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TO ERLE:
FROM RICH

I'M NOT SURE
THAT WILL MAKE ANY
SENSE - GIVE ME A
CALL

RICH
~

PCR
SOUTHERN ANALYSIS

A. ISOLATION AND RESTRICTION OF GENOMIC DNA FROM CULTURED CELLS:

- SKIP*
1. Wash 1-3x10⁷ cells in PBS.
 2. Resuspend in 2-3 ml 50mM Tris (pH8.0), 50mM EDTA, 1% SDS, 200 ug/ml protease K (made fresh), in 15ml polypropylene tube. Pipet vigorously (a bulb works best) to shear the DNA a bit.
 3. Incubate ^{37°} 4hr-0.N.
 Extract with sec-butanol to 0.4-0.5 ml volume, and transfer to Eppendorf tube. All solutes get concentrated in the aqueous phase. Bear this in mind during subsequent extractions.
 4. Add 1/10 volume 3M NaAc.
 5. Extract 2x with equal volumes phenol/chloroform or any interface clean.
 6. Extract 2x with chloroform. If there is a white interface, there is residual SDS. ~~It should be removed with a few more phenol/chloroform extractions before proceeding.~~ *remove w/ mult. pptns.*
 7. Extract 1x with water saturated ether. Aspirate the bulk of the upper phase w/ a pipet being careful not to suck up the viscous lower DNA phase. Cook off the residual ether @ 68c x 15'.
 8. Ethanol ppt with 2 vol 70% ETOH. DO NOT use 95% ETOH for the first pptn. The DNA should precipitate without chilling and look like white or dirty white cobwebs. *ADN NaACETATE*
 9. Spin DNA in microfuge for 10' @ 15000 ¹⁵ 40°. Pour off the supt. and resuspend pellets (without washing) in 300ul T10E1(pH8.0) + 50ug/ml RNase A. Incubate @ 37c x 1hr. on a multivortexer if possible or with occasional pipetting to homogenize the DNA.
 10. Reprecipitate DNA using NH4Ac and 95% ETOH. Spin and wash with 70% ETOH.
 11. Resuspend in 100-300 ul T10E1. Pipet up and down to homogenize. Spin briefly in microfuge to pellet insoluble crud. *Reprecipitate 1-2x if desired to remove SDS.*
 12. Dilute 1:200 and measure OD.

I DON'T BOTHER TO OD ANYMORE.

I DO NOT CUT λ WITH MY DNA

- 14. To 10ug genomic DNA, add 2.5ug lambda DNA (or other appropriate DNA that is cut with restriction enzyme to be used), and incubate with 2-3 fold excess of restriction enzyme, i.e. 25-35 units, for 2-3 hours. Remember to set up control digestion with lambda DNA alone. The extent of digestion is monitored via the appearance of completely digested marker DNA.
- 15. Check cutting on minigel. Load 1/20th of total digestion. Repeat digestion if needed.

