

Genomic (Plant) DNA: Restriction Digestion for Southern Blotting

Background on the art of getting beautiful Southern:

There are several things to consider. (1) Digesting enough DNA. (2) The DNA must be digested to completion; i.e., no partial digests because they make interpretation difficult if not impossible. (3) The resolution of fragments. This is determined by both the thickness of the gel comb and the speed at which the gel is run.

For Arabidopsis use about 2 μ g of total DNA; for other plants; e.g., alfalfa, tomato, potato and soybean use 10 μ g of total DNA. To avoid partial digests the DNA should be relatively clean. Performing the digestion in a very large volume (150 μ l) with an excess of enzyme also reduces partial digests because it dilutes salts and other potential contaminants. A 1mm thick comb and an overnight run at 30 to 35 V for 14 to 16 hours using TEA gel buffer (TAE) produces highly resolved bands in the size range of about 500bp to about 10kb. To do it this way however requires a period of several days:

Timetable:

- Day 1: Digest the DNA
Ethanol-precipitate the digested DNA
Resuspended the DNA in a very small volume (~15 μ l) of TE overnight
- Day 2: Pour and load the gel
- Day 3: Photograph and blot the gel
(Include a ruler in the photograph)
- Day 4: Disassemble the blot
UV crosslink (Using the Stratalinker) and/or bake the membrane
Go directly to pre hybridization or store filter.

A few additional considerations:

An excess number of samples may preclude that gained by the 1mm comb and the need to ethanol precipitate the digested DNA in order to end up with a very small volume (~15-20 μ l) that will fit into the well. If this is the case do the restriction in 25-30 μ l, skip the ethanol precipitation step and use a 2mm thick gel well comb. The 3mm thick combs do not provide good resolution and should be avoided. The 2mm combs allow greater loading volume and are a good compromise.

In my (Eric's) experience gels run overnight using TEA buffer produce more highly resolved bands than do TBE buffered gels. The problem with TAE is that it has a low buffering capacity. As a consequence the buffers will become polarized; I have experienced the upper reservoir hitting pH>10; when this happens the gel begins to melt, which is not good. To get around this either run the gel using a gel apparatus that holds a large volume of buffer and/or with TEA overnight in one of the gel boxes that allows buffer exchange between the cathode (-) and anode (+) buffer reservoirs.

If many samples will be run that are identical except for one component; e.g., the DNA or the enzyme, prepare a cocktail of all the common components and then aliquot this cocktail out to the different tubes (keeping everything on ice the entire time).

Basic Digest (perform everything on ice):

Genomic DNA (2-10 μ g)	_____ μ l
10X Restriction Digestion buffer	15.0 μ l
10mg/ml BSA (if needed)	1.5 μ l
10mg/ml RNase A (boiled)	1.5 μ l
ddH ₂ O to final volume (150 μ l)	_____ μ l
Enzyme (~50 units; usu 2.5 to 5.0 μ l)	_____ μ l
Incubate at temperature	~1h
Add an additional 50 units of enzyme	_____ μ l
Return to incubation temperature for	~1h

At the conclusion of the digest either (a) Ethanol precipitate the DNA (which can also be stored at -20°C to spin at a later date), (b) Load it on the gel (c) Freeze it away at -20°C to be processed later or (d) Add loading dye (if DNA is at the desired volume) and load on gel or freeze at -20°C .

Hint: If you are digesting multiple samples, prepare a cocktail of all components minus the one unique component. For example if you want to digest one particular DNA with 5 different enzymes, prepare a master mix cocktail that contains everything except the enzyme. Of course this assumes that all enzymes chosen will cut in the same buffer (BSA does not matter; i.e., you may include it without any negative consequences for any enzyme). Dole out the cocktail to the tubes on ice and then add the DNA. Generally it is wisest to prepare slightly more than needed; i.e., if eight DNA samples will be digested, then prepare enough master-mix cocktail for 10 samples, etc.

