

Zakian Lab 2D gel protocol

Solutions and preparation (Day 1):

1. 500 ml of sterile ddH₂O. Autoclave. Store @ 4°C.
2. 500 ml yeast medium per strain (500ml = 3 gels for rDNA, 1 gel for single copy)
3. 25 ml 1X TE buffer pH 8.0. Autoclave. Store @ 4°C.
4. 100 ml TEN buffer pH 8.0:

5 ml of 1M Tris pH 8.0	(50 mM)
10 ml of 0.5M EDTA	(50 mM)
2 ml of 5 M NaCl	(100 mM)
75 ml of ddH ₂ O	
pH to 8.0, then add ddH ₂ O up to 100ml	
Autoclave and store @ 4°C.	
5. 400 ml of NIB buffer pH 7.2 (Nuclear Isolation Buffer for 8 X 0.5L cultures)

68 ml of 100% glycerol	(17% v/v)
4.2 g of MOPS free acid	(50 mM)
5.88 g of KoAc	(150 mM)
0.8 ml of 1M MgCl ₂	(2 mM)
20.8 mg of Spermine	(150 μM)
200 λ of 1M Spermidine	(500 μM)
add ddH ₂ O to 375 ml. pH to 7.2 with KOH. add ddH ₂ O to 400 ml.	
DO NOT AUTOCLAVE. Store @ 4°C.	
6. Make 0.1 g/ml SodiumAzide mix: (5mls/500ml culture)

For 8 samples = 42.5 ml ddH₂O + 4.25 g NaAzide. Vortex. Store @ 4°C.
7. Weigh out 0.75 g Sarkosyl and place in a 15 ml conical tube.
8. Weigh out 12 mg of Proteinase K (for 8 reactions) and place in eppi tube @ 4°C.
9. If you really want to be organized:
 - Make sure you have at least 50 ml of glass beads.
 - Make sure there is enough 5 mg/ml of Hoechst Dye (1 ml for 8 samples).
 - Make sure you have 5:1 Isopropanol:ddH₂O (50 mls for 8 rxns).
 - Make sure you have 3 M KoAC, 100% Isopropanol, and 70% EtOH.
 - Make sure you have 1 opti-seal Beckman ultracentrifuge tube per strain.
 - Label 2 X 50 ml conical tubes / strain
 - Label 2X 15 ml conical tubes / strain
 - Label 3 X 1.5 ml Eppi tubes / strain (protein precipitation)
 - Label 2 X 1.5 ml Eppi tubes / strain (DNA precipitation)
 - Label 1 X 30 ml Oakridge tube / strain.
 - Organize 500 ml Nalgene bottles for spinning down cultures.

Day 3: Harvesting yeast cells for DNA isolation

1. Treat 500 ml cultures with 5 ml of 0.1g/ml Sodium Azide (4.25 g NaAzide in 42.5 ml sterile H₂O). Swirl well. Place on ice for 5-10 minutes.
2. Spin down cultures in 500 ml Nalgene bottles for 7 minutes @ 2700 RCF (4000 RPM in GS3 rotor). Discard Supernatant.
3. Add 35 ml of pre-chilled (4°C) sterile ddH₂O. Transfer to 50 ml conical tube on ice. Rinse the bottle with another 10-15 ml ddH₂O and fill the conical tube to 50 ml.
4. Spin down 5 minutes @ 2800 RPM and 4°C in the Swinging bucket centrifuge. Discard Sup.
5. Resuspend pellets in 5 ml of ice-cold NIB.
6. Place cultures @ -80°C if doing the prep another day OR proceed to step 2 of the Genomic DNA purification protocol (below).

Day 3: Genomic DNA Purification

1. Thaw cultures from -70 freezer (frozen in NIB in 50 ml conical tubes with a volume of 5-6 ml).
2. Add 7 ml glass beads to each sample. Vortex samples in cold room using maximum vortexing power. Vortex samples 6 times for 1 minute each time. Check for ~80-90% lysis under the microscope. Vortex until at least 80% lysis is seen.
3. Add 5 ml ice-cold NIB, mix with the beads, and transfer the top (no glass beads) to oak-ridge tubes. Add 10 ml NIB to the glass beads, mix, and transfer again to the same oak-ridge tube. Repeat with 10 ml NIB.
4. Spin samples 20 minutes at 4 degrees and 7600 RCF (7300 RPM in SA-600 rotor).
5. While samples are spinning, prepare 0.6 ml of 20 mg/ml Proteinase K.
6. While samples are spinning, make a 7.5% w/v solution of Sarkosyl (10 ml TEN buffer + 0.75 g of Sarkosyl). Resuspend by gentle rocking to avoid too many bubbles.
7. Carefully discard supernatant from step 4. Resuspend pellet in 1 ml of ice-cold TEN buffer. **IMPORTANT:** Resuspend the pellet using a p1000 tip to "mush" the pellet into solution. **DO NOT VORTEX. DO NOT PIPETTE UP AND DOWN.**