DON'T BOTHER DOING

~~I CUT EN~~

I CUT EN ⁵⁰ ~~50~~ λ FOR
 A COUPLE OF HOURS THEN
 ADD ANOTHER 30 λ OF
 BUFFER, H₂O & ENZYME

B. SOUTHERN BLOTTING OF GENOMIC DNA:

*FOLLOW
POST-BLOT
PROTOCOL*

O. WEAR GLOVES!

1. Load 10ug/well of genomic DNA on prep gel of appropriate agarose concentration, and run OT at 40 v. Remember to load size markers. (If lanes are to be compared quantitatively, repeat O.D. on equal aliquots of digested DNAs and marker control. Subtract marker contribution and calculate genomic DNA concentration in each sample).
2. Photograph with ruler. Transfer gel to a glass plate and place into a large Pyrex baking dish.
3. Add sufficient 0.25M HCl to cover gel (about 700 ml) and incubate 20' @ 25c, gently shaking on an orbital shaker. The BPB dye should have turned yellow. This treatment nicks the DNA to facilitate transfer of larger fragments.
4. Discard HCl (most easily done by lifting gel on plate from dish) and denature in 1.5M NaCl, 0.5M NaOH for 1hr as above.
5. Neutralize 1hr or more in 1M Tris(pH8.0), 1.5M NaCl.
6. Fill a second baking dish about half way with 10x SSC, and place plexiglass slab or glass plate across the rim. Wet several sheets of 3MM Whatman with 10x SSC, and place on Plexiglass (excluding air) to act as wick. Remove plate and gel from neutralizing bath. Wet another piece of filter paper and place on top of gel. Place another glass plate on top, and invert. Remove first plate (slide, don't lift), pick up gel by filter paper under it, and transfer to wick. Cut top right hand corner to orient after blotting.
7. Cut Genescreen to gel size +1 cm around. Wet in dH2O, then 10xSSC with shaking for 10'. Place wet convex side on gel ("B" side as per Genescreen protocol). Frame gel with Parafilm. Cover with one sheet filter paper (wet with 10xSSC) cut to same size as Genescreen. Place 2-3" of paper towels on top, then glass plate, and small weight (empty 100 ml bottle).
- 8: Allow transfer to proceed OT, change towels and continue 4-6h.
9. Invert genescreen, gel and bottom filter paper onto a glass plate. Remove filter paper, cut genescreen exactly at gel origin and at nicked corner.
10. Place Genescreen in .4M NaOH for thirty seconds. Neutralize with 2x SSC, 0.2M Tris(pH 7.5) for several minutes. Lay onto dry 3MM to blot gross liquid from filter.
11. Dry filter paper clipped flat between two sheets of 3MM for 1hr. in 80c vacuum oven. Dried blot may be stored.

C. HYBRIDIZATION ON GENESCREEN:

1. Transfer dried blot to a seal-a-meal bag and wet with dH₂O. Incubate minimum 3hrs. in ~~30-50~~ 50mls prehybridization solution @ 68c or other suitable temperature.

For 50 mls. add IN ORDER:

- 2.0ml sheared, boiled salmon sperm DNA (5mg/ml)
 200ul 5M NaOH - mix to denature
 200ul 5M HCl - mix to neutralize*
 1.0ml 50x Denhardt's solution (see Maniatis)
 2.5ml 20% SDS (final=1.0%)
 15ml 20x SSC
 50mM TrisHCl (pH 7.5) to 50ml 2.5 ml

* a persisting white ppt indicates excess acid

2. Prepare a suitable probe and determine total cpm's. To 0.5ml SS carrier DNA add q.s. of probe to give $1-4 \times 10^{-6}$ cpm/ml of prehyb. soln. Denature together as above and add to blot in prehyb.

3. Incubate ^{OVERNIGHT} at 68 degrees.

4. Wash 2 x 10' in 200 ml 2X SSC 25c.
 Wash 2 x 30' in 200 ml 2X SSC + 1% SDS, at 68c.
 Wash 2 x 30' in 200 ml 0.1X SSC, at 68c (stringency).

5. Check blot with Geiger counter. If unacceptably hot, continue washes in 0.1X SSC.

6. Do not allow to dry completely if blot is to be stripped: Remove from stringency wash and lay onto prewet (water) 3MM sheet small enough to fit into a film cassette. Wrap in plastic and expose to film.

7. Blots can be stripped as follows:

Incubate in 0.4M NaOH for 30' @ 65c
 Neutralize and wash in 2X SSC/1% SDS 1hr. @ 65c
 Repeat as needed

I WRT WITH H₂O ACCORDING TO

GENE SCREEN PROTOCOL BOOK

I DO NOT HAVE SS DNA ON MY HYBRIDIZATION

SPECIFICATIONS FOR THE PRESSURE CONTROL STATION

1. Weight: 13 lbs
2. Dimensions: 28 cm W x 18 cm H x 16.5 cm D

SPECIFICATIONS FOR THE POSIBLOT PRESSURE BLOTTER

1. Weight:

Small (Cat # 400170): 1.8 kg
Large (Cat # 400171): 4.2 kg

2. Dimensions:

Small:

Outer: 19.5 cm W x 10 cm H x 19.5 cm D
Inner: 14 cm x 14 cm (gel support area)

Large:

Outer: 35.5 cm W x 11 cm H x 25 cm D
Inner: 28 cm x 18 cm (gel support area)

COMPONENTS

Top Lid: Durable acrylic lid compresses the gaskets evenly.

