Preparation of chromosomal DNA in agarose plugs

Materials

- 100 ml Sabouraud Liquid Medium with 100 U penicillin and 100 µg streptomycin
- 1 litre PB buffer : 1 M NaCl; 0.01 M $MgCl_2$;
  Autoclave, then add: 72 ml sterile 1 M KH_2PO_4; 28 ml sterile 1 M K_2HPO_4 to give a final pH of 5.8
- Lysing enzyme (Sigma L1412)
- 2 x plastic funnels for filtering, each lined with a folded household J-cloth, covered in foil, autoclaved and dried in an oven.
- 100 ml GMB buffer, store at 4ºC: 0.9 M sorbitol; 0.125 M EDTA; 0.01M Tris-Cl, pH 7.5. Filter sterilise.
- InCert agarose (Cambrex)
- Proteinase K
- NDS buffer, made up fresh as required: 0.5 M EDTA; 0.01 M Tris-Cl, pH 8.0; 2% Sarkosyl®
- 200 ml 0.5 M EDTA
- Whatman 54 paper

Equipment

- 250 ml baffled conical flask autoclaved
- 250 ml conical flask autoclaved
- 37ºC shaking incubator
- 34ºC shaking incubator
- 37ºC water bath
- 50ºC oven
- Buchner funnel
- Sidearm flask attached to a vacuum line
- Stirring block
- Haemocytometer and microscope

- Disposable moulds for agarose plugs (Bio-Rad)
- Wash adaptor for 50 ml centrifuge tubes (Bio-Rad)
- 50 ml centrifuge tubes

Protocol

1. Inoculate 100 ml Sabouraud Liquid Medium with 4 x 10^8 conidia and place at 37ºC, shaking at 225 rpm for 16 hours.
2. Harvest mycelium by filtration. Place a piece of Whatman 54 filter paper in a Buchner funnel on top of a sidearm conical flask attached to a vacuum pump. Pour cell suspension slowly onto paper, allowing the medium to be pulled through into the conical flask.
3. Wash the cells with 100 ml PB buffer. Dry the mycelium between another piece of Whatman filter paper and paper towels. Scrap the mycelium into a 50 ml centrifuge tube
4. Weigh the cells and resuspend in PB buffer to give 40 mg/ml wet weight. Transfer to a conical flask.
5. Add lysing enzyme to a final concentration of 12 mg/ml and then incubate at 34ºC with very slow shaking. Check the yield of protoplasts using a haemocytometer. Continue the incubation until a yield of > 1 x 10^6/ml is achieved, but do not incubate for more than 2.5 h.
6. Pre-wet the J-cloth in the filter funnels with PB buffer. Filter the protoplasts through the J-cloth into 50 ml centrifuge tubes and collect by centrifugation at 800 x *g* for 10 min.
7. Wash protoplasts with 50 ml cold PB buffer. Resuspend as gently as possible using, for instance, a 10 ml pipette and pipette controller. Count protoplasts again and calculate actual yield.
8. Meanwhile, melt 1.0 % InCert agarose in GMB solution using a boiling water bath. Cool agarose to 37ºC.
9. Centrifuge protoplasts and resuspend in GMB buffer. Warm cells to 37ºC and gently add agarose to give a final concentration of 0.5 %. The final concentration

of protoplasts should be 1-2 x 10^8 protoplasts/ml as determined from the calculation in step 7.

10. Mix cells and agarose and pipette into disposable plug moulds. Leave to set at 4ºC
11. Once set, place into a 50 ml centrifuge tube and treat protoplasts by adding proteinase K to a final concentration of 2 mg/ml in NDS buffer and rocking gently at 50ºC for 24 h. Use 0.5 to1 ml buffer for each plug. A change of proteinase K and NDS buffer can be carried out halfway through this step if desired.
12. Wash agarose plugs 4 x 1 hr in 50 ml 0.5 M EDTA at room temperature. Carry out additional washes if necessary to ensure complete removal of detergent.
13. Store agarose plugs in 0.5 M EDTA at 4ºC.

Timetable

Growth of cells (1) 16 h

Production of protoplasts in agarose plugs (2 – 11) 4-6 h

Proteinase K digestion (11) 24 h

Washing of plugs (12) 4 h

Tips and General Comments

1. We would recommend processing at least 4 overnight flasks of culture to get a sufficient yield of protoplasts for a decent number of plugs
2. Protoplasts should be treated as gently as possible with minimal manipulation. For instance, for the final resuspension in step 9, we have found that a 10 min incubation at 4ºC after an initial dispersal makes it easier to obtain a more even suspension afterwards. Also, we prefer using a magnetic stirrer to ensure precise control during protoplast generation rather than relying on finding a shaking incubator set at the appropriate rpm.
3. If there are problems with DNA degradation, then the pH of the NDS buffer can be increased to 9.5 (using a different buffer from Tris). The O/N proteinase K treatment can also be repeated. This additional step should result in the plugs becoming clearer.

4. We use a hybridization oven with a rocking platform for the proteinase K treatment stage (11) and a blood rotator for washing the plugs in EDTA (12)

References

modified from Tobin *et al.*, Diagn. Microbiol. Infect Dis. 1997; 29:67-71