OptiPrep™ Application Sheet S36

Separation of membrane vesicles and cytosol in a self-generated gradient

- OptiPrep™ is a 60% (w/v) solution of iodixanol in water, density = 1.32 g/ml
- The **OptiPrep™ Reference List RS13** "**The resolution of soluble cytosolic proteins from membrane vesicles and organelles: a bibliography**" provides a list of all published papers reporting the use of OptiPrep™: to access return to the initial list of Folders and select "**Reference Lists**". **The references are divided into cell or particle type.**
- **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

There are many situations where it is necessary to provide an effective separation of membrane vesicles from cytosolic proteins. The discontinuous gradient flotation strategy, **see Application Sheet S35**, uses a swinging-bucket rotor and allows the dense cytosolic proteins to remain in a dense load zone, while lower density membranes float to the top of a lower density solutions layered on top of the sample. This application sheet describes a similar strategy, i.e. the sample is adjusted to a high density and a lower density solution is layered on top, but in this case the use of a small volume high performance fixed-angle rotor permits the formation of an almost linear gradient by self-generation [1]. Since a linear gradient is formed, the method may allow a more detailed analysis of the membrane other than its simple resolution from cytosolic proteins. The method was developed by Du and Novick [1] for determining whether the GTPase activating protein Gyp1p was membrane-bound in yeast, but the gradient is more widely applicable to any protein in any cell type. The method used by Du and Novick [1] is ideally suited to multiple samples because of simple tube filling using open-topped (1 ml) polycarbonate tubes for a fixed-angle rotor.

The method employs the usual sorbitol based solutions characteristic of yeast, but this can be adapted to mammalian cells (an example is given in **Section 2.2**). Note that the protocol below, adapted from ref 1 does not contain details of spheroplast or cell lysis.

2. Solutions required

2.1 Yeast (see Section 5.1)

A. OptiPrep™

B. OptiPrep™ diluent: 2.4 M sorbitol, 6 mM EDTA, 120 mM tetraethylammonium acetate, pH 7.2.

C. Working Solution (50% iodixanol): mix 5 vol. of OptiPrep™ with 1 vol. of Solution B.

D. Lysis buffer: 0.4 M sorbitol, 1 mM EDTA, 20 mM tetraethyl-ammonium acetate, pH 7.2.

2.2 Mammalian cells (see Sections 5.1, 5.2 and 5.3)

E. OptiPrep™

F. OptiPrep™ diluent: 0.25 M sucrose, 6 mM EDTA, 120 mM Hepes-NaOH, pH 7.4

G. Working Solution (50% iodixanol): mix 5 vol. of OptiPrep™ with 1 vol. of Solution B.

H. Homogenization buffer: 0.25 M sucrose, 1 mM EDTA, 10 mM Hepes-NaOH, pH 7.4

Keep the following stock solutions at 4°C:

1 M TEAOAc•4H_2O	26.1 g in 100 ml water OR
1 M Hepes (free acid)	23.8 g in 100 ml water
100 mM EDTA (Na_2•2H_2O)	3.72 g in 100 ml water

Solution B: Dissolve 43.6 g sorbitol in 50 ml of water; add 12 ml and 6 ml respectively of TEAOAc and EDTA stocks; adjust to pH 7.2 and make up to 100 ml.

Solution D: Dissolve 7.28 g sorbitol in 50 ml of water; add 2 ml and 1 ml respectively of TEAOAc and EDTA stocks; adjust to pH 7.2 and make up to 100 ml.

Solution F: Dissolve 8.5 g sucrose in 50 ml water; add 12 ml and 6 ml respectively of Hepes and EDTA stocks; adjust to pH 7.4 and make up to 100 ml.

Solution H: Dissolve 8.5 g sucrose in 50 ml water; add 2 ml and 1 ml respectively of Hepes and EDTA stocks; adjust to pH 7.4 and make up to 100 ml.

3. Ultracentrifuge rotor requirements (**see Section 5.4**)
Small-volume fixed angle rotor: Beckman TLA120.2, TLA100.2, Sorvall S120-AT2, S150-AT or equivalent

4. Protocol
Carry out all operations at 0-4°C.

1. Centrifuge the spheroplast lysate or cell homogenate at 1000 *g* for 5 min to remove nuclei and unbroken cells.

2. Remove the supernatant and adjust to 40% (w/v) iodixanol by mixing with Solution C or G (1 + 4 vol. respectively).

3. Prepare a solution of 35% (w/v) iodixanol by diluting Solution C (or G) with Solution D (or H); place 0.9 ml in a tube for the ultracentrifuge fixed-angle rotor and underlayer it with the post-nuclear supernatant in 40% iodixanol.

