OptiPrep™ Application Sheet S53

Fractionation of ER, Golgi, TGN, endosomes and vacuoles 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 **2020SMemapp** file and select the appropriate S-number.
- Important technical notes, information regarding alternative methodologies and membrane analysis are contained in the "Technical Notes and Review" section (Section 5).

1. Background

This application sheet is concerned with the use of iodixanol gradients in an analytical mode to study the membrane localization of a particular protein or function. Continuous gradients are best suited to this task. One of the protocols described in this application sheet starts with a discontinuous gradient [1], but since the gradient is centrifuged at 174,000 *g* for 16 h it will become continuous by diffusion. Some sedimentation of the iodixanol (self-generated gradient formation) will also occur and contribute further to the creation of a more or less linear density profile in the top three quarters of the gradient. Kim et al [2] used a pre-formed continuous gradient, also for 16 h, but at a lower RCF (100,000 g). Iodixanol will sediment rather less at this RCF and so the shape of density profile will change relatively little. Note that the discontinuous iodixanol gradients described in Application Sheet S50 were centrifuged for much shorter times.

Shintani et al [1] used a gradient between 10 and 50% (w/v) iodixanol, while that used by Kim et al [2] had a lower density range (0-40%). The two groups also used different spheroplast lysis buffers and protocols. Choice of buffer will depend on the subsequent analysis of the gradient fractions; in the following description only a basic buffer is described (**see Section 5.1**).

- A method for producing spheroplasts from a yeast culture is provided in Application Sheet S50.
- Two options are provided for the iodixanol gradient, a discontinuous or a continuous one, although the former will becomes continuous during the centrifugation.

2. Solutions required (see Section 5.1)

A. OptiPrep™

B. Spheroplast lysis buffer: 0.2 M sorbitol, 1 mM EDTA, 20 mM Pipes-KOH, pH 6.8

C. OptiPrep™ diluent: 0.6 M sorbitol, 6 mM EDTA, 120 mM Pipes-KOH, pH 6.8

D. Iodixanol (50% w/v) working solution: mix 5 vol. of OptiPrep™ + 1 vol. of Solution B

E. **Discontinuous gradient solutions**: 40%, 30%, 25%, 20%, 15% and 10% (w/v) iodixanol (dilute Solution D with Solution B)

F. **Continuous gradient**: 40% (w/v) iodixanol (dilute Solution D with Solution B)

Keep the following stock solutions at 4°C:
100 mM EDTA (Na_2•$2H_2O$): 3.72 g per 100 ml water
500 mM Pipes (free acid): 15.1 g per 100 ml water (pH 10-11 with KOH)

Solution B: Dissolve 3.64 g of sorbitol in 50 ml of water; add 1 ml and 4 ml respectively of EDTA and Pipes stocks; adjust to pH 6.8 and make up to 100 ml.

Solutions C: Dissolve 10.9 g of sorbitol in 50 ml of water; add 6 ml and 24 ml respectively of EDTA and Pipes stocks; adjust to pH 6.8 and make up to 100 ml.

3. Ultracentrifuge rotor requirements (see Section 5.2)

Ultracentrifuge with swinging-bucket rotors to accommodate 13 ml tubes (e.g. Beckman SW41Ti, Sorvall TH641 or similar

4. Protocol (adapted from refs 1 and 2).
Carry out all operations at 0-4°C.

1. Prepare a spheroplast lysate using standard procedures (**see Section 5.3**).

2. Sediment cell debris and nuclei by centrifugation at 500 *g* for 5 min.

3. Remove the supernatant by aspiration and centrifuge this at 100,000 *g* for 20-30 min to prepare a total membrane fraction (**see Section 5.4**)

4. Resuspend the pellet in 1-2 ml of Solution B.

5. In tubes for the swinging-bucket rotor EITHER form a 12 ml linear gradient (using a two-chamber gradient maker or a Gradient Master®) from Solution B and 40% (w/v) iodixanol OR form a discontinuous gradient from 0.5 ml of 50%, 1.5 ml of 40% and 30%, 2.0 ml of 25%, 3 ml of 20% and 15%, and 2 ml of 10% (w/v) iodixanol (**see Section 5.5**)

6. Layer 1 ml of the 100,000 *g* pellet suspension over the gradient to fill the tube (**see Section 5.5**).

7. Centrifuge at 100-180,000 *g* for 16 h and allow the rotor to decelerate from 2000 rpm without the brake or use a controlled slow deceleration program.