Mask: Verilon™ mask is easily cut to fit gels.

Membrane Support: Cleans easily with warm water to stay free of contaminants.

Buffer Collection Base: Durable acrylic and easily washable.

Draw Latch: Stainless steel latch which is adjustable to assure a tight seal.

Drain Valve: Allows for easy draining of reservoir.

Inlet port: Durable nylon barb connector attaches to Pressure Control Station and allows pressurizing the unit.

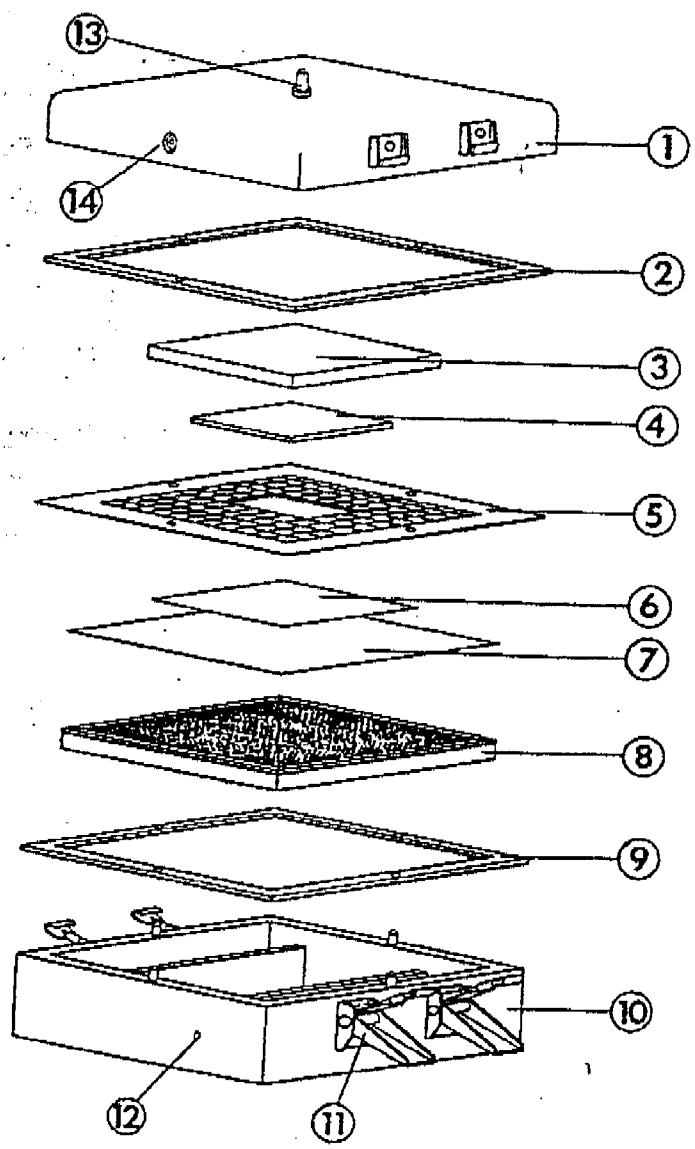
Pressure Relief Valve: Protects unit from overpressurization.

Cellulose Sponge Reservoir: Reusable cellulose matrix keeps gel well hydrated with transfer buffer. It is packed in a compressed, desiccated state and must be rehydrated before use (see section C step1).

DIAGRAM

1. Top Lid
2. Gasket
3. Sponge
4. Gel
5. Mask
6. Membrane
7. Filter Paper

8. Membrane Support
9. Gasket
10. Buffer Collection Base
11. Draw Latch
12. Vent
13. Inlet
14. Pressure Relief Valve



OPERATING INSTRUCTIONS

Introduction

The following guidelines were determined using Stratagene's line of hybridization membranes. When compared to the standard capillary technique, the PosiBlot pressure blotter will decrease transfer time from 12 hours to less than 1 hour while maintaining excellent resolution and sensitivity. Procedures are performed at room temperature.

