

Vacuole preparation II
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1. Inoculate culture into one liter of medium in a two liter flask. Cells can be grown in YPD or minimal medium.
2. Harvest cells when O.D. is at least 0.7 and less than 1.5. It is best to start with approximately 2,000 O.D.s of each strain (volumes below given for 2,000 O.D.s) but the procedure works for as little as 100 O.D.s.
3. Spin in 500 ml tubes at 5K RPM, 5 min.
4. Discard S/N and R/S pellets in DTT/PIPES buffer (100 mM PIPES/KOH, pH 9.4, 10 mM DTT) 1 using 100 ml per strain. Pool and combine into one tube each.
5. Incubate at 30°C for 10 min. and spin at 5K RPM, 5 min.
6. R/S pellets in spheroplasting buffer (0.6 M sorbitol, 50 mM potassium phosphate, pH 7.5, in 0.2X YPD) by shaking using 45 ml per strain. Check O.D. of 10 μ l in 1 ml water.
7. Add oxalyticase. You will need approximately 2 to 4 mg per tube. Aliquot into three 40 ml polypropylene tubes each. Incubate at 30°C for 25 minutes. Check O.D. of 10 μ l in 1 ml water. O.D. should be less than 10% of starting O.D.
8. Spin at 3K RPM, 1 min, 3.5K RPM, 1 min.
9. Remove S/N by pipetting (do not decant as pellet is not stable).
10. R/S pellet by shaking. Add 2.5 ml 15% ficoll (in 10 mM PIPES/KOH, pH 6.8, 200 mM sorbitol) to each tube and shake again to R/S. Transfer ficoll suspensions to one tube by pouring. If pellets are not completely resuspended, pipette gently up and down. Keep tubes on ice.
11. Add DEAE dextran. Prepare a 0.4 mg/ml solution (in 10 mM PIPES/KOH, pH 6.8, 200 mM sorbitol, 15% ficoll). Add approximately 300 μ l to 600 μ l (you will need to determine the amount that works best for your strain background empirically). Mix gently by shaking (do not vortex) and by pouring between two tubes. Hold on ice 2 min.
12. Place tubes at 30°C for 2.5 min. with gentle shaking then transfer back to ice.
13. Aliquot into three SW41 rotor (swinging bucket) tubes per strain (approx. 3.5 ml per tube). Overlay with 3 ml 8% and 3.5 ml 4% ficoll. Fill with 0% ficoll (all ficoll solutions in 10 mM PIPES/KOH, pH 6.8, 200 mM sorbitol).
14. Balance tubes and spin at 30K RPM, 90 min at 4°C. Collect vacuoles at 4%/0% interface using pasteur pipette.