

Vacuole Prep
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1. Start 20 ml cultures in Wickerham's minimal proline medium with 2% glucose (can use YPD). Doubling time is approximately 6 hours.
2. Inoculate 200 ml media and grow to an $O.D._{600} = 0.5-1.0$.
3. Pre-cool SW-41 rotor.
4. centrifuge 100 OD of cells at 5K RPM, 5 min.
5. Wash cells with 10 ml of 10 mM NaN_3 and transfer to 40 ml tubes.
6. Resuspend in 10 ml of 10 mM Tris- SO_4 , pH 9.4, 10 mM DTT (add DTT just before use; 15.4 mg/10 ml).
7. Shake for 20 min at 30°C.
8. Centrifuge 5 min at 5K RPM.
9. Resuspend in 25 ml of 0.6 M sorbitol, 0.2 M imidazole, pH 6.5, 1.5 mg zymolyase.
10. Incubate at 30°C until a 1:10 dilution has an $O.D._{600} < 0.07$ (usually 20-30 min).
11. Cool on ice for 5 min.
12. Centrifuge 2 min at 3K RPM.
13. Remove 1 ml supernatant for lysis control invertase assay.
14. Resuspend in 3.5 ml of 0.2 M sorbitol, 0.2 M imidazole, 15% ficoll (do this carefully with a 10 ml pipet and avoid generating air bubbles to minimize lysis).
15. Add approximately 75 μ l (should be titrated to give the best lysis of cells and yield of vacuoles) of 0.4 mg/ml DEAE-dextran (in 0.2 M sorbitol, 0.2 M imidazole, 15% ficoll) and mix very gently.
16. Incubate 1 min at 0°C and 5 min at 30°C. Cool to 0°C.
17. Remove 0.3 ml for load assays.
18. Determine volume (load volume).
19. Place remaining lysate (load) in SW-41 tube (Beckman ultra clear).
20. Carefully layer 3 ml of 8% ficoll, then 4 ml of 4% ficoll and 2 ml of 0% ficoll in 0.2 M sorbitol, 0.2 M imidazole (all at 4°C) over the lysate as quickly as possible.
21. Centrifuge at 30K RPM in SW-41 rotor for 1.5 hr at 8°C.
22. Remove vacuole fraction at interface between 0% and 4% ficoll layers.
23. Measure volume of vacuole fraction. Recovery should be 30-50% of total -mannosidase in the vacuole fraction.

Wickerham's Minimal Salts (10X)

	<u>1 liter</u>
KH ₂ PO ₄	10 g
NaCl	1 g
MgCl ₂	8.3 g
dH ₂ O	to 1 liter

Add trace elements and autoclave.

Wickerham's Minimal Proline Medium with Low Sulfate

	<u>Stock</u>	<u>50 ml</u>	<u>150 ml</u>
dH ₂ O		35 ml	105 ml
glucose	50%	2 ml	6 ml
Wickerham's Minimal Salts	10X	5 ml	15 ml
proline	20%	2.5 ml	7.5 ml
vitamins	200X	0.25 ml	0.75 ml
amino acids	10X	5 ml	15 ml
0.1 mM MgSO ₄	1 M	5 µl	15 µl
trace elements	1000X	50 µl	150 µl