OptiPrep™ Application Sheet S55

Isolation of peroxisomes 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.
- Recent published papers reporting the use of iodixanol gradients are given in Section 7.

1. Background

Wild-type yeast strains contain rather few peroxisomes, however they can be induced to proliferate and there are a number of peroxisome gene-deletion strains of the organism [1,2]. Yeast systems are therefore a popular means of investigating peroxisome development.

Although Percoll® has been used for peroxisome isolation from mammalian cells and yeast spheroplasts, the organelles are contaminated by both Golgi and smooth ER membranes, which have similar low densities in this medium. In both iodixanol and Nycodenz® however peroxisomes are denser than any other organelle in the light mitochondrial fraction and contamination by ER and Golgi is never a problem. Nycodenz® gradients have been widely used for the isolation of peroxisomes, but in the last eight years there has been increasing use of OptiPrep™ for both mammalian and yeast peroxisomes. Although Nycodenz® has remained a popular choice for yeast peroxisomes, the availability of iodixanol as a 60% (w/v) solution (OptiPrep™) makes gradient solution preparation much easier than with Nycodenz®. Iodixanol and Nycodenz® solutions of the same % (w/v) concentration have almost identical densities, but only iodixanol permits the banding of peroxisomes under more or less isoosmotic conditions and it is only the OptiPrep™ option that is presented in this Application Sheet.

Spheroplasts are homogenized in a sorbitol-containing medium using a Dounce homogenizer. Some variation exists in the sorbitol concentration in this medium; Watkins et al [2] used 0.6 M, while Crane et al [3] used 1.0 M. The iodixanol methodology is adapted from refs 4-5, 7-10.

2. Solutions required (see Box)

A. Spheroplast wash solution: 1.2 M sorbitol, 20 mM phosphate buffer, pH 7.4

B. Spheroplast lysis buffer: 0.6 M sorbitol, 1 mM EDTA, 1 mM KCl, 0.1% (v/v) ethanol, 5 mM MES-NaOH pH 6.0

C. OptiPrep™

D. OptiPrep™ diluent: 18% (w/v) sucrose, 3 mM EDTA, 3 mM KCl, 0.3% ethanol, 15 mM MES-NaOH, pH 6.0

E. Gradient diluent: 18% (w/v) sucrose, 1 mM EDTA, 1 mM KCl, 0.1% ethanol, 5 mM MES-NaOH, pH 6.0

Add protease inhibitors to solutions as required.

Keep the following stock solutions at 4°C:

1 M KCl	7.45 g per 100 ml water
1 M MES, purchased ready-made solution	
100 mM KH_2PO_4	1.36 g per 100 ml water
100 mM $K_2HPO_4 \bullet 3H_2O$	2.28 g per 100 ml water
100 mM EDTA ($Na_2 \bullet 2H_2O$)	3.72 g per 100 ml water

Solution A: Mix 40 ml of K_2HPO_4 stock with 10 ml of KH_2PO_4 stock; check the pH is 7.4 and adjust if necessary. Dissolve 21.8 g of sorbitol in 50 ml of water, add 20 ml of the phosphate buffer and make up to 100ml

Solution B: Dissolve 10.9 g of sorbitol in 50 ml of water; add 0.1 ml, 1 ml and 0.5 ml of the KCl, EDTA and MES stocks respectively; adjust to pH 6.0 with 0.1 M NaOH; add 0.1 ml of ethanol and make up to 100 ml.

Solution D: Dissolve 18 g of sucrose in 50 ml of water; add 0.3 ml, 3 ml and 1.5 ml of the KCl, EDTA and MES stocks respectively; adjust to pH 6.0 with 0.1 M NaOH; add 0.3 ml of ethanol and make up to 100 ml.

Solution E: As Solution C using one third of the volumes of KCl, EDTA, MES and alcohol

3 Preparation of gradient solutions

Mix 2 vol. of OptiPrep™ with 1 vol. of Solution D to produce a 40% (w/v) iodixanol stock solutions and then dilute further with Solution E to produce solutions of 2.25% and 24% (w/v) iodixanol (**see Section 6, Notes 1 and 2**)

4 Ultracentrifuge rotor requirements

Vertical rotor with a tube capacity of approx 36 ml tubes (e.g. Beckman VTi50) with appropriate sealed tubes (**see Section 6, Note 3**).