8. Collect the gradient in 0.5-1.0 ml fractions by tube puncture, upward displacement with a dense medium or aspiration from the meniscus. For more information on gradient harvesting **see Application Sheet S08**.

♦ Some examples of the resolution that can be achieved with these gradients are given in **Section 5.6**. This section also contains in Table 1 a summary of the gradient and centrifugation conditions reported in other papers and the analysis that was carried out.

5. Technical Notes and Review

5.1 Lysis media and gradient solutions

The lysis buffer used by Shintani et al [1] contained in addition to the listed reagents in Solution B, 50 mM sodium acetate. On the other hand that used by Kim et al [2] contained in addition 1 mM DTT and 1 mM $MgCl_2$ (see Note 2). Protease inhibitors should also be added as required.

The preparation of gradient solutions is carried out by dilution of a 50% (w/v) iodixanol working solution (Solution D) that contains the same concentrations of EDTA (1 mM) and buffer (20 mM PIPES) as the lysis buffer and 0.1 M sorbitol. The concentration of sorbitol is lower than in the lysis buffer because the iodixanol is also contributing to the osmolality of the solution. To account for the additional components in the lysis buffer, Solution C might also contain 6x their normal concentration as well (e.g. 300 mM sodium acetate or 6 mM DTT + 6 mM $MgCl_2$). In this manner the concentration of EDTA, buffer and either acetate or DTT + $MgCl_2$ remain constant in the gradient. If however this is not considered important (or even desirable) then the gradient solutions may simply be prepared by diluting OptiPrep™ with the lysis buffer. A description of the preparation of gradient solutions for yeast spheroplasts is given in **Application Sheet S02**.

Protease inhibitors should be added to Solutions B and C as required.

5.2 Ultracentrifuge rotors

The method may be scaled up or down as required to the use of larger or smaller volume rotor. It might be adaptable to a vertical or near-vertical rotor, in which case centrifugation times can be considerably reduced.

5.3 Spheroplast lysing

To disrupt the spheroplasts Shintani et al [1] extruded the spheroplast suspension through a filter (3 µm pore size); other workers use the standard liquid shear homogenization devices such as Potter-Elvehjem or tight-fitting Dounce homogenizer or differential lysis [3]. Whatever method is used the aim must be to disrupt the spheroplasts as gently as possible to avoid damage to delicate organelles but at the same time achieve at least 90% breakage.

5.4 Differential centrifugation

A centrifugation of the 500 *g* supernatant at 10,000 *g* for 10 min to pellet most of the larger organelles might be interposed if required. Alternatively, if the volume of the lysate is small, the 100,000 *g* step could be omitted and the entire 500 *g* supernatant applied to the gradient. Cytosolic proteins in this fraction however will both diffuse and sediment into the gradient during the overnight centrifugation.

5.5 Gradient construction and sample layering

Some of the options for layering discontinuous gradients and for preparing continuous gradients are described in **Application Sheet S03**. An alternative to layering the sample on top of the gradient might be to layer the sample in a dense medium beneath the gradient – strategy often used with mammalian cell membrane fractionation that often produces improved resolution (**see Section 5.7**)