Best results are obtained when gels are 0.5 cm thick or less.

A. Pre-treatment of Gels

Pre-treatment of gels prior to Southern transfer

1. Following electrophoresis, stain gel in 5 µg/ml ethidium bromide in water; destain in water and photograph the gel.

2. DEPURINATION

1.5 mm or 3 mm vertical gels:

Treat agarose gels with 0.25 N HCl for 5 minutes with gentle shaking. HCl solution should cover the gel completely.

5 mm horizontal gels:

Treat gels with 0.25 N HCl for 15 minutes with gentle shaking

10 mm horizontal gels:

Treat gels with 0.25 N HCl for 30 minutes with gentle shaking.

NOTE: The bromophenol blue dye should turn to green by the end of the HCl treatment.

3. DENATURATION

Pour off the HCl and add 0.5 N NaOH, 1.5 M NaCl. Add enough solution to cover gel.

1.5 or 3.0 mm gels:

Treat gels for 15 minutes

5 mm or 10 mm gels:

Treat gels for 30 minutes to 1 hour

4. NEUTRALIZATION

Pour off the denaturing solution and add 1 M Tris pH 7.5, 1.5 M NaCl. Add enough solution to cover gel.

1.5 mm or 3.0 mm gels:

Treat gels for 15 minutes

5 mm or 10 mm gels:

Treat gels for 30 minutes to 1 hour.

Pre-treatment of gels prior to Northern transfer (formaldehyde gels)

1. Following electrophoresis, stain gel in 5 µg/ml ethidium bromide in water; destain in water and photograph the gel.
2. Denaturation - treat gels in 0.05 N NaOH - 0.15 M NaCl for 30 minutes to 1 hour.
3. Neutralization - pour off the denaturing solution and add 0.1 M Tris pH 7.5 - 0.15 M NaCl for 30 minutes to 1 hour.

B. Preparation of the membrane

1. Depending on your application, any of Stratagene's high quality hybridization membranes may be used. Always handle membranes with gloved hands.

DURALOSE UV™ MEMBRANES (Cat# 420111-420115)

Duralose UV™ membranes are reinforced nitrocellulose membranes that combine the strength of nylon membranes with the lower background generally observed with nitrocellulose membranes (especially with non-radioactive methods). Duralose UV membranes are 500 times stronger than ordinary nitrocellulose membranes, making them ideal when reprobing Southern, Northern, dot and slot blots as well as colony and plaque lifts. Duralose UV membranes bind 80 - 100 µg of DNA or RNA per cm².

NITROCELLULOSE MEMBRANES (Cat# 420106-420110)

Stratagene's nitrocellulose membranes are an ideal choice when performing Southern, Northern, slot and dot blot analysis. The convenient range of sizes also makes them ideal for colony and plaque lifts. Each lot of nitrocellulose membranes binds 80 - 100 µg of DNA or RNA per cm².

DURALON UV™ MEMBRANES (Cat# 420100-420105)

Duralon UV™ membranes are uncharged nylon membranes that exhibit exceptional strength and increased sensitivity when compared to traditional nitrocellulose membranes. Duralon UV membranes are an ideal choice for hybridization of low copy number sequences or when multiple reprobing is required. Duralon UV membranes will exhibit high background with color development assays and, therefore, should not be used with non-radioactive probes. Each lot of Duralon UV membranes binds up to 500 µg of DNA or RNA per cm².

2. Pre-wet the membrane by first soaking in H₂O for 20 minutes and then in transfer buffer; 10X SSPE or 10X SSC and soak for an additional 10 minutes. Alternatively, 25 mM sodium phosphate pH 6.5 can be used for Southern type transfers.