8. Add 2 ml of ice-cold TEN buffer.
9. Add 0.8 ml of 7.5% (w/v) sarkosyl and mix gently.
10. Add 60 λ of 20 mg/ml Proteinase K (final concentration of 300 μ g/ml) and mix gently.
11. Incubate 1 hour at 37 degrees with occasional gentle swirling of the tube.
12. Transfer the precipitated solution using large orifice p1000 tips (very viscous) in 1.3 ml aliquots (2 X 650 λ) into 3 labeled Eppi tubes. Pellet the precipitated protein by spinning 10 minutes @ 10,000 RPM and 4°C in the eppi centrifuge.
13. Using large orifice p1000 pipette tips, slowly transfer sup to 15 ml conical tubes. Remove all air bubbles and bring volume up to ~3.95 ml with ice-cold TEN buffer (meniscus should be right below the 4 ml marker).
14. Slowly pour solution into 50 ml conical tubes containing 4.15 g optical grade CsCl (1.05 g/ml of solution).
15. Rock tubes VERY SLOWLY AND GENTLY at room temp until all the CsCl has gone into solution. This can take up to 1 hour.
16. Add 0.025 volumes of 5 mg/ml Hoechst Dye #33258 (add 125 λ) and mix gently on the rocker approximately 10 minutes.
17. Carefully transfer (using cut-off p1000 tips) solution into ultracentrifuge tubes. This involves pipetting a 980 λ volume 5 times (4.9 ml total per tube). Make sure there is no liquid layer at the neck of the tube.
18. Balance tubes to within 0.025 grams (25 mg). Seal with caps.
19. Place tubes in vTi65.2 rotor and tighten caps to a torque of ~120 lbs/inch²
20. Spin tubes 16-20 hours at 55,000 RPM and 17°C.

Day 4: Isolation of DNA from the CsCl gradient:

1. Organize the following materials: (1 per tube)---1CC syringes, 16.5 gauge needles, 18 gauge needles, a ring stand, and 15 ml conical tubes pre-labeled with sample names.
2. Stop ultracentrifuge spin. Carefully carry rotor into the dark room (**KEEP LEVEL--DO NOT TILT!!**). Remove caps slowly with care taken not to tilt the rotor.
3. Transfer tube to ring stand and secure tightly.
4. Poke a hole in the top of the tube by inserting an 18 gauge needle (leave needle in tube).

5. Shine hand-held UV lamp on the tube and mark tube (with a sharpie) ~5-7 mm below the genomic DNA and rDNA band. Turn off the UV lamp while extracting DNA (avoid nicking).
6. Attach a 16.5 gauge needle to a 1CC syringe. "Loosen" the seal on the syringe by pulling back. Insert the needle into the tube at the site of the mark. Be careful not to go all the way through to the other side of the tube. **SLOWLY AND METHODICALLY** draw the DNA into the syringe. Typically you pull off ~ 0.7 ml per tube (stopper on syringe reads ~0.75 to 0.8). When done, remove the needle on top and dispose of properly. Pull the needle off the syringe (dispose of tube + needle) and slowly transfer your DNA into the labeled 15 ml tube.
7. Extract Hoechst Dye using a 5:1 Isopropanol:H₂O mix. Add 1 ml, rock gently 10-15 minutes, let stand upright ~ 2 minutes, and discard top layer into waste. Repeat for a total of 5 times.
8. Using cut-off p1000 tips, gently aliquot 350 λ of DNA into 1.5 ml eppi tubes (should have 2 eppis per ultracentrifuge tube. Precipitate DNA by adding 1.1 ml of ice-cold 70% (v/v) ethanol. Mix gently. **DO NOT VORTEX**. Spin 20 minutes at 14,000 RPM and 4°C.
9. Slowly pour off sup. Wash pellet 2 times with 1.2 ml of 70% (v/v) ethanol. Spin 5 minutes after each ethanol addition and discard sup carefully. On the final wash, remove 980 λ of the sup, bump down the sample in the mini-fuge and remove the rest of the sup (without touching the pellet) with a p200.
10. Air dry the pellet on the bench top for ~ 10 minutes.
11. Resuspend pellet in a total volume of 400 λ 1X TE buffer (make sure pH is = 8.0). (2 tubes = 200 λ /tube). Resuspend gently. **DO NOT VORTEX!!!** It is ok to **SLOWLY** pipette up and down using large orifice tips to promote mixing.
12. Gently bump sample down and store at 4°C for ~24 hours.

