S.cerevisiae genomic DNA

Including an accessory protocol for making DNAse-free RNAse A (p2)

SAFETY: When working with phenol always take extreme care. Make sure lids are fully closed on the ependorffs you are vortexing – phenol burns are not pretty.

NOTE 1: Protocol is suitable for the isolation of yeast genomic DNA and the analysis thereof by Southern (Screening option A) or PCR (Screening option B). Option B is quicker, much less work and much more useful; there are few times when option A is preferable.

Extracting genomic DNA:

1. Grow overnight 5ml culture to confluence (loopful from fresh plate into media the evening before, harvest the next AM). Next morning allow tubes to sit on the bench for about 20 minutes while setting up. This is a handy method of observing possible bacterial contamination: if the prep is yeast only they will settle quickly and the top few mm of the culture will clarify.

2. Transfer to centrifuge tube and collect 3000rpm, 5', RTº. Discard supernatant and transfer pellet (packed cell volume ≈ 350µl) with ddH_2O to an ependorff tube. Flash spin (to 10K) in a microfuge to collect, and remove supernatant.

3. Resuspend pellet in 200µl breaking buffer. Add an equal volume of glass beads (Sigma G8772) tube and 200µl PCI. Vortex high speed 3' (1' vortex, 1' on ice, repeat x 2) (check: if the tube is leaking the ink on the cap will smudge). Carefully pop the lid and add 200µl TE pH8 (10mm Tris pH8, 1mM EDTA pH8). Vortex to mix and centrifuge 15000rpm, 10', 4ºC.

4. Transfer aqueous top layer to a clean tube (avoiding the white protein phase). Add 1ml EtOH (-20ºC), mix well and centrifuge 15000rpm, 10', RTºC. Discard the supernatant and resuspend the pellet in 400µl TE pH 8 (it will quickly go into solution if you dislodge it from the bottom of the tube with a pipette tip). Add 3µl 10mg/ml RNAseA (see Accessory Protocol 1 below) and incubate 5' 37ºC.

5. Add 10µl 4M NH_4OAc and 1ml EtOH (-20ºC). Mix well and place on ice for 10'. Spin in microfuge 14000rpm 10', RTºC. The quality of the pellet will be different; some at the bottom of the tube, but a significant amount coating the side. Keeping this in mind carefully remove the supernatant. Wash with 750µl 70% EtOH (-20ºC) and 14000rpm 10', RTºC. Discard supernatant, 50ºC 5' to dry and resuspend DNA in 100µl TE pH8.

Breaking buffer
2% Triton X-100
1%SDS
100mM NaCl
10mm Tris.Cl pH8
1mM EDTA pH 8
(Stable indefinitely at RTº)

PCI **(25:24:1)** 25 PhOH (Tris equilibrated)
24 $CHCl_3$
1 IsoAmyl alcohol
(Store at 4ºC)

Accessory Protocol 1: Preparation of DNAse free RNAseA (from *Molecular Cloning: A laboratory manual*, 3rd Ed Sambrook and Russell)

Dissolve bovine pancreatic RNAse A (eg. **US Biological** R2011, 250mg) in 0.01 M NaAc pH5.2 (Rather than weighing out the hygroscopic powder, it's easiest to do an entire bottle at a time and aliquot). Heat to 100ºC for 15 mins and allow to cool slowly to room temperature. Adjust the pH by adding 0.1 volume 1M Tris.CL pH 7.4. Dispense into 500µl aliquots and store at -20ºC. **Note that RNAse A precipitates when concentrated solutions are heated to 100ºC at neutral pH.**

Screening Option A: Southerns

NOTE 2: In this protocol you digest the genomic DNA from above with a restriction enzyme(s), run it out on a gel and probe. The map of fragment sizes tell you what's going on with your region of interest. There are a few things to consider:
(i) Restriction enzyme choice: obviously determined by the map of the region but there are always a few enzymes preferred (because they're cheap, relatively efficient, and 6-base cutters): EcoRI, BamHI, HindIII, XbaI, EcoRV.
(ii) Resolution: assuming a 0.8 – 1% gel (30cm), you will get efficient separation in the range 500bp – 8kB (don't think you'll be able to see the difference between a 12 and 15kB fragment)
(iii) Think it through: A southern should tell you in a second if it worked or not (**eg.** 2kb fragment Neg, 5kB Pos). If you do a assay that takes three days and can't interpret the results because you don't know what you're supposed to see then you shouldn't have done the experiment.

