Subcellular Fractionation Using Accudenz Gradient to Separate ER/Golgi in Yeast

Bio-protocol Editor*

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[Abstract] This protocol describes how to separate the endoplasmic reticulum (ER) and Golgi apparatus in yeast cells using a subcellular fractionation approach with an Accudenz gradient.

Materials and Reagents

1. Accudenz (Accurate Chemical & Scientific Corporation)
2. Protease inhibitors
   a. Phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich, catalog number: 78830-5G)
   b. Aprotinin (Sigma-Aldrich, catalog number: A3428-10MG)
   c. Pepstatin A (Sigma-Aldrich, catalog number: P5318-5MG)
3. NaN_3
4. Sodium fluoride (NaF)
5. Tris-HCl
6. Beta-mercaptoethanol
7. TCA
8. Sorbitol
9. HEPES-KOH
10. Spheroplasting buffer (see Recipes)
11. Lysis buffer (see Recipes)

Equipment

1. Dounce homogenizer (Cole-Parmer)
2. Refractometer (Bausch & Lomb Incorporated)
3. Beckman rotor

Procedure

A. Preparation of cell lysate

1. Collect 50 OD of cells and wash once in 10 mM NaN_3, 1 mM sodium fluoride (NaF), 50 mM Tris-HCl (pH 7.5), to poison energy-dependent processes.
2. Incubate them in 10 OD/ml 100 mM Tris-HCl (pH 9.4), 50 mM beta-mercaptoethanol, 10 mM NaN_3 at room temperature (RT) for 10 min to reduce disulfide bonds in the cell wall.
3. Wash and resuspend in Spheroplasting buffer 30 ~50 OD/ml and incubate at 37 °C until 90% of the cells are converted to spheroplasts (30 ~ 40 min).
4. Centrifuge at 3,000 *x g* for 5 min to collect spheroplasts.
5. Resuspend in 2 ml of Lysis buffer. Lyse cells in a dounce homogenizer (tight pestle, 15 strokes).
6. Centrifuge lysates at 500 *x g* for 5 min to clear unbroken cells. Centrifuge twice if necessary.

B. Preparation of Accudenz gradient
1. Prepare gradient solutions in lysis buffer. Generate gradients with the following weight/volume amounts of Accudenz: 1 ml 43%, 1 ml 37%, 1 ml 31%, 1.5 ml 27%, 1.5 ml 23%, 1.5 ml 20%, 1 ml 17%, 1 ml 13% and 1 ml 8%.
2. Measure the refractive index of the standard Accudenz gradient using a refractometer and convert these values to grams per milliliter based on a standard curve generated by five weighed standards.
3. Measure the refractive index of the collected fractions to determine their densities.

C. Fractionation by equilibrium sedimentation
1. Intracellular membranes can be separated on the basis of their characteristic densities, and cofractionation of the protein of interest with a known membrane marker protein can be examined.
2. Load the cleared lysates at the top of the Accudenz gradient (8% - 43%) and centrifuge to equilibrium in a Beckman rotor for 18 h at 170,000 *x g* at 4 °C. Use slow break.
3. Collect 12 fractions from the top of the gradient; precipitate proteins with 10% TCA.

D. Western blot

Catalog number or source (MP biomedicals)	**Yeast gene name**	**Yeast antigen tecognized by antibody**	**Yeast organelle in which antigen resides**	**Monoclonal or polyclonal, host**	**Western blots (g/ml)**
A-6427	Vma 2	V-ATPase 60 kDa subunit	Vacuole membranes		
A-6429	Dpm 1	Dol-P-Man Synthase	ER		
A-6457	PGK	3-Phosphoglycerate Kinase	Cytoplasm		

Recipes

1. Spheroplasting buffer

Spheroplasting buffer	100 ml	200 ml
1 M sorbitol (FW 182.17)		
10 mM NaN_3		
10 µg/µl oxolyticase or 20~40 U/OD oxolyticase		
40 mM HEPES-KOH (pH 7.5)		

2. Lysis buffer

Lysis buffer	100 ml	200 ml
0.2 M sorbitol (FW 182.17)		
50 mM KOAc		
2 mM EDTA		
20 mM HEPES-KOH (pH 6.8)		

3. Add protease inhibitors to final concentration (20 µg/ml PMSF, 5 µg/ml antipain, 1 µg/ml each of aprotinin, leupeptin, and pepstatin, and 10 µg/ml alpha$_2$-marcroglobulin).

Acknowledgments

This protocol has been modified and adapted in the Espenshade Lab, Johns Hopkins School of Medicine. Funding to support different projects that have used this protocol has come from NIH – National Heart, Lung, and Blood Institute, National Institute of Allergy and Infectious Diseases, the Pancreatic Cancer Action Network, and the American Heart Association.

References

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