Fractionation of vacuoles, pre-vacuoles, vacuolar, subvacuolar vesicles, secretory vesicles and Cvt vesicles from yeast spheroplasts

- OptiPrep™ is a 60% (w/v) solution of iodixanol in water, density = 1.32 g/ml
- To access other Application Sheets referred to in the text return to the Subcellular Membranes Index; key Ctrl "F" and type the S-Number in the Find Box.
- Important technical notes, information regarding alternative methodologies and membrane analysis are contained in the "Technical Notes and Review" section (Section 5).
- See also the related **Application Sheet S51**

1. Background

Spheroplasts are prepared from yeast by a standard zymolase digestion. They are then lysed (or permeabilized) in a low concentration sorbitol buffer [1,2] from which a high-speed particulate and a soluble fraction are obtained. The vacuoles are first isolated from the particulate fraction in a discontinuous polysucrose (Ficoll) gradient and subsequently exposed to a hypoosmotic medium. A discontinuous iodixanol gradient is used to resolve vacuolar, subvacuolar and other subfractions [2]. This is described in Parts A-C of the Application Sheet.

Satyanarayana et al [3] also used a discontinuous iodixanol gradient to resolve the vacuole and Cvt vesicles from a yeast spheroplast lysate and also used the same gradient to analyze the vacuole fraction isolated on a polysucrose gradient, in a similar manner to that described by Harding et al [1] and by Scott et al [2]. This is described in Part D.

Section 4 of this Application Sheet describes the following procedures

a. Formation of yeast spheroplasts (adapted from ref 1)
b. Isolation and vesiculation of the vacuoles (adapted from ref 2)
c. Separation of the vacuolar and subvacuolar vesicles (adapted from ref 2)
d. Separation of vacuoles and Cvt vesicles from a yeast spheroplast lysate (adapted from ref 3)

2. Solutions required (see Section 5.1)

A. OptiPrep™
B. Wash buffer: 10 mM DTT, 10 mM Tris-SO_4, pH 9.4
C. Spheroplast buffer: 1 M sorbitol, 20 mM Pipes-KOH, pH 6.8.
D. Spheroplast lysis medium: 200 mM sorbitol, 20 mM Pipes-KOH, pH 6.8
E. Polysucrose solutions: 4%, 10% and 12% (w/v) polysucrose in Solution D.
F. Vesiculation medium: 20 mM Pipes-KOH, pH 6.8

Keep the following stock solutions at 4°C:
100 mM Tris: 1.21 g per 100 ml water.
100 mM DTT: 1.54 g per 100 ml water
100 mM Pipes (free acid): 3.02 g per 100 ml water (pH10-11 with KOH)

Solution B: Mix 20 ml each of DTT and Tris stocks; adjust to pH 9.4 with 1 M H_2SO_4 and make up to 200 ml.

Solutions C (and D): Dissolve 18.2 g (3.6 g) sorbitol in 50 ml water; add 20 ml Pipes stock; adjust to pH 6.8 and make up to 100 ml.

Solution F: To 50 ml water; add 20 ml Pipes stock; adjust to pH 6.8 and make up to 100 ml.

3. Ultracentrifuge rotor requirements (see Section 5.2)

For Protocol 4c: Swinging-bucket rotor to accommodate approx 2 ml tubes (e.g. Beckman TLS55)
For Protocol 4d: Swinging-bucket rotor to accommodate 13 ml tubes (e.g. Beckman SW41Ti, Sorvall TH641 or similar)

4. Protocol

4a. Spheroplast isolation

1. Harvest cells (20 OD_{600} units) at OD_{600} of 0.8-1.2 (scale up or down as required) and wash once in Solution B.

2. Resuspend in solution B (OD_{600} = 2.0) and incubate at 30°C for 15 min with shaking.

3. Harvest the cells and resuspend in Solution C (OD_{600} = 1.0).

4. Dissolve Zymolase 20T (0.2 mg) by gentle inversion and incubate at 30°C for 15 min with occasional gentle shaking.

5. Harvest the spheroplasts at 3000 *g* for 3 min.

6. Resuspend the pellet in Solution C and transfer to microcentrifuge tubes.

7. Centrifuge the spheroplast suspension at 3000 *g* for 3 min.

4b. Isolation of vacuoles

For 700-800 OD_{600} units of spheroplasts

1. Resuspend the spheroplast pellet in 1 ml of Solution D.

2. Incubate at 23°C for 5 min (inverting once at 2.5 min).

3. Sediment the vacuole-containing fraction by centrifugation at 5000 *g* for 5 min.

4. Suspend the vacuolar pellet in 4 ml of the 12% polysucrose solution.

5. In 13 ml tubes for the swinging-bucket rotor layer 4 ml each of the sample in 12% polysucrose, 10% and 4% polysucrose and 1 ml of Solution D.

6. Centrifuge at 100,000 *g* for 90 min at 8°C.

7. Collect the vacuoles from the 4% polysucrose/Solution D interface (see Section 5.3).

8. Dilute the vacuole fraction with 2 vol. of Solution D and harvest by centrifugation at 60,000 *g* for 15 min.

4c. Separation of vacuolar and subvacuolar vesicles

1. Gradient solutions: dilute Solution A with Solution F to give 11% and 22% (w/v) iodixanol (1.06 and 1.12 g/ml respectively).

