Protoplast method for Chromosome prep with Zymoseptoria tritici

Pat Martinez

Abstract

Preparation of Zymos*eptoria tritici* isolates for extraction of chromosomes via pulsed-field electrophoresis

Pat Martinez 1990 (Bruce McDonald lab) (Simplified from Cooley, et al, Curr. Genet. 13:383-389.)

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Protocol

Prepare spores

Step 1.

Grow single-spore isolate in 40 ml yeast-sucrose broth, **without chloramphenicol**, until cells are in log phase, (5-8 days at room temperature) in shaker at 150-200 rpm. **Do not grow cells past log phase!** In stationary phase, protoplasts do not form as easily.

Transfer culture to sterile 50 ml Falcon tube. Harvest spores and mycelium by centrifuging at top speed in IEC clinical centrifuge for 5 minutes. Pour off supernatant.

Make protoplasts

Step 2.

Add 40 ml of 600 mM MgSO4 pH 5.8 (sterile) and resuspend cells completely in solution. Harvest washed cells by centrifuging at top speed for 5 minutes in IEC clinical centrifuge.

Make protoplasts

Step 3.

Resuspend pellet in 20 ml of a 1.2 M MgSO4 pH 5.8 solution containing 3.0 mg/ml of Novozyme 234 (this solution must be filter sterilized, mix Novozyme with 1.2 M sltn and vortex to mix before filter sterilizing). Sterilize by forcing solution slowly through 5 ml syringe-mounted Nalgene filter. Transfer spore-Novozyme solution to a 40 ml glass Corex centrifuge tube.

Make protoplasts

Step 4.

Incubate at 30° C for 2 hours without agitation. Check for formation of protoplasts after two hours, and again at 15 minute intervals. **Do not agitate tube any more than necessary!**

Harvest protoplasts

Step 5.

Once protoplasts have formed, centrifuge in IEC clinical at top speed for 3 minutes. Should find that the majority of pinkish cells are floating on top of liquid; these are protoplasts and some undigested spores. Pipette away cloudy, brown liquid underneath protoplast layer and save top layer.

Harvest protoplasts

Step 6.

To wash cells, add 20 ml of 1.2 M sorbitol and resuspend cells. Harvest cells by spinning at top speed, IEC clinical for 5 minutes. Discard supernatent and save pellet of protoplasts. Repeat this wash step again (**two washes total**). After second wash, resuspend protoplasts in 0.4 ml of 1.0 M sorbitol and quantify protoplast concentration with hemocytometer (probably will need to make 1:10 dilution of protoplast prep). Add 1.2 M sorbitol to achieve protoplast concentration of between 5-10 x 10^8 protoplasts per ml.

Harvest protoplasts

Step 7.

Add equal volume of **2.2%** low melting point (LMP) agarose (in TE) to protoplasts and mix well but gently. Keep solution liquid in 37° C water bath. Tape closed one end of plug molds to receive protoplast solution. Use pasteur pipette to pipette protoplast-LMP solution into each well of plug mold (push pipette end to bottom of well and work upward as protoplast solution enters well) and allow to solidify for **two hours** at 4° C.

Harvest protoplasts

Step 8.

Push out plugs with flame-blunted pasteur pipette into a 15 ml Falcon tube containing 10 ml lysis buffer (1% sarkosyl, 450 mM EDTA, 1 mg/ml proteinase K). Incubate tubes at 50° C for 48 hours, replacing lysis buffer once after 24 hours.

Harvest protoplasts

Step 9.

Rinse plugs in 500 mM EDTA and store in 500 mM EDTA at 4° C