5. Protocol

Carry out all operations at 0-4°C

1. Prepare spheroplasts from 1 litre of yeast culture, grown in YPD medium (OD_{600} = 0.5-1.0).
2. Suspend the spheroplasts in Solution A (10-15 ml).
3. Centrifuge in an 8x50 ml fixed-angle rotor (high-speed centrifuge) at 4000 *g* for 5 min.
4. Remove the supernatant and repeat steps 1 and 2.
5. Suspend the spheroplasts in 35 ml of Solution B and homogenize in a tight-fitting Dounce homogenizer (Wheaton Type A), using 10 up-and-down strokes of the pestle.
6. Centrifuge the homogenate at 1,500 *g* for 10 min.
7. Using a syringe and metal cannula aspirate and retain the supernatant on ice (**see Section 6, Note 4**).
8. Resuspend the pellet in 35 ml of Solution B and repeat steps 5-7.
9. Combine the two supernatants and centrifuge at 25,000 *g* for 30 min.
10. Resuspend the light mitochondrial pellet in 6 ml of Solution B using 10 gentle strokes of the pestle of the small volume loose-fitting (Wheaton Type B) Dounce homogenizer (**see Section 6, Notes 5 and 6**).
11. Using a two chamber gradient maker or Gradient Master™ prepare 30 ml linear gradients from equal volumes of the 2.25 and 24% (w/v) iodixanol in tubes for the vertical rotor (**see Section 6, Notes 7 and 8**).
12. Underlayer the gradient with 1.0 ml of 0.5 ml of OptiPrep™.
13. Layer the sample on top of the gradient to fill the tube and seal it (**see Section 6, Note 9**).
14. Centrifuge at 40,000 *g* for 90 min. Use a controlled acceleration and deceleration programs to ensure a smooth reorientation of the gradient. If these are not available, turn off the brake below 2000 rpm.
15. Collect the gradient dense end first in 0.5 ml fractions; the peroxisomes band close to the bottom of the gradient. For more information on gradient harvesting and analysis **see respectively Application Sheets S08 and S09**.

6. Notes

1. In the gradient EDTA was omitted in the method of ref 7.
2. Originally the gradient covered a wider density range 15-36% (w/v) iodixanol [4,5], but more recently the 2.25-24% or 2.25-22.5% [7-14] iodixanol gradients have become more popular. Discontinuous iodixanol gradients are less often [15,16]; the light mitochondrial fraction was adjusted to 23.5% (w/v) iodixanol and layered over 35% (w/v) iodixanol and centrifuged at 110,000 *g* for 2 h. The peroxisomes band at the interface.
3. The use of a vertical rotor is very common in many organelle purifications. The short sedimentation path length of the rotor means that the particles reach their banding density very

quickly and the low hydrostatic pressure in the gradient preserves organelle integrity. A smaller rotor such as the VTi65.1 is permissible. If a vertical rotor is not available, fixed-angle rotor or swinging-bucket rotors may be substituted, but the centrifugation times will need to be increased.

4. Metal cannulas (i.d. approx 0.8 mm) can be obtained from most surgical instrument supply companies.
5. To avoid damage to the delicate organelles only use very gentle strokes of the pestle.
6. A post-nuclear supernatant was used instead of a light mitochondrial fraction according to ref 5.
7. If neither of these devices is available then first construct a discontinuous gradient from equal volumes of 15%, 25%, 30% and 36% (w/v) iodixanol and allow them to diffuse. For more information on preparing gradients **see Application Sheet S03**.
8. For smaller rotors scale down all volumes proportionately.
9. There are a variety of sealed tubes that are commercially available but the easiest sealed tubes to use are Beckman Optiseal™ tubes.

7. Recent publications

In a Cold Spring Harbor Protocols publication Cramer et al [17] provided a detailed and interesting comparison of sucrose gradients, iodixanol gradients in which the OptiPrep™ was diluted with a simple buffer and iodixanol gradients in which the OptiPrep™ was diluted with a standard buffered sucrose homogenization medium and found that the latter clearly gave superior results. Wróblewska et al [18] using an iodixanol gradient containing 18% (w/v) sucrose, based on the methods described by Cramer [17] were able to resolve not only very pure peroxisomes, but also a population of vesicles that contained a subset of peroxisomal membrane proteins. Effelsberg et al [19] used a 15.5-36% (w/v) iodixanol gradient, also containing 18% (w/v) sucrose. Mindthoff et al [20] and Effelsberg et al [21], used the 2.25-25% (w/v) iodixanol gradient previously described by Grunau et al [9].

8. References

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