate.
Quick spin the tube to pull all remaining ethanol down to the bottom of the tube.
Remove this ethanol with a pipette.
Air dry the DNA pellet ~15-30 minutes on the bench top (cover it with foil or a kim wipe)
(Alternatively minimally dry the DNA pellet in speed vacuum; i.e., 5-7 min.)

Resuspend in 10µl dd$H_2O$.

Heat to 65°C and allow the DNA to slowly cool to RT. To do this fill a test tube with 65°C $H_2O$, place the microfuge tube in the test tube full of 65°C $H_2O$ and allow the temperature to slowly drop to RT.

Quick spin the microfuge tube to pull all liquid to the bottom.

Place the microfuge tube on ice. This heating and slow cooling process should assist the annealing of the λ COS sites such that when this DNA is added to ligation reaction, the COS sites will ligate creating a 29kb fragment, which in theory should be much harder to clone than the λ inserts that are ~9 to 23kb.

**Ligation of *Not*I digested clones to pGEM11Z+ *Not*I/ΔP/GI**

| | Per Rxn |
|---|---|
| pGEM11Z+ NotI/ΔP/GI (~50ng/µl)* | 1.0µl |
| 10X T4 ligase buffer | 0.5µl |
| T4 DNA ligase | 1.0µl |

(Prepare a ligation cocktail mix if working with multiple inserts, and then aliquot 2.5µl of cocktail to each tube prior to adding the insert DNAs.)

Place on ice.

Add 2.5µl λ DNA from the previous step.

Include a negative control by substituting 2.5µl $T_{10}E_{0.1\ (pH\ 7.9)}$ for the insert (to determine background self-ligation of the vector.

Incubate at RT (or 16°C) for 1h to O/N.

Transform ligated DNAs into E. coli** and plate onto $LB_{Amp150}$ plates augmented with 2% X-gal and ____IPTG.

*See Shotgun Library construction for the preparation of vector DNAs.
**We have used lab prepared DH5α competent cells and commercially prepared (Stratagene) XL10-Gold competent cells, which are marketed as an excellent transformable host for very large (e.g., BAC size) insert DNAs.

Media and other stuff

**SM**

| | 500ml |
|---|---|
| 5M NaCl | 10.0ml |
| $MgCl_2$ | 5.0ml |
| Tris (pH7.5) | 25.0ml |
| Gelatin | 0.050g (50mg) |
| Autoclave | |

**NZY**

| | 1000ml |
|---|---|
| NZ Amine | 10.0g |
| Yeast Extract | 5.0g |
| NaCl | 5.0g |
| $MgSO_4 \cdot 7H_2O$ | 2.0g |
| $dH_2O$ | 1000ml |
| Adjust pH to | 7.5 with NaOH (~2 pellets) |
| Autoclave | |

**20% Maltose**

| | 100ml |
|---|---|
| Maltose | 20.0g |
| dd $H_2O$ | 100ml |
| Autoclave | |

**NZY supplemented with 0.2% maltose**

Prepare 1L NZY.
Aliquot it into four 250ml bottles.
Autoclave the bottles containing the NZY.
Add 2.5ml of sterile 20% maltose to each bottle.

**NZY Agar Plates**

| | 1000ml |
|---|---|
| NZ Amine | 10.0g |
| Yeast Extract | 5.0g |
| NaCl | 5.0g |
| $MgSO_4 \cdot 7H_2O$ | 2.0g |
| $dH_2O$ | 1000ml |
| Adjust pH to | 7.5 with NaOH (~2 pellets) |
| Agar | 15.0 g |
| Stir Bar | |
| Autoclave | 30 minutes |
| Cool and pour plates | |

**NZY Top Agarose**

| | |
|---|---|
| NZY liquid medium | 250ml |
| Agarose | 1.75g (the same stuff used for DNA gels) |
| Autoclave | |

**NZY Agarose Plates for Plate Lysates (this yields about 12 plates using 80ml/plate)**

| | 1000ml |
|---|---|
| NZ Amine | 10.0g |
| Yeast Extract | 5.0g |
| NaCl | 5.0g |
| $MgSO_4 \cdot 7H_2O$ | 2.0g |
| $dH_2O$ | 1000ml |
| Adjust pH to | 7.5 with NaOH (~2 pellets) |
| **Agarose** | 15.0 g |
| Stir Bar | |
| Autoclave | 40 minutes |
| Cool and pour plates | |

# Bacteriophage λ Plant Genomic Library Construction

**Qiagen Buffers**

**Buffer L1**

| Stock | Quantity to use | Final concentration |
|---|---|---|
| 5M NaCl | 6.0ml | 300mM |
| 1M Tris pH 7.4 | 10.0ml | 100mM |
| 0.5M EDTA | 2.0ml | 10mM |
| $ddH_2O$ | 82.0ml | |
| 10mg/ml BSA | | 2mg/ml |
| RNase A (Boiled; See Molecular Cloning) | | 20mg/ml |
| DNase I | | 6mg/ml |

**Buffer L2**

| | | |
|---|---|---|
| PEG (MW 6000 or 8000) | 30.0g | 30% |
| 5M NaCl | 60.0ml (or 17.5g) | 3M |
| $ddH_2O$ | to 100ml | |

**Buffer L3**

| | | |
|---|---|---|
| 5M NaCl | 2.0ml | 100mM |
| 1M Tris pH 7.4 | 10.0ml | 100mM |
| 0.5M EDTA | 5.0ml | 25mM |
| $ddH_2O$ | 83.0ml | |

**Buffer L4**

| | | |
|---|---|---|
| 10% SDS | 40.0ml (or 4.0g stock) | 4% |
| $ddH_2O$ | to 100ml | |

**Buffer L5**

| | | |
|---|---|---|
| Potassium Acetate | 49.2g | 3M Potassium/5M Acetate |
| $ddH_2O$ | 60ml | |
| Glacial acetic acid | 11.5ml | |
| $ddH_2O$ | 60ml | |
| Filter through 0.4μm filter | | |
| Autoclave | | |
| Store RT | | |