Two-dimensional Gel

(From the Zakian laboratory)

Day 1: <u>Solutions and preparation</u>

Sterile ddH_2O

- 500 ml

Autoclave, store at 4° C.

Yeast medium

- 500 ml per strain (500 ml = three gels for rDNA, one gel for single copy.)

1X TE buffer

- 25 ml

pH to 8.0, autoclave, store at 4° C.

TEN buffer

- 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_20

pH to 8.0, add ddH_20 to 100ml, autoclave and store at 4° C.

NIB buffer — nuclear isolation buffer (for 8 x 0.5 L 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_20 to 375 ml, pH to 7.2 with KOH, add ddH_20 to 400 ml. **Do not autoclave.** Store at 4° C.

Sodium azide (0.1 g/ml, 5 ml/500 ml culture)

- For 8 samples: 42.5 ml ddH_20 + 4.25 g Na-azide

Vortex, store at 4° C.

Sarkosyl

- 0.75 g

Put in a 15 ml conical tube.

Proteinase K

- 12 mg (for 8 reactions)

Put in Eppendorf tube at 4° C.

If you really want to be organised

1. Make sure you have at least 50 ml of glass beads.
2. Make sure there is enough 5 mg/ml of Hoechst Dye (1 ml for 8 samples).
3. Make sure you have 5:1 isopropanol:ddH_20 (50 mls for 8 rxns).
4. Make sure you have 3 M KoAC, 100% isopropanol, and 70% EtOH.
5. Make sure you have 1 opti-seal Beckman ultracentrifuge tube per strain.
6. Label 2 X 50 ml conical tubes/strain.
7. Label 2X 15 ml conical tubes/strain.
8. Label 3 X 1.5 ml Eppendorf tubes/strain (protein precipitation).

9. Label 2 X 1.5 ml Eppendorf tubes/strain (DNA precipitation).
10. Label 1 X 30 ml Oakridge tube/strain.
11. Organize 500 ml Nalgene bottles for spinning down cultures.

Days 1-2: <u>Yeast culture growth</u>

1. Pick a single colony or part of a single colony. Inoculate 5 ml cultures (or 10 ml if 1L cultures are grown) and grow for 24 h (30° C strains) or 48 h (ts strains, 37° C or 23° C) in the appropriate medium at the appropriate temperature.
2. Spec your small cultures and record the OD_{660} in the space below:

Colony name	**OD_{660}**	**Colony name**	**OD_{660}**

3. Make dilutions of your cultures such that they will grow overnight and be at an OD_{660} of ~0.6 (1.2 on our spec) to 1.0 (2.0 on our spec) when they are to be harvested (during log phase growth). Use the following equation to dilute your samples:

Desired OD_{660} / Actual OD_{660} x Volume of new culture = Amount to be added

4. Inoculate large cultures and grow until they reach log phase. Follow growth progress by monitoring OD_{660} over time. Harvest as described in the following section the appropriate time.

Time ⟶								
Strain	**OD_{660}**	**OD_{660}**	**OD_{660}**	**OD_{660}**	**OD_{660}**	**OD_{660}**	**OD_{660}**	**OD_{660}**

Day 3: <u>Harvesting yeast cells for DNA isolation</u>

1. Treat 500 ml cultures with 5 ml of 0.1g/ml sodium azide (4.25 g Na-azide in 42.5 ml sterile H_2O). Swirl well. Place on ice for 5-10 min.
2. Spin down cultures in 500 ml Nalgene bottles for 7 min at 2700 RCF (4000 rpm in GS3 rotor). Discard supernatant.
3. Add 35 ml of pre-chilled (4° C) sterile ddH_2O. Transfer to 50 ml conical tube on ice. Rinse the bottle with another 10-15 ml ddH_2O and fill the conical tube to 50 ml.

4. Spin down 5 min at 2800 rpm and 4° C in the swinging bucket centrifuge. Discard supernatant.
5. Resuspend pellets in 5 ml of ice-cold NIB.
6. Place cultures at −80° C if doing the prep another day OR proceed to step 2 of the genomic DNA purification protocol (below).

Day 3: <u>Genomic DNA purification</u>

1. Thaw cultures from −70° C 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 min 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 min at 4° C 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 µg/ml) and mix gently.
11. Incubate 1 h at 37° C 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 Eppendorf tubes. Pellet the precipitated protein by spinning 10 min at 10000 RPM and 4° C in the Eppendorf 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 (miniscus 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 temperature until all the CsCl has gone into solution. This can take up to 1 h.
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/inch^2
20. Spin tubes 16-20 h at 55000 rpm and 17° C.