Ethanol precipitation (assuming 150 μ l volume):

DNA sample	150 μ l
3M NaOAc (pH ~5)	15 μ l (1/10 volume)
95% EtOH	350 μ l (2 to 2.5 volumes)
Thoroughly mix by inverting tube	
Spin in microfuge, RT, Max RPM	5 minutes
Decant supernatant	
Wash pellet 70% EtOH	~1ml
Spin in microfuge, RT, Max RPM	5 minutes
Decant off EtOH washate.	
Dry pellet in speed vacuum for about 10min.	

Resuspend the DNA pellet overnight in 15 μ L (or any convenient volume) of T₁₀E_{0.1}. (With or without loading dye, I don't think it matters). Some people simply incubate the T₁₀E_{0.1} and DNA at 60-65 $^{\circ}\text{C}$ ~20 minutes to resuspend the DNA.

Electrophoresis and Blotting

Load the gel.

Run for approximately 500V-h overnight. This is a general rule of thumb. For example 35V for 14h = 490 V-h works great to give beautiful Southern Blots.

Photograph the gel. Include a ruler in the photograph. It is probably easiest if the ruler "0" mark is aligned at the origin (the well). The markings on the gel should be visible in the photograph. If you want you can use one of the several rulers in the lab that fluoresce when put on the *uv* box. The problem with these is that often there is too much fluorescence from the ruler.

Determine the dimensions of the gel that need to be blotted. This would be from the origin to the bottom, and the width of the gel. If only a subset of lanes were loaded then those would be the dimensions (plus a little extra).

Place the gel into a container slightly larger than the gel itself. This can be anything from a glass pyrex baking dish to a plastic Rubbermaid container.

Add approximately 250ml of "*denaturation solution*," enough to cover the gel and allow movement of the gel solution. Place the gel on a shaker platform and gently shake for about 15 minutes at RT. Decant off the denaturation solution. An easy way to do this is to hold the gel in the tray with a gloved hand and tip the container over the sink.

Add a second 250ml volume of *denaturation solution* and incubate for approximately another 15minutes.

Decant off the *denaturation solution*.

Rinse the gel by filling the container with tap DI water and decanting off the water.

Add approximately 250ml of "*neutralization solution*." Return the gel to the shaker platform and gently shake for about 30 minutes at RT.

Decant off the *neutralization solution*.

Add a second 250ml volume of *neutralization solution* and incubate for approximately another 30 minutes.

Meanwhile, while the gel is incubating in the *denaturation/neutralization solutions*, prepare the Hybond N transfer membrane (bottom left drawer opposite the -80°C) and the S&S Gel Blot papers (above the Stratalinker in room 201A). Note that we have both Hybond N (for nucleic acid transfers) and Hybond P (for protein transfers) in the lab. Wear gloves when handling both transfer membrane and the S&S Gel Blot papers. If oils from your hands contaminate them (especially the Hybond N), transfer will be inhibited. Transfer membranes are VERY EXPENSIVE!

Transfer membrane:

Obtain a new razor blade and a clean ruler (wipe the ruler with 70% Ethanol). Unroll the Hybond with the bottom blue sheet protecting the membrane from the lab bench top. You will probably need to weigh the membrane down so it does not roll back upon itself, just don't contaminate the unused portions. Measure out the size of membrane needed for the transfer. This should be a bit larger than the gel to be blotted; e.g., if the gel is 14cm long (measured at the origin) and 10cm wide, measure out a piece 14.25 X 10.25 cm, or so. Using the clean ruler as a straight edge and the new razor blade, cut out only that portion of membrane you need; i.e., try to minimize unnecessarily cutting into unused sections of the membrane. Transfer membranes are VERY EXPENSIVE! Return the unused membrane to its container. Gingerly label the membrane with a pencil. I generally label membranes in the lower left corner with pertinent info:

experiment and date. Place the cut out section of membrane into ddH₂O to wet it. Decant off the ddH₂O then add a small volume of 10X SSC and let the membrane “equilibrate” alongside the shaking gel.