A.1 RE digest genomic DNA from above. To each 20µl reaction add:
- 5µl DNA (assume this to be 5µg DNA),
- 2µl 10X RE buffer (RE dependent)
- 0.2µl BSA (10mg/ml)
- 2µl enzyme (this will usually be 20 – 30U depending on RE; massive excess)
- H_2O to 20µl

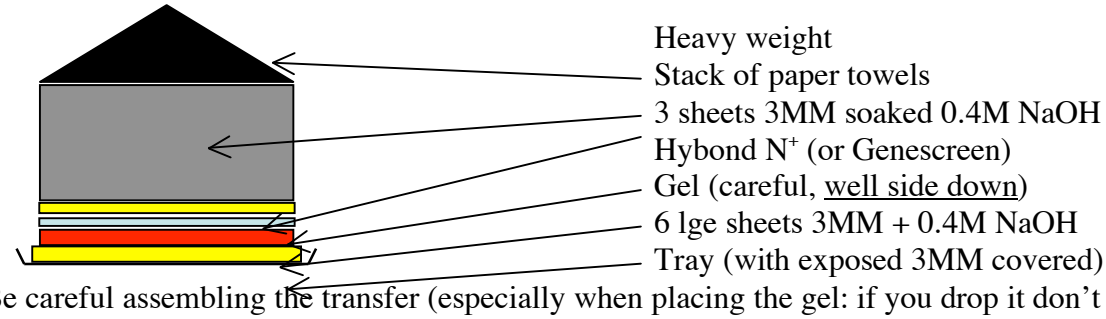
Digest 3hrs, 37ºC. Add another 10µl containing:
- 1µl appropriate 10X RE buffer
- 0.1µl BSA (10mg/ml)
- 1µl enzyme (this will usually be 20 – 30U depending on RE; massive excess)
- H_2O to 10µl

Digest another 2hrs, 37ºC (**Do not worry about * activity**). Add 6µl 6xGel loading buffer III (0.25% w/v Bromophenol blue, 0.25% w/v Xylene Cyanol FF, 30% glycerol) and use immediately or store at –20ºC.

A.2 Pour a 30cm 1% Agarose gel in 1 x TGE (with 2µl 10mg/ml EtBr/200mls gel mix). Choose a well size that allows you to load at least 20µl). Re. Agarose concentration: at lower % (0.7%) gels are very difficult to handle, at higher % (1.4%) the transfer of large fragments is not as efficient (although if you've chosen 1.4% what do you care). Place the gel in the tank and add buffer to the lip of the gel (do not submerge it completely). Pipette buffer into the wells. Then load the sample (**DO NOT FORGET MARKERS**). The advantage of dry loading is that some genomic DNA preps shoot out of the wells – this will avoid that.

A.3 Run samples: 150V 30'. Add sufficient buffer to submerge gel completely. Run 100V 3hrs or 20V overnight (latter gives slightly better resolution). Carefully transfer gel onto Saran wrap and photograph on short wave UV (with a fluorescent ruler to line up the markers to a distance). Break the DNA backbone by 90s high-power UV (avoids the HCl depuriniation step of some protocols).

Assembled in order (top --> bottom):



A.4 Be careful assembling the transfer (especially when placing the gel: if you drop it don't freak out, I've successfully transferred from re-assembled jig-saws). Ensure the weight on top is evenly distributed (and <1KG). Transfer overnight.

A.5 Disassemble apparatus carefully. Mark wells on the membrane with a pencil (aids alignment later). Depending on the transfer buffer and membrane (**check the product manual**) you may need to crosslink DNA to membrane with UV, or baking at 80ºC, or not at all. Once dried membrane can be stored at RTº between clean sheets of 3MM.

A.6 Pre-hybridize the membrane >3hrs at 65ºC.

A.7 Make 400bp – 1kb probe, although primers or fragments >1kb are occasionally used.

Option A.7.i (PCR amplification). As easy as it sounds: do a PCR with some radiolabelled nucleotide (rough conditions could be those used in the **ChIP protocol**). You can get rid of the unincorporated radiolabel by passing the PCR product through a hand-packed 1ml G50-size exclusion column (below), although it's not really necessary.