2. Suspend the vacuole fraction in 0.2 ml of Solution F.

3. In approx 2 ml tubes for the TLS55 swinging-bucket rotor layer 0.5 ml of 22% (w/v) iodixanol, 1.3 ml of 11% (w/v) iodixanol and 0.3 ml of the sample (see Section 5.2).

4. Centrifuge at 160,000 *g* for 60 min at 12°C.

5. Vacuolar vesicles band at the Solution F/11% iodixanol interface and subvacuolar vesicles at the 11%/22% iodixanol interface (see Sections 5.3 and 5.4).

4d. Separation of vacuoles and Cvt vesicles from a yeast spheroplast lysate

1. Make up a 50% (w/v) iodixanol working solution (WS) containing 10 mM K-Pipes, pH 6.8 and dilute with the same buffer to give 30%, 25% and 19% (w/v) iodixanol gradient solutions.

2. Lyse spheroplasts (from Protocol 4a) in water; adjust lysate to 10 mM K-Pipes, pH 6.8 and mix with WS so that the final iodixanol concentration is 37% (w/v).

3. In tubes for a Beckman SW41Ti, Sorvall TH641 (or similar) overlayer 3 ml of this lysate with 2 ml of each gradient solution and K-Pipes buffer to fill the tube.

4. Centrifuge at approx 80,000 *g* for 4 h at 4°C.

5. The vacuoles band at the buffer/19% interface and the Cvt vesicles at the 37%/30% interface (see Section 5.3).

5. Technical Notes and Review

5.1 Lysis media and gradient solutions

Protease inhibitors should be added to solutions used in the lysis of the spheroplasts and in solutions for subsequent operations as required.

5.2 Ultracentrifuge rotors

The Beckman TLS55 is accommodated in the Beckman Table-top ultracentrifuge and is used in Protocol 4c. A 5 ml rotor (e.g. Beckman SW50.1 or similar) with 3.0-3.5 ml adapted tubes is an alternative. In this case it will be necessary either to increase the volume of the sample in order to fill the tube or a cushion of 30% iodixanol could be included. Alternatively it may be acceptable simply to scale up all the volumes proportionately for larger volume tubes. The use of alternative rotors needs to be validated.

5.3 Harvesting the banded material from the gradients

In separations as well-resolved as these, the material of interest can simply be aspirated into a syringe fitted with a flat-tipped metal cannula (i.d approx 0.8 mm). If it is considered useful to unload the gradients in a series of equal volume fractions then for more information see **Application Sheet S08**. Note that the small volume tubes are probably best unloaded by tube puncture or aspiration from the meniscus.

5.4 Analysis of results

The fractions from the small volume discontinuous iodixanol gradients (Protocol 4c) were run on SDS gels and probed for Precursor API (prAPI) and ALP (subvacuolar and vacuolar markers respectively). See ref 2 for more information on the analysis of this material. Figure 1 shows an analysis of the crude vacuole fraction (V) and the banded material at the sample/11% iodixanol interface which is enriched in vacuolar vesicles (VV), the 11/22% iodixanol interface, enriched in subvacuolar vesicles (SV) and also the two 11% and 22% iodixanol layers (LD and HD respectively).

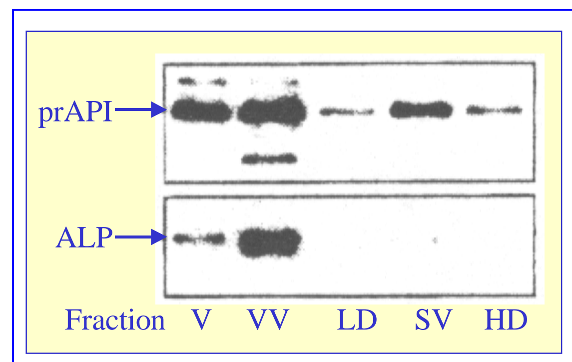

Figure 1 SDS-PAGE gels of gradient fractions probed with prAPI and ALP; see text for details (From ref 2 with kind permission of the authors and The Rockefeller University Press)

5.5 Vacuole and pre-vacuole separations

The continuous iodixanol gradient system devised by Chen and Kaplan [4] and subsequently reported in a number of other papers [5-9] and used primarily for the study of the uptake of iron into yeast mitochondria, also separates vacuole and pre-vacuole fractions. It is described in **Application Sheet S17**.

In a study of yeast Myo2p, Chang et al [10] used a small volume self-generated iodixanol gradient. Differential centrifugation of a 2000 *g*/10 min supernatant of a yeast lysate in 150 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 0.2% TX100, 2 mM DTT, 20 mM HEPES-NaOH, pH 7.2 was carried out at 2000 *g* for 10 min, 30,000 *g* for 30 min and 100,000 *g* for 1h. The final centrifugation included a 0.1 ml 60% (w/v) sucrose cushion. The 100,000 *g* pellet was resuspended in 0.75 ml of the lysis medium (plus the cushion) and 0.85 ml of OptiPrep™. This was centrifuged at 287,000 *g* for 2 h in a small volume rotor (Beckman TLA 120.2). Although vertical (or near-vertical) rotors are usually used for the creation of self-generated gradients, this small volume fixed-angle rotor has an ideal low sedimentation path length (approx 15 mm). The vacuole banded close to the top of the gradient, in secretory vesicles and ribosomes also showed distinctive banding patterns,

6. References

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Application Sheet 50; 8th edition, October 2016

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