Gel Blot Paper:

You will need four sections of S&S Gel Blot Paper cut to dimensions slightly larger than the transfer membrane; e.g., the above gel and membrane would require four sections approximately 15 X 11 cm. Set these aside.

You will need one section of gel blot paper that will serve as the “wick.” This piece will drape over a support (e.g., a clean piece of glass) into a buffer chamber. The support will support the gel. You can also use a sponge for this purpose; Esther does it this way and can show you how to do it; I have never done it this way. See Fig 2.9.1 on page 2.9.4 of Ausubel, Current Protocols in Molecular Biology (The Red Book) for a diagram of both methods.

Assembling the Blot:

Determine what the buffer chamber will be. Locate a support. Cut the wick such that it will drape over the support into the buffer chamber.

Prepare enough 10X SSC transfer buffer by diluting 20X SSC 1:1 with ddH₂O. For a standard 15 X 10 cm gel you will need about 1000ml, ~500ml for the buffer chamber plus some additional buffer to equilibrate the transfer membrane and wet the Gel Blot Papers.

Fill the buffer chamber with about 500ml 10X SSC.

Wet the wick with the 10X SSC and drape it over the support such that two ends are immersed in the buffer chamber. The wick should be flat against the support such that there are no air bubbles trapped between the wick and the support. The support should rest on the top edge of the buffer chamber.

Place the gel on top of the wick. The gel should be flat against the wick such that no air bubbles are trapped between the gel and the wick.

Grasp the membrane edges approximately half way down with both hands rolling both edges slightly upward. Allow the top edge of the membrane to adhere to the gel just below the gel origin. Gingerly lay the rest of the membrane down onto the gel by letting adhesion suck it down. Once the membrane is laid on top of the gel do not remove it and set it back down. The transfer of DNA begins IMMEDIATELY! If you lift up and reposition the membrane you will have two imprints on that membrane. The Hybond N membrane should be flat against the gel such that no air bubbles are trapped between the membrane and the gel. Anywhere there are air bubbles, no transfer will occur. Often these areas also contribute to background noise on the autoradiogram. If there are air bubbles trapped below the surface of the Hybond N transfer membrane then simply massage them out with a gloved hand. Do not lift up the membrane and set it back down!

Seal the edges of the membrane with Saran or other plastic wrap. Simply tear off a (narrow) sheet of Saran and lay it over about one cm of membrane edge. Repeat this for the other three edges. This seals the system so that the buffer in the chamber will move through the gel and not around it.

Next lay the four sections of S&S Gel Blot Paper, which have been cut to dimensions slightly larger than the transfer membrane, on top of the transfer membrane. To do this take one of the four, dip it into 10X SSC and then lay it on top of the Hybond N membrane. Again there should be no air bubbles trapped between the Gel Blot Paper and the Hybond N membrane. Repeat this for each of the remaining three sections of Gel Blot Paper.

Set a 4-inch stack of paper towels on top of the Gel Blot Paper.

Set a flat support on top of the paper towels.

Set a 500g weight (e.g., an Erlenmeyer flask with 500 ml H₂O) on top of the flat support. Go home.

Disassembling the Blot:

Disassemble the blot. Discard the Saran, the Gel Blot Paper and any paper towels that are wet. Remove the transfer membrane from the gel, invert it so that it is now DNA side up and place it in a small volume of 2X SSC. Massage off any pieces of agarose stuck to the Hybond N membrane. Discard the 2X SSC and incubate the Hybond N membrane in the 2X SSC with gentle shaking for a few minutes.

Lay the Hybond N membrane DNA side up on a piece of Saran wrap.

Crosslink the DNA to the membrane by placing it and the Saran wrap into the Stratalinker. Turn on the Stratalinker. Press AUTOCROSS LINK. Press START. In about 20 seconds the DNA is permanently crosslinked to the Hybond N membrane. Seal the membrane in the plastic wrap and store it away in a drawer until you are ready for the hybridization. A week at RT is probably okay.