Option A.7.ii (Random priming). Bit tougher: need to isolate the probe DNA (either a PCR product or excised fragment). Estimate concentration and use 100ng/reaction. Separate probe from the unincorporated P^{32} nucleotide with a G50-size exclusion column. Size exclusion: Remove the plunger from a 1ml syringe and pack the base of the barrel with a glass-wool plug. Pack a 1ml deep G50 resin bed and equilibrate with TEN (10mM Tric.Cl pH 8, 1mM EDTA pH 8, 100mM NaCl). Assemble the following: place ependorff tube (with lid removed) in a 15ml Falcon, then put in the 1ml syringe/column. Apply 50µl random prime reaction + 50µl TEN to top of column. Spin 1200rpm 5' in a benchtop centrifuge. About 50% of the counts should be in the eluate (in the ependorff tube): this is the probe. The counts in the column are smaller unincorporated nucleotide.

A.8 Pre-hybridize the membrane >3hrs at 65ºC. When ready discard the pre-hyb, and add about 8mls Hyb buffer. Incubate 10 mins at 65ºC and add the boiled single-stranded probe. Hybridize overnight at 65ºC with rotation and ensure that no part of the membrane dries out (that would be v. bad).

A.9 Carefully remove the hybridization solution and save in a 50ml Falcon behind a radiation shield. It can be stored at RTºC and I've used it a week or two later. Before reuse puncture the cap (probe has to be denatured again) and place in a beaker of boiling water (over a bunsen burner) for 10 mins. Hybridize as in **A.8**.

A.10 Wash the membrane sequentially with:

2 x SSC / 0.1% SDS 50mls RTº 5 min
1 x SSC / 0.1% SDS 20mls 65ºC 10 min
0.5 x SSC / 0.1% SDS 20mls 65ºC 10 min

Check the membrane with a Geiger counter – if counts still dispersed try further washes ..

0.5 x SSC / 0.1% SDS 20mls 65ºC 10 min
0.1 x SSC / 0.1% SDS 20mls 65ºC 10 min

Check the membrane with a Geiger counter between these washes. When it sounds like probing up some bands expose to an ECL screen (or film). Calculate where the bands are on the membrane and use the knowledge gleaned from **Step A.5** to determine size.

Screening Option B: PCR amplification

NOTE 3: There are two main reasons you'd use this protocol: **(i)** Screen integrants, as when knocking sequences in or out. It is theoretically possible to do this without isolating genomic DNA (either by putting yeast, or even zymolase treated yeast, straight into the PCR reaction). In practice the PCRs are unpredictable under these conditions, making them next to useless. **(ii)** PCR out sequences to use for transformation. You'd think a high fidelity enzyme would always be better for this, but not really. Gel purification of the products is not a bad idea but not usually done.

NOTE 4: Re. Enzyme choice, I generally use Denville TaqPro (marketed as low fidelity; according to Tim the error rate is $\approx 1x10^{-6}$ per cycle). The higher-fidelity Denville SurePol is always great if you're worried, although the yield tends to be lower (are you really that worried?). The maximal comfortable product size is <3kb (smaller is better). Bigger (up to 4kb-ish) is doable, but strongly recommended against, especially if you're screening (when 1kb-ish is optimal).

NOTE 5: You obviously need some primers. Since you're PCRing against the entire yeast genome BLAST each to ensure it doesn't amplify multiple regions. Primers usually ≈22nt, AT° ≈ 55°C, **ALWAYS** a G/C at the 3' end.

B.1 Following conditions are for **Denville TaqPro**. For PCR screening I usually do a 20µl reaction, 50µl when amplifying regions for transformation. Following volumes are for a 50µl reaction, for smaller volumes scale down accordingly. A hot start is essential. To a PCR tube add 1µl genomic DNA and 10µl ddH_2O and place at 94°C for 5 mins. Then pause the machine, put tube on ice, and add following:

x10 Rb°	5µl
2mM dNTP	5µl
$MgCl_2$	2µl
2.5µM primer mix	5µl
Denville TaqPro	0.5µl
ddH_2O	to 39µl

Flash spin to collect. Place back on the machine, and PCR (35 cycles):

T_1	94°C	45s
T_2	55°C	45s
T_3	72°C	90s (allow 1min/kb)
T_4	16°C	∞

B.2 If PCR screening, run the reaction and look for the appropriate size fragment: you should be able to easily distinguish a targeted integrant from the WT. If amplifying a fragment for transformation, run some of the product (10µl) to confirm the appropriate size fragment. Store the remainder at –20°C and use about 4µl per transformation. You could gel purify the product but I usually don't bother.