DSB Analysis in rDNA

1. Suspend 5 x 10^7 cells in 50 µl of cold L buffer (0.1 M EDTA, pH 8, 0.01 M Tris, pH 7.6, and 0.02 M NaCl) in an Eppendorf tube containing 5 µl of cold zymolyase (zymolyase-20T, *Arthrobacter luteus*, 20000 U/gm; ICN) at a stock concentration of 20 mg/ml.
2. Warm cell suspension by incubating the tube in a 42° C water bath for a few seconds and mix briefly with 50 µl of 1% low melting agarose prepared in L buffer cooled down to 42° C.
3. Placed mixture in an ice water bath.
4. Tranfer gel plug to a 20 ml round-bottomed glass tube containing 5 ml of 0.5 M EDTA, pH 8.0 and 0.01 M Tris, pH 7.6 plus 1% β-mercaptoethanol. (The gel plug can be easily flushed out into the glass tube with the buffer).
5. Incubate the gel plug at 37° C for 24 h.
6. Remove buffer and add 0.5 ml of the same buffer containing 0.5 mg/ml zymolyase.
7. Incubate the gel plug at 37° C for 24 h.
8. Remove buffer and add 1 ml of L buffer containing 1 mg/ml proteinase K and 2.5% Sarkosyl.
9. Incubate the gel plug at 50° C for 24 h.
10. Cool gel plug on ice, remove buffer and add 0.5 ml of the same buffer containing proteinase K and Sarkosyl.
11. Incubate at 50° C for 24 h.
12. Incubate the gel plug in 5 ml of Tris-EDTA (pH 7.6) containing 40 µg/ml phenylmethysulfonyl fluoride at 50° C for 1 h.
13. Incubate in 5 ml of Tris-EDTA at 50° C for 1 h.
14. Incubate the plug with 100 µg/ml RNase A in 1 ml restriction enzyme buffer at 50° C for 3 h.
15. Remove buffer and incubate the plug in 0.5 ml of restriction enzyme buffer containing BglII (100 units) at 37° C overnight.
16. Soak the gel plug in 5 ml of Tris-EDTA at 4° C for 0.5 h.
17. Drain buffer, add 20 µl of 6X loading buffer (28) to the gel plug and incubate on ice for 0.5 h.
18. Load the gel plug into the well of a 1% agarose gel and run in TBE buffer at 1.5-2 V/cm at 4° C for 24 h.
19. Stain gel in 1 µg/ml ethidium bromide for 2-4 h, inspect under UV and use for Southern blot.

Probes and labelling-probe DNA was prepared by PCR from a rDNA plasmid with the following pairs of primers: probe A, ER5A-up, 5'-GCC ATT TAC AAA AAC ATA ACG-3' and ER5A-lower, 5'-GGG CCT AGT TTA GAG AGA AGT-3'; probe B, X35S-up, 5'-ATA TCA ACC CTG ACG GTA GAG-3' and X35S-lower, 5'-CAT GGT ATA ACT GTG GTA ATT CTA GAG-3'; probe C, BglII end-up, 5'-ACA GAT GTG CCG CCC CAG CCA AAC TCC-3' and BglII end-lower, 5'-CCT GGA TAT GGA TTC TTC ACG GTA ACG-3'. The PCR products were gel-purified. Random-primed labelling kit (Roche Applied Science GmbH) was used to label the probe DNA in the presence of four [α-32P]dNTPs.