Day 4: <u>Isolation of DNA from the CsCl gradient</u>

1. Organize the following materials: (1 per tube) — 1 cc 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:H_2O mix. Add 1 ml, rock gently 10-15 min, let stand upright ~2 min, 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 Eppendorf tubes (should have two Eppendorfs per ultracentrifuge tube. Precipitate DNA by adding 1.1 ml of ice-cold 70% (v/v) ethanol. Mix gently. **Do not vortex**. Spin 20 min at 14000 rpm and 4° C.
9. Slowly pour off supernatent. 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 supernatent, 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 min.
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 okay 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 h.

Day 5: <u>Resuspension and pooling of genomic DNA</u>

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

Day 6: <u>Digestion of genomic DNA and starting the first dimension</u>

1. Use the spectrophotometer to determine the approximate concentration of your DNA:
   Dilute DNA: 10 λ DNA + 490 λ ddH_20. Spec and determine concentration:
   Concentration = OD_{260} x 0.05 µg/λ x 50 (dilution factor)

   1.

   2.

   3.

   4.

   5.

   6.

   7.

   8.

2. Set up to digest ~10 µg of DNA per sample in a 600 λ reaction volume:
   – DNA + ddH_2O should equal a total volume of 150 λ, so add the appropriate volume of DNA to each tube to make 10 mg 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 h 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 h 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 at 55° C for about 30-40 min to cool to a good pouring temperature. 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 1.6-1.8 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) below.

7. Precipitate digested DNA:
    a. Add 60 λ of 3 M KoAc (pH 5.5).
    b. Add 700 λ of 100% isopropanol.
    c. Mix gently by slowly inverting the tube several times.
8. Spin down your precipitated DNA 20 min at 14000 rpm and 4° C. Pour off the supernatent and wash the pellet 2X with 1 ml of 70% EtOH (5 min 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 h. After 1 h 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 h at 22 V at room temperature.
11. Set up for the next dimension by pre-chilling 2 L of 1X TBE for every 4 samples to be run. Place 4 L of buffer in the cold room. N.B. Add 60 λ of 10 mg/ml ethidium bromide to every 2 L of pre-chilled 1X TBE (0.3 µg/ml ethidium bromide final concentration) either now or right before using.

Day 8: <u>Starting the second dimension</u>

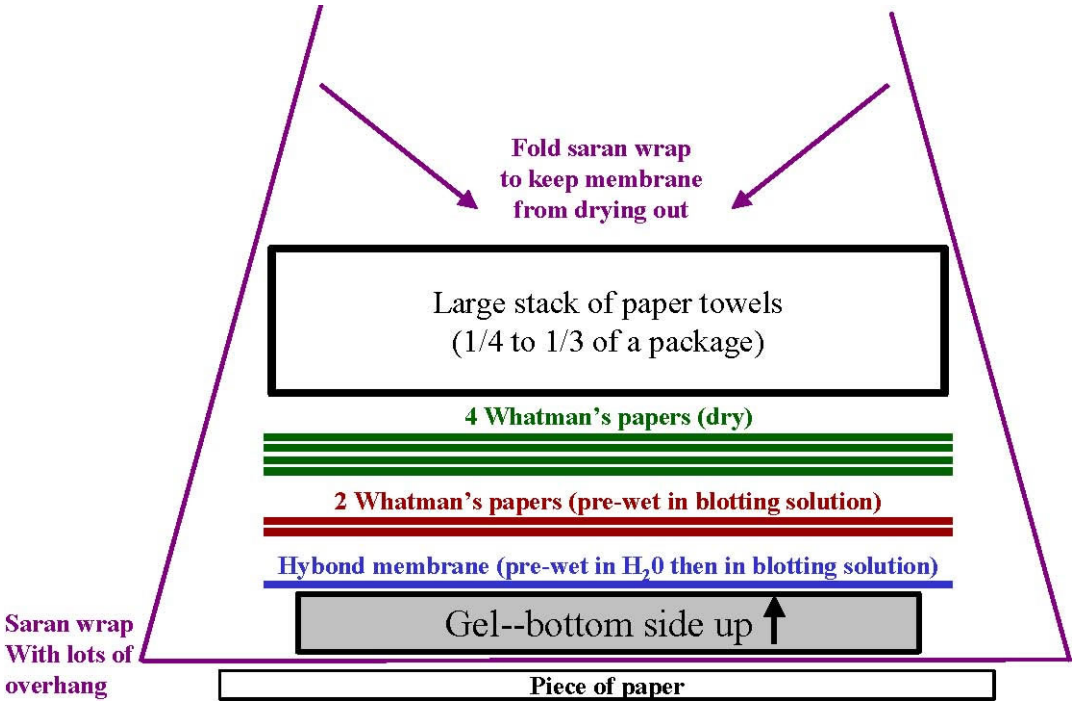
1. Make your 0.95% (w/v) agarose second dimension gel mixes: 500 ml of 1X TBE + 4.75 g of agarose + 15 λ of 10 mg/ml ethidium bromide (0.3 µg/ml final concentration). Mix in a 1 L glass bottle. Microwave on high until all agarose has gone into solution. Check by swirling the bottle and looking at the bottom. There should be no denser agarose visible. Store at 55° C until ready to pour the second dimension.
2. At the appropriate time stop the first dimension. Carefully soak the gel in a glass baking dish with 750 ml of 1X TBE with 0.3 µg/ml ethidium bromide to stain the DNA. This should take 30-40 min. The gel is very fragile, so be extra careful.
3. Excise the KB ladder lane and photograph on the eagle-eye. Photograph a ruler alongside the ladder and overlay the two. Determine which 9-10 cm slice of the gel to run in the second dimension and excise by cutting between the lanes and with the right length. It is easiest to cut out the 9 cm slices first and then to cut between the lanes while on the UV light box. Try to cut with the UV setting on preparative (long wave) to avoid nicking. You can show the KB ladder and ruler in the space provided below:

4. Transfer the slices to the 2D gel apparatus tray (4 samples per apparatus — Fischer self-circulating gel box). Place the DNA so that the higher molecular weights are to the left. Set up the apparatus in the cold room and level it.
5. Seal the edges of the gel and seal the slices in place with agarose. Pour the second dimension gel and make sure the gel slices do not move. Allow to solidify approximately 45 min to 1 h. Remove the gel dams and add the pre-chilled 2 L of 1X TBE and run the gel 18-30 h (depending on fragment size) at 130 V.
6. You can monitor the progress in the second dimension with a hand-held UV lamp. Run the gel so that the smallest fragments on the arc of linear molecules is just reaching the bottom of the gel (lower right hand corner of the gel).

Day 9: <u>Stop second dimension and Southern transfer</u>

1. Stop the gels. Cut the four gels apart in the middle, leaving you with two sets of two gels. Place in glass baking dishes and be sure to label the glass baking dishes with what is on each gel. Photograph the gels on the eagle eye (or UV light box).
2. Paste gel photo(s) here:

3. Nick DNA by placing the entire gel (in a tray is recommended) in the Strata-linker. Be careful not to touch/hit the electrical probe in the back of the Strata-linker. Use the autocrosslink function.
4. Treat each gel 30 min with denaturation solution (1.5 M NaCl, 0.5 N NaOH).
5. Treat each gel 30 min with 500 ml blotting solution (1.5 M NaCl, 0.25 N NaOH).
6. Transfer the gels 12+ h as outlined below. Use Hybond N+ nylon membranes.



Day 10: <u>Hybridisation using Church's buffer</u>

Church's buffer (recipe for 1 L)

- 2 ml of 0.5M EDTA pH 8.0 (1 mM)
- 2 ml of 85% phosphoric acid (H_3PO_4)
- 67 g of anhydrous sodium phosphate dibasic (0.5 M Na_2HPO_4)
- 70 g of SDS (lauryl sulfate) (7%)
- ddH_2O to 1 L

Gently heat on stir plate while mixing to get everything into solution (can take 2-3 h). Store at room temperature. **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 two times in 250-500 ml 2X SSC or two 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 dH_2O 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-hybridise membranes with 15 ml pre-heated Church's buffer for at least 5 min (longer is okay).
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 ml of stock to each

15 ml of Church's) to fresh Church's buffer at 65° C. Pour off pre-hybridisation (can go down drain) and add hybridisation solution with your probe and carrier DNA to the tube.

7. Incubate tube overnight at 65° C in hybridisation oven.

Day 11: <u>Washing blots and putting down on film</u>

1. If you wish to save your probe for future hybridisations, pour back into 50 ml conical tube and store at 4° C (it will turn into a solid at this temperature — this is okay). Heat to 65° C before the next hybridisation.
2. Wash the blots in blot wash I (1X SSC 0.1% SDS) in the tubes at 65° C. Do three washes of at least 5-10 min each. The first wash should go into liquid waste, the other washes can go down the drain. Next, wash blots two times for 10 min 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.

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