C. Assembly of pressure blotter (see diagram)

1. The cellulose sponge serves as a buffer reservoir and is shipped desiccated and compressed. It must be expanded before use by soaking in a tray of water for about 5 minutes. Once the sponge is fully rehydrated, wring out the water before saturating with buffer (step 7). This rehydration procedure needs to be performed only once per sponge, since the sponge will remain expanded.
 2. Carefully cut out a rectangular hole in the plastic mask (5) that is slightly smaller than the area of the gel that will be subjected to the pressure gradient. Imagine a rectangle that is formed by the distance from the wells to the bottom of the gel in one dimension and the width of the gel in the other dimension. The hole that should be cut in the mask should be smaller than this rectangle by at least 0.3 cm on all 4 sides.
 3. Place the membrane support (8), screen side up in the buffer collection base (10).
 4. Cut both the membrane (Wearing gloves) and a piece of 3 MM Whatman paper such that they are both larger than the hole in the plastic mask. Prewet both the membrane and the Whatman paper as described in section B and place on the membrane support (8); 3 MM paper first, then the membrane. Smooth out the membrane so that no wrinkles or air bubbles are present.
 5. Place the plastic mask(5) over the box and secure it via the alignment pins. Make sure that the edges of the plastic that form the rectangular hole overlap both the 3MM paper and the membrane on all four sides.
 6. Place the treated gel over the mask such that the upper edge of the rectangular hole lines up below the row of wells and the other 3 edges are overlapped by the gel. Make sure there are no air bubbles trapped under the gel.
 7. Place the cellulose sponge (3) in a tray filled with transfer buffer. Saturate the sponge by squeezing it and allowing it to absorb the buffer. Raise the sponge out of the fluid and hold it horizontally* until excess buffer leaving the sponge changes from a stream to a drip. At this point gently lay the soaked sponge over the gel assembly.
- *The sponge must be held horizontally in this step. Otherwise, too much buffer will drain out resulting in poor transfer.
8. Place the lid (1) on top of the apparatus using the pins for alignment and clamp down.
 9. Attach the hose from the Pressure Control Station* to the blotter inlet port. Make sure the pressure control knob is turned fully counter-clockwise. Turn on the Pressure Control Station and adjust pressure to 75 mm Hg. If the pressure exceeds 150 mm of Hg the pressure relief valve is clogged. Turn the Pressure Control Station off and clean the lid with warm water.

*Do not attach the PosiBiot pressure blotter to anything other than Stratagene's Pressure Control Station.

10. Blot 15-30 minutes for thin gels (<3mm) and 30 to 60 minutes for thicker gels(>5mm).

Note: Blotting times vary for different gels and depend on the following:

- Amount and size of DNA
- Size, thickness and percent of gel
- Total surface area
- Depth of gel wells
- Volume of sample loaded on gel

11. After the allotted blotting time, turn off the Pressure Control Station and disconnect the hose. Unclamp the lid and remove the sponge. Using a pencil, pierce the gel at the lanes and mark the position of the wells on the membrane, then remove the gel. The gel may be stained and destained as in section A to check the efficiency of transfer.
12. Remove the membrane from the device and place on clean Whatman 3MM paper to allow excess buffer to be absorbed. Once the membrane is free of standing liquid, but not dry, place the membrane and Whatman paper in the Stratalinker™ UV crosslinker (Cat # 400071) and crosslink for 30 seconds. Alternatively, dry the membrane in a 80°C drying oven for 1 to 2 hours. Rinse the membrane in deionized water to remove excess salt before prehybridizing.

CARE AND HANDLING

The PosiBlot pressure blotter and Pressure Control Station were designed to be durable and long lasting units; however, care must be taken to ensure proper operation and reliable performance. Failure to maintain the PosiBlot pressure blotter and Pressure Control Station in the proper condition may reduce their effectiveness.

Do not use the blotter without cutting a window in the mask.

Always be sure the PosiBlot pressure blotter and Pressure Control Station are on a flat, stable surface.

Do not allow buffer to dry up inside unit as salt crystals will clog pressure relief and drain valves. Wash the PosiBlot pressure blotter in lukewarm water after use and dry with a clean cloth. Do not use acetone, alcohol or chloroform.

Do not over-tighten the latches, since this may warp or break the lid. Leave the draw latches unlocked when storing the unit. Store the mask outside the unit on a flat surface under a book.

Rinse the sponge in fresh water after each use, then wring out the water and let dry.