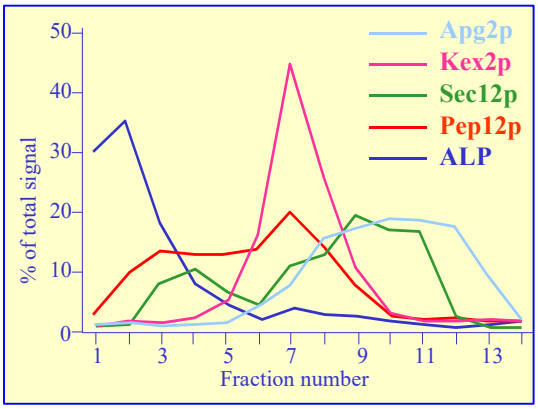

Figure 1 Distribution of Apg2p and markers in gradient (low density on left); fractions analyzed by SDS-PAGE and electroblots probed with antibodies to Apg2p, ALP (vacuole), Kex2p (late Golgi), Pep12 (endosomes) and Sec12p (ER). Figure adapted from ref 1 with kind permission of the authors and The American Society for Biochemistry and Molecular Biology

5.6 Gradient resolution

Examples of the resolving power of the iodixanol gradients are given in Figures 1 and 2. Shintani et al [1] used the 10-50% gradient (formed by diffusion of a discontinuous gradient) to study the distribution of a protein (Apg2p), which is essential for autophagosome formation. From Figure 1, it is clear that the Apg2p did not precisely co-localize with any of the other recognized markers, although it overlapped the ER marker, some denser fractions containing Apg2p were devoid of the ER marker (for more information see ref 1). **For information on autophagosomes see Section 5.9**

Kim et al [2], studying the transport of cytoplasmic material to the vacuole, identified a cytoplasm-to-vacuole targeting (Cvt) pathway. In particular the gradient (continuous 0-40% iodixanol) was used to determine the localization of Cvt9, a protein required for the selective delivery of prAPI to the vacuole. The Cvt9 did not co-localize to the vacuole, endosomes, TGN or ER; indeed it had its own very distinctive distribution pattern (for more information see ref 2).

See Table 1 for a summary of the types of density gradient reported in some other publications, all using overnight centrifugation at a minimum of 100,000 *g*. **See Section 5.8 re centrifugation time**.

By making the gradient span a smaller range of density Sakakibara et al [16] were able to improve the resolution of some of the membrane compartments; the gradient was constructed from 16-60% (v/v) Optiprep™, which is approx. equivalent to 9-36% (w/v) iodixanol, which was centrifuged at 150,000 *g* for 16h after top-loading the 500 *g* supernatant. The gradient was able to provide very distinctive banding of the vacuole, endosomes, endoplasmic reticulum, *cis*-Golgi and mitochondria. Interestingly the banding from the wild-type yeast was different to that of the *opi3Δ* variant.

Membranes identified[1]	Grad[2]	RCF (time)	Ref #
Vac, ER, Golgi, end	0-40	100,000*g* (16 h)	6
Vac, ER, Golgi, end	10-50[d]	180,000*g* (16 h)	1
Vac, end, *cis*-Golgi, ER	10-55	100,000*g* (12 h)	10
Vac, ER, mit, PM	10-50[d]	180,000*g* (16 h)	4
Vac, pre-vac, ER	ns	140,000*g* (16 h)	15
Vac, end, mit	10-50[d]	180,000*g* (16 h)	7
Vac, end, Golgi, ER	10-55	100,000*g* (12 h)	11,12
Vac, end, TGN, ER	0-40	100,000*g* (16 h)	2
Vac, end, Golgi, ER	0-40	100,000*g* (16 h)	5
Vac, ER, end,	0-40	100,000*g* (16 h)	8
Vac, end, early/late Golgi	0-60	100,000*g* (16 h)	13
Vac, median density mem, cytosol	0-45	190,000*g* (24 h)	9
Vac, Golgi	0-30	200,000*g* (18 h)	14

Table 1 Selected papers describing the use of pre-formed iodixanol gradients, centrifuged at ≥ 100,000*g* for buoyant density separation of yeast membranes
[1] Vac = vacuole, ER = endoplasmic reticulum, end = endosomes, mit = mitochondria, TGN = *trans*-Golgi network, mem = membrane
[2] d = set up as a discontinuous gradient, ns = not stated

More recently Sakakibara et al [26] using 0.2 M sorbitol, 5 mM EDTA, 20 mM HEPES-KOH buffer (pH 7.2) to produce a 15-60% (v/v) OptiPrep™ gradient (150,000 *g* for 16 h) obtained distinctive profiles for vacuoles, endosomes, ER, mitochondria and *cis*-Golgi.