An alternative method to crosslink the DNA to the membrane is to layer it between a few kim wipes (don't use plastic in this case), wrap the kim wipes plus membrane in aluminum foil and then bake this in a vacuum oven (under vacuum) for 3h at +80°C. (This is a good way to preserve the membrane for long periods.)

Hybridization:

Transfer the membrane to a small volume of 2X SSC and incubate with shaking at RT for about 15 minutes.

Begin preparing the pre-pre-hyb, pre-hyb and hyb solutions; you will need approximately ~0.2 ml/cm², ~0.1 ml/cm², and ~0.05 ml/cm² of membrane surface area respectively. See the table.

<u>Hybridization solutions</u> (10 X 15 cm filter)			
Stock Solutions	Pre-Pre-Hyb (~0.2 ml/cm ²)	Pre-Hyb (~0.1 ml/cm ²)	Hyb (~0.05 ml/cm ²)
Formamide	-	5.0 ml (50%)	2.5 ml (50%)
SSC (25X)	0.8 ml (1X)	2.0 ml (5X)	1.0 ml (5X)
Na-PB pH 6.8 (2M)	-	0.25 ml (50 mM)	0.05 ml (20 mM)
Denhardt's (100X)	-	0.5 ml (5X)	0.05 ml (1X)
SDS (10%)	1.0 ml (0.5%)	0.2 ml (0.2%)	0.05 ml (0.1%)
Dextran sulfate (50%)	-	-	1.0 ml (10%)
ssDNA (10 mg/ml)	-	0.2 ml (200 ug/ml)	0.05 ml (100 ug/ml)
dH ₂ O	18.2 ml	1.45 ml	

Add the Pre-Pre Hyb solution and incubate the membrane with shaking at ~60°C for ~ 1h. You can do this either in one of the glass bottles or in a flat sealable Rubbermaid container. (Note that some people skip the step entirely.)

Discard the pre-pre hyb solution.

If the pre pre hyb was performed in a Rubbermaid type container, then transfer the membrane to the appropriate sized glass hybridization bottle (in Hot Room).

Boil the HS DNA for 10 minutes and quick chill it on ice.

Add the HS DNA to the pre-hyb solution and add the pre-hyb solution to the glass hybridization bottle containing the membrane.

Incubate at 42°C with shaking for 4 h to overnight.

Discard the pre-hyb solution into an appropriate waste container. Formamide is targeted to the reproductive organs and is very toxic.

Add the probe to the appropriate quantity of HS DNA, boil for 2- 10 minutes and quick chill on ice.

Add the hyb solution to the glass hybridization bottle containing the membrane. Then add the boiled probe/HS DNA to the bottle.

Incubate at 42°C with shaking overnight.

Discard the hyb solution into an appropriate ³²P 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.

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.

	<u>Wash solutions</u>	
Stock solution [direct from shelf]	Low (250 ml)	High (250 ml)
SSC (20X)	25 ml (2X)	2.5 ml (0.2X)
SLS (10%)	1.25 ml [0.125 g] (0.05%)	1.25 ml [0.125 g] (0.05%)
Na-PPi (5%)	1.0 ml [0.05g] (0.02%)	0.5 ml [0.025g] (0.01%)
dH ₂ O	222.75 ml	245.75 ml

Notes: The above wash volumes are approximated for a 10 X 15 cm filter.

Lay the membrane on a piece of paper towel or a kim wipe to blot the excess moisture away.

Prepare a “mount” for the membrane 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. No other brand will work. There is something about other brands of plastic wrap that cause high backgrounds in autoradiograms.

Lay the membrane on top of the Saran wrapped mount.

Cover this with another piece of Saran Wrap.

Place into film cassette. If you use an intensifying screen the film and membrane need to go into the -80°C . (This process increases signal intensity approximately three-fold.)

