

**Protoplast Preparation from Oval Spores  
Modified Hanau Lab Method  
(Panaccione et al., 1988)**

This method for making protoplasts was developed by Dan Panaccione in the Hanau laboratory. M1.001 makes abundant oval spores in submerged culture, and these protoplast really well. They release large individual protoplasts, all of which will have nuclei, and there is very little debris. The method is not suitable for strains or species of *Colletotrichum* that do not produce a lot of oval spores, for those you should use the mycelial method. If possible though it is best to use oval spores because of the cleaner prep, with less debris. Debris, as I understand, can hang up DNA and reduce efficiency. Furthermore, the fact that nearly all the protoplasts will have nuclei means that they will all be transformable.

1. Isolate spores from 2-3-wk-old PDA cultures of M1.001 grown at 23°C under continuous white light. To isolate the spores, add 10 mls of sterile water to each plate, scrape GENTLY with a rubber policeman or a spatula, filter through several layers of cheesecloth, and rinse the spores twice in 50 ml sterile water to remove the mucilage. Dilute to  $1 \times 10^6$  spores per ml.
2. Add the spore suspension to one liter of Fries Complete Medium (500 mls in each of two 1L flasks) to a final volume of  $1 \times 10^5$  spores per ml, and incubate at 30°C for 36-48 hours on a gyratory shaker at 200 rpm (no faster, since excessive aeration will tend to produce mycelium rather than spores). The culture is ready to use when there are no more falcate spores and there are a lot of oval spores. Don't let the culture get too old. If you want more spores, make more flasks.
3. Filter the cultures through several layers of cheesecloth to remove mycelium, and collect the oval spores by centrifugation at 10,000 x g for 15 minutes.
4. Add the spores to a solution of lysing enzymes prepared as follows: Add Lysing Enzymes (100 mg), Driselase (500 mg) and Chitinase (1 mg) (all from Sigma) to 20 mls 1.2 M KCl, mix thoroughly (will be cloudy), and filter sterilize. Add the solution to the oval spore pellet, pour into a Petri dish, and incubate with very slow agitation (80-100 rpm) for 60-120 minutes at 30°C. FROM THIS POINT ON treat the suspension very gently because protoplasts are fragile.
5. Filter the suspension through a sterile Nytex membrane.
6. Pellet the protoplasts at 3000 x g at 4°C and resuspend in 15 mls of ice-cold 1.2 M KCl. From this point, keep the protoplasts cold.
7. Pellet again and resuspend in 5 mls of ice-cold STC (1.2 M Sorbitol, 10 mM Tris-Cl, pH 7.5, and 10 mM CaCl<sub>2</sub>) and count them on a hemacytometer.
8. Resuspend at a concentration of  $1 \times 10^8$  per ml.
9. If you have enough, aliquot 300 microliter portions and freeze them at -80. Each aliquot will be enough for three transformations. They will lose their efficiency (about half) after freezing, so use fresh if possible. You can store the protoplasts overnight in the refrigerator, but don't store them for longer as they will begin to regenerate cell walls.