5.7 Flotation separation

Mitsui et al [17] suspended spheroplast lysate membranes in (0.8% sorbitol, 10 mM triethanolamine, 1 mM EDTA, pH 7.4. The suspension was adjusted to 35% (w/v) iodixanol and overlaid with a continuous 12–30% (w/v) iodixanol density gradient and centrifuged 100,000 *g* for 16 h. An easier alternative is to prepare the gradient first and then underlay with the sample. Late endosome and vacuole fractions were recovered at the top of the gradient and these were very well resolved from plasma membrane, which peaked about a third the way down the gradient. Cytoplasmic proteins remained in the original sample zone.

5.8 Shorter centrifugation time methods

There are several examples of the use of both shorter times and lower *g*-forces for the analysis of yeast membranes. Some examples of these separations are outlined below. The gradients often, but not exclusively, span a smaller and lower density range.

1. A 16,000*g* pellet from a spheroplast homogenate was resolved on a 0-25% (w/v) iodixanol gradient centrifuged at only 14,000*g* for 2 h. The gradient system was able to resolve ER, vacuole and late endosomes fractions and was used to locate Bph1p to late endosomes and vacuolar structures in a study on cell wall formation and protein sorting [18].
2. Welker et al [19] used a 2.25-24% (w/v) iodixanol gradient in a vertical rotor, centrifuged at 48,000 *g* for 1.5 h. Distinctive, but overlapping profiles of vacuole, plasma membrane, ER, endosomes, mitochondria and peroxisome markers. A stress protein (Hsp12) localized mainly to the PM and endosomes.
3. A similar gradient demonstrated that Yke4p (a zinc transporter) associated with the ER rather than the Golgi [20].
4. Diaz et al [21] tracked the bromovirus replication protein 1a using a flotation gradient. The spheroplasts were lysed in 150 mM NaCl, 5 mM EDTA, 30 mM Tris-HCl, pH 7.5. After clarification at 500 *g* for 5 min, the lysate was adjusted to 40% (w/v) iodixanol and 0.6 ml overlaid with 1.4 ml of 30% (w/v) iodixanol (and topped up with lysis buffer). After centrifugation at approx. 200,000 g_{av} for 2 h, the ER had floated to the top of the gradient. Lack of expression of either the CAP or HEL fragments of 1a considerably reduced the ER flotation efficiency.
5. Work on PM-ER contact sites by Toulmay and Prinz [22], identified (using more or less the same methodology) the low density banding of the plasma membrane marker Pma1p and observed that

the synaptogamin-like-mitochondrial-lipid binding proteins (SMP) domains shifted the GFP from a high- to a low-density

5.9 Autophagosomes

Autophagosomes were resolved on a very shallow gradient by Yamamoto et al [23]. A clarified lysate was layered on to an 4.5-18% (w/v) iodixanol gradient and centrifuged at 200,000 *g* for 1 hr. Golgi membranes banded sharply in the top two fractions; endosomes banded in fractions 1-7 while autophagosomes were recovered principally from fractions 8-10. More recently these particles have also been identified in the long-spin gradients described in Section 4 above: a 0-30% (w/v) iodixanol gradient was centrifuged at 100,000 *g* for 20 h [24]

5.10 Self-generated gradients

In their study of vesiculogenic membranes, a 10,000 *g* pellet was suspended in 1 ml of 35% (w/v) iodixanol and layered under an equal volume of 30% (w/v) iodixanol, with 1 ml of lysis buffer on top, in tubes for a Beckman TLA 100.3 rotor. The samples were centrifuged at approx. 150,000*g* for 18 h [25]. The gradients that were formed occurred partly by diffusion and partly by self-generation.

6. References

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