OptiPrep™ Application Sheet M12

Analysis of proteins in small volume self-generated gradients

- 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 Macromolecules and Macromolecular Complexes Index; key Ctrl "F" and type the M-Number in the Find Box.**
- For more background information on the use of iodinated density gradient media for protein analysis see the companion **Application Sheet M11**

1. Background

Continuous sucrose gradients in swinging-bucket rotors are still used for the fractionation of proteins on the basis of sedimentation rate. Continuous gradients are generated by diffusion of discontinuous gradients or by a gradient making device. Centrifugation times are usually at least 4 h and may be as long as 16 h and if, as is often the case, these gradients are used for analyzing the formation of protein-protein and protein-nucleic acid complexes, these macromolecular complexes may be insufficiently stable to survive these long times. The stability of protein complexes is also affected by the hydrostatic pressure that is generated in a gradient [1,2] and in a standard swinging-bucket rotor this pressure can be considerable. Basi and Rebois [3] adapted these procedures to the use of OptiPrep™ in small volume (200 µl) self-generated gradients.

The strategy is to centrifuge a uniform concentration of iodixanol at approx 350,000 *g* to form a self-generated gradient; then to layer the proteins on top of the gradient and recentrifuge for no more than 1 h. Thick-walled open-topped polycarbonate tubes must be used to allow these operations. Such small volume tubes pose a problem for the subsequent harvesting and must be carried out low-density end first using a Brandel Microfractionator™ or a Labconco Auto Densi-flow™.

The molecular mass of membrane proteins solublized in either dodecyl-maltoside or octyl-glucoside has been analyzed by analytical ultracentrifugation, using dense Nycodenz® stock solutions for density adjustment in sedimentation velocity and sedimentation equilibrium runs. For more details of this specialized methodology see ref 4.

2. Solutions required

A. OptiPrep™

B. Protein solution: 20 mM HEPES-NaOH, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 2 mM $MgSO_4$ and 0.1% Lubrol PX.

C. Diluent: 40 mM HEPES-NaOH, pH 8.0, 2 mM EDTA, 2 mM dithiothreitol, 4 mM $MgSO_4$ and 0.2% Lubrol PX (see Note 1).

D. Protein solution (1.0-1.2 mg/ml)

Prepare 100 ml of each of the following stock solutions and keep at 4°C:

500 mM HEPES (free acid)	11.9 g
100 mM $MgSO_4 \bullet 7H_2O$	2.46 g
100 mM DTT	1.54 g
100 mM $EDTANa_2 \bullet 2H_2O$	3.72 g
1% (w/v) Lubrol PX	

To make 100 ml of Solution B:
To 50 ml of water; add the following stock solutions: 4 ml of HEPES, 1 ml of EDTA, 1 ml of DTT, 2 ml of $MgSO_4$, 10 ml of Lubrol, adjust to pH 8.0 with NaOH and make up to 100 ml.

To make 100 ml of Solution C:
To 50 ml of water; add the following stock solutions: 8 ml of HEPES, 2 ml of EDTA, 2 ml of DTT, 4 ml of $MgSO_4$, 20 ml of Lubrol, adjust to pH 8.0 with NaOH and make up to 100 ml.

3. Ultracentrifuge rotor requirements

Beckman TLA100, TLA 100.1 or TLA120.1, Sorvall S100-AT3, RP100-AT3, S120-AT3. Larger volume rotors such as the Beckman TLA100.2 or Sorvall S150-AT may be suitable (see Note 2).

Only open-topped tubes are suitable for this application, sealed tubes cannot be re-sealed once they have been centrifuged. Moreover such tubes cannot be easily unloaded low-density end first.

4. Protocol

1. Mix equal volumes of OptiPrep™ and Solution C to produce a 30% (w/v) iodixanol working solution. This can either be used directly as the gradient forming solution or diluted further with Solution B as required.

2. Fill a thick-walled open-topped centrifuge to the recommended level with 30%.

3. Centrifuge at 350,000 g_{av} for 2-3 h to self-generate the gradient (see Note 3).

4. Allow the rotor to decelerate from 2000 rpm using a controlled deceleration program to allow a smooth reorientation of the gradient.

5. Layer a small volume (10 µl on top of a 0.2 ml gradient; 50 µl on top of a 2.0 ml gradient).

6. Recentrifuge the gradients at approx 250,000 g_{av} for approx 1 h (see Note 4). Use controlled programs for acceleration to and deceleration form 2000 rpm.

7. Collect the gradient using a Brandel Microfractionator™ or some other appropriate system (see Note 5).

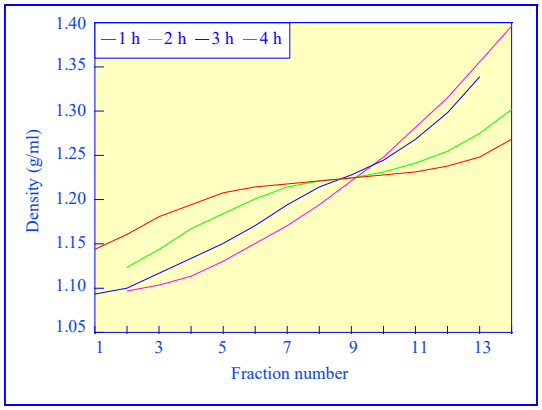

Figure 1 Gradient profiles generated at approx 270,000g from 0.2 ml of 30% iodixanol. Data adapted from ref 3, with kind permission of the authors and Academic Press

5. Notes

1. The protein solution and diluent can contain any low concentrations of reagents that will aid protein stability without materially affecting their density. Some glycoproteins may need slightly higher starting concentrations of iodixanol. For more information about the preparation of gradient solutions **see Application Sheet M01**.
2. Basi and Rebois [3] used an adapted Beckman TLA 120.2 rotor to reduce the tube volume to 0.2 ml, but there are many other standard Beckman and Sorvall rotors (see **Ultracentrifuge rotor requirements**) that accommodate similar volumes and will produce linear gradient profiles of the type shown in Figure 1 (2/3h). Larger volume rotors are also capable of producing approximately linear gradients at higher RCFs. For more information on the formation of self-generated gradients **see Application