Day 5: Resuspension & pooling of genomic DNA:

1. At least three times during this time gently flick the tubes to promote resuspension of the genomic DNA. It is ok to **SLOWLY** pipette up and down using large orifice tips.
2. After 24-30 hours pool all the tubes for each sample (large orifice tips) and store at 4°C for an additional 12+ hours. Mix by slow pipetting up and down using large orifice tips.

Day 6: Digestion of genomic DNA & starting the first dimension:

1. Use the spectrophotometer to determine the approximate concentration of your DNA:

Dilute DNA: 10 λ DNA + 490 λ ddH₂O. Spec and determine concentration:

Concentration = OD₂₆₀ X .05 μ g/ λ X 50 (dilution factor)

- 1.
- 2.
- 3.
- 4.
- 5.
- 6.
- 7.
- 8.

2. Set up to digest ~10 μ g of DNA per sample in a 600 λ reaction volume:

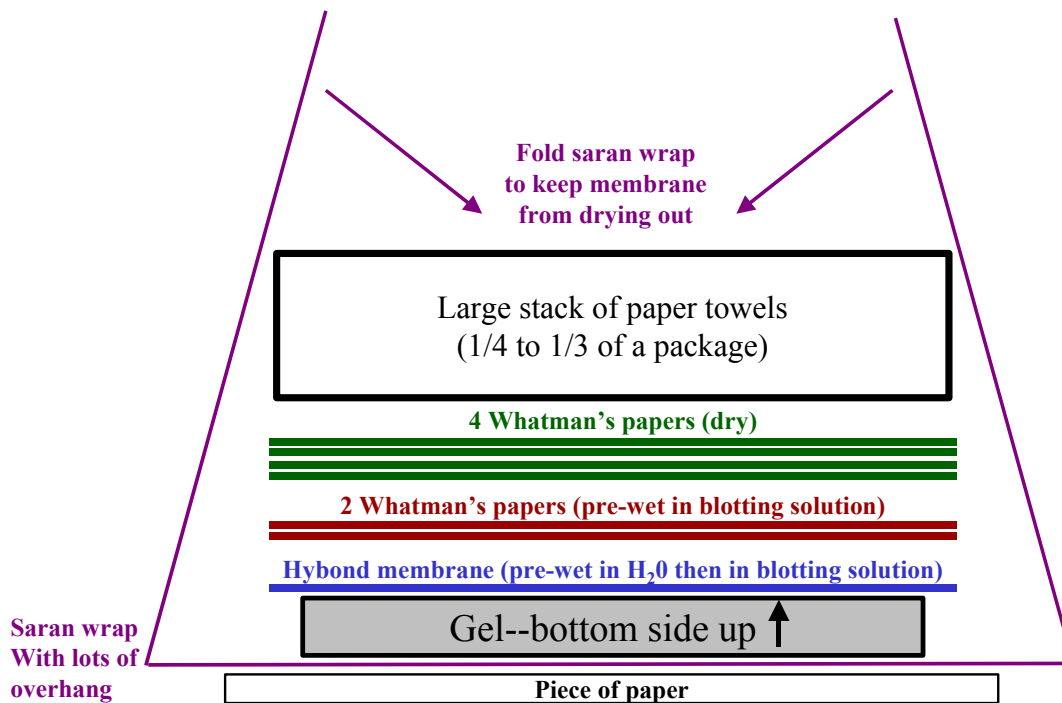
DNA + ddH₂O should be equal a total volume of 150 λ , so add the appropriate volume of DNA to each tube to make 10 μ g and then bring the volume up to 150 λ .

Enzyme mix should be in a volume of 450 λ per sample. DNA is typically digested with 300-400 units of enzyme for 5-6 hours at 37°C.