4. Transfer to the ultracentrifuge fixed-angle rotor and centrifuge at 100-120,000 rpm for 3 h (**see Section 5.5**).

5. Unload the gradient using an automatic pipette or a Labconco Auto Densi-flow fractionator in approx. 0.1 ml fraction (**see Section 5.6**).

5. Technical Notes and Review
5.1 Preparing solutions
If Solution D (or H) contains low concentrations of other reagents such as DTT or MgOAc then these can be included in the OptiPrep™ diluent (Solution B or F) at 6x the normal concentration. Thus the 50% (w/v) iodixanol working solution (Solution C or G) and all the gradient solutions produced from it by dilution with Solution D or H, will contain these reagents at the same concentration as in Solutions D or H. Strategies for preparing working solutions for mammalian cells and for yeast are given respectively in **Application Sheets S01 and S02**.

Protease inhibitors may be included in Solutions B, D, F and H at the operator's discretion.

5.2 Homogenization media and gradient solutions for mammalian cells
The homogenization medium often has to be tailored to the tissue or cell type and it is not known if the composition of the HM is relevant to the separation. Organic osmotic balancers such as sucrose, mannitol and sorbitol were introduced for their compatibility in functional studies on subcellular membranes; moreover these low ionic strength HMs and gradient solutions permit the direct use of fractions for SDS-PAGE. Although 0.25 M sucrose buffered with either Tris, Hepes, Tricine or triethanolamine (at 10-20 mM concentration) and containing 1 mM EDTA is still a widely used HM, supplementation with inorganic salts is becoming increasingly common and can reduce ionic interactions, aggregation between membranes and combat any raised viscosity of the homogenate due to cytoskeletal proteins. Some media that omit sucrose entirely use either NaCl or KCl or both as the principal osmotic balancer(s). The composition of the HM should also be compatible with any subsequent analytical process. The inclusion of divalent cations can guard against nuclear breakage; stabilize membranes generally, but may lead to aggregation. If a hypoosmotic medium is required to swell the cells in order to achieve an adequate degree of homogenization it is important to return the homogenate to isoosmotic conditions as soon as possible. Some examples of homogenization media for mammalian cells are given in **Application Sheet S06**.

5.3 Homogenization

The homogenization protocol should be tailored to the cell (or tissue) type. Potter-Elevhjem homogenization for tissues and Dounce homogenization for cells used to be the standard procedures. For cells use of 5-15 passages through a 27- or 25-gauge syringe needle, sometimes preceded by Dounce homogenization, is more common. The ball-bearing homogenizer ("cell cracker") is now widely regarded as one of the most effective and reproducible of devices. Ideally the procedure should be as gentle and reproducible as possible, the aim being to cause at least 95% cell disruption without damage to the major organelles, particularly the nuclei and lysosomes. The type and severity of the homogenization process will have consequences for the integrity of the organelles. Some hints on homogenization are given in **Application Sheets S05 (tissues) and S06 (cells)**.

5.4 Ultracentrifuge rotor

Almost any high-performance fixed-angle rotor can be used, as long as the sedimentation path length of the tube is less than 20 mm. Information on the formation of self-generated gradients is given in **Application Sheet S04**.

5.5 Centrifugation

With such small sedimentation path length rotors it is very likely that the centrifugation time could be reduced to 2 h without seriously affecting the resolution of the gradient. Rotors with a maximum speed of 100,000 rpm may need a slightly longer centrifugation time but the gradient formation and particle banding should be satisfactory after 3 h at the lower RCF with these short path length rotors.

5.6 Analysis

Cytosolic proteins will remain in the load zone, while membrane vesicles will band in the top half of the gradient. For more information on harvesting gradients **see Application Sheet S08**. Compare the protocol in this Application Sheet with that in **Application Sheet S37** that describes specifically the separation of membranes from large protein complexes.

6. References

1. Du, L-L. and Novick, P. (2001) *Yeast Rab GTPase-activating protein Gyp1p localizes to the Golgi apparatus and is a negative regulator of Ypt1p* Mol. Biol. Cell, **12**, 1215-1226

serumwerk bernburg

Serumwerk Bernburg AG • Hallesche Landstraße 105 b • 06406 Bernburg • Germany
phone: +49 3471 860 429 • diagnostic.serumwerk.com • diagnostic@serumwwerk.com • serumwerk.de