The membrane may be stripped of probe and hybridized a second and thirdetc times by:

Stock Solutions for DNA/Southern (and RNA) blot analyses

	<u>Stock Solution</u>		<u>Conc</u>
	<u>1L</u>	<u>20L</u>	
(1) <u>Denaturation Solution</u>			
NaOH	20g	400g	0.5M
NaCl	58.4	1,168g	1.0M
ddH ₂ O to	1L	20L	

(2) <u>Neutralization Solution</u>			
NaCl	175.4	3,508g	3.0M
Tris Base	60.6	1,212g	0.5M
ddH ₂ O to	~0.9L	~19L	
pH with HCl to	~7.4	~7.4	
ddH ₂ O to final volume	1L	20L	

(3) <u>20X SSC</u>			
NaCl	175.25	3,505	3.0M
Na-Citrate	88.2	1764	0.3M
dH ₂ O to just under	1L	20.0L	
pH to	~7.0	~7.0	
ddH ₂ O to final volume	1L	20.0L	

(4) Formamide

Formamide stock solution is deionized by stirring with AG 501-X8 (D) ion exchange resin (BIORAD) for 30 min and subsequently filtering/decanting. USE CAUTION when handling Formamide. Absolutely wear gloves. Formamide is toxic. It is targeted to the reproductive organs.

(5) 25X SSC

	<u>Stock</u>	<u>Conc</u>
1.	NaCl 43.8g	3.75M
2.	Na-Citrate 22.1g	0.375M
3.	pH w/HCl to 7.0	
4.	ddH ₂ O to 250ml	
5.	f, A/C	

(6) 2M Phosphate Buffer (2M PB)

1.	Na ₂ HPO ₄ 28.3g
2.	ddH ₂ O 160ml
3.	Mix until dissolved (heat ok)
4.	NaH ₂ PO ₄ 27.5g
5.	Mix until thoroughly dissolved
6.	Bring vol to 200ml
7.	F, A/C

(7) 100X Denhardt's

1. BSA 0.5g
2. PVP 0.5g
3. Ficoll 0.5g
4. ddH₂O to 25.0ml
5. Filter through cinder-glass millipore
6. Store at -20°C

(8) 10% SDS

1. SDS 10g
2. ddH₂O to 100ml
3. Sterile filter

(9) 50X Dextran sulfate

1. Dextran sulfate 25g
2. Sterile H₂O to 50ml
3. Heat/Vortex to dissolve
4. Store -20°C

(10) Fish (Herring Sperm) DNA

1. Dissolve 100mg of Herring sperm DNA in 10 ml ddH₂O. A combination of vortexing, heat and time are required to get the DNA to completely dissolve.
2. Shear the DNA by vigorously passing it through a small gauge needle fitted onto the appropriate sized syringe. Do this 20 or more times.
3. Add an equal volume TE equilibrated phenol.
4. Vortex and spin.
5. Transfer the aqueous layer to a new tube.
6. Add an equal volume of TE equilibrated phenol – Sevag (50:50).
7. Vortex and spin.
8. Transfer the aqueous layer to a new tube.
9. Add an equal volume of Sevag.
10. Vortex and spin.
11. Transfer the aqueous layer to a new tube.
12. Add 1/10 volume 3M NaOAc (pH 5.2-6.0).
13. Add 2X volume EtOH.
14. Invert to mix well and spin for 1-2 min.
15. Decant off supernatant.
16. Wash pellet with 70% EtOH.
17. Spin, Wash and Dry the DNA pellet.
18. Resuspend DNA in T₁₀E_{0.1} pH 7.5 to ~20mg/ml.
19. Boil 10 minutes to denature.
20. Place on ice; it is now ready for use. Store at -20°C.
(Alternatively boil just before use.)

(11) 5% PPi

1. Sodium pyrophosphate 7.5g
2. ddH₂O to 150ml
3. F, A/C