3. Mix DNA with enzyme mixes by gently pipetting up and down using large orifice tips. **DO NOT VORTEX YOUR SAMPLES.**
4. Digest DNA 5 hours at 37°C. Every hour gently mix DNA in tube by lightly flicking the tube and the bumping down and placing back at 37°C.
5. While DNA is digesting, make your 0.35% (w/v) agarose gel mix: 400 ml 1X TBE + 1.4 g Agarose in a 1 Liter glass bottle. Heat in the microwave until all agarose has gone into solution. Check for complete resuspension by swirling the bottle and looking at the bottom for agarose that is not fully in solution. Store @ 55°C for about 30-40 minutes to cool to a good pouring temp. Pour the gel in the cold room. The gel tray should be 15 cm X 25 cm and should be leveled within the BioRad Sub-cell GT gel box. 1 gel allows for up to 8 samples. Once solidified, move gel to room temp. Set up for first dimension by adding the 1.6L - 1.8 L of 1X TBE needed to fill the gel box and then remove the comb.
6. Confirm complete digestion by running an agarose gel of your digested DNA alongside undigested genomic DNA on a small 0.5% gel. Room for photo of gel(s) on next page.

7. Precipitate digested DNA:
 - Add 60 λ of 3 M KoAc (pH 5.5)
 - Add 700 λ of 100% Isopropanol.
 - Mix gently by slowly inverting the tube several times
8. Spin down your precipitated DNA 20 minutes @ 14,000 RPM and 4°C. Pour off the sup and wash the pellet 2X with 1 ml of 70% EtOH (5 minute spins each time as above). Remove as much of the second wash as possible with a p200. Dry on the bench-top ~ 10 minutes. Resuspend with 16 λ of 1X TE pH 8.0. **DO NOT VORTEX**. Resuspend by flicking the tube very gently and leaving on ice or at room temp for ~ 1 hour. After 1 hour add 7 λ of 5X dyes, mix gently and bump down.
9. Load KB ladder in the first lane. Skip two lanes and then load 20 λ of the DNA samples in every other lane.
10. When done loading, start the gel. Run the gel 42-48 hours @ 22 volts @ room temp.
11. Set up for the next dimension by pre-chilling 2 L of 1X TBE for every 4 samples to be run. Place 4L of buffer in the cold room. Note: Add 60 λ of 10 mg/ml EtBr to every 2L of pre-chilled 1X TBE (0.3 μ g/ml EtBr final concentration) either now or right before using.

6. Transfer the gels 12+ hours as outlined below. Use Hybond N+ nylon membranes.



Day 10: Hybridization using Church's Buffer:

How to make Church's Buffer (Recipe for 1 liter)

2 ml of 0.5M EDTA pH8.0 (1 mM)
 2 ml of 85% Phosphoric Acid (H₃PO₄)
 67 g of anhydrous sodium phosphate dibasic (Na₂HPO₄) (0.5M)
 70 g of SDS (laurel sulfate) (7%)
 ddH₂O to 1 Liter

Gently heat on stir plate while mixing to get everything into solution (can take 2-3 hours).
 Store at room temp. **Heat to 65°C before using.**

1. Pre-heat Church's Buffer to 65°C (7.5 ml for small tube and 15 ml for large tube)
2. Take down the Southern transfer and gently rinse blots 2 times in 250-500 ml 2X SSC or 2 times in 250-500 ml of 1:10 blotting solution.
3. X-link DNA to blots using the Stratalinker and the autocrosslink function.

4. Pre-wet blot(s) with dH₂O and place them into large hybridization oven tubes back to back (DNA sides facing away from each other—it is fine to have the DNA from one of your blots in direct contact with the glass).
5. Pre-hyb membranes with 15 ml pre-heated Church's buffer for at least 5 minutes (longer ok).
6. Label probe with *Rediprime II* random prime labeling kits. Denature probe 5 min. at 95°C then snap cool on ice. Purify out unincorporated counts using the G-50 spin columns. Add probe and denatured herring sperm DNA (final concentration of 100 µg/ml--add 150 µl of stock to each 15 ml of Church's) to fresh Church's buffer at 65°C. Pour off pre-hyb (can go down drain) and add hyb solution with your probe and carrier DNA to the tube.
8. Incubate tube overnight at 65°C in hyb oven.

Day 11: Washing blots & putting down on film:

1. If you wish to save your probe for future hybridizations, pour back into 50 ml conical tube and store at 4°C (It will turn into a solid at this temp-this is ok). Heat to 65°C before the next hybridization.
2. Wash the blots in blot wash I (1X SSC 0.1% SDS) in the tubes at 65°C. Do 3 washes of at least 5-10 minutes each. The first wash should go into liquid waste, the other washes can go down the drain. Next, wash blots 2 times for 10 minutes each in 65°C blot wash II (0.1X SSC 0.1% SDS) in baking dishes with gentle agitation. Two blots, back-to back, can go in the same baking dish as long as they are not stuck together during washing. Wrap blots and place on film.

Misc. Comment: Stringency can be increased or decreased by altering the temperatures of hybridization and/or washes as well as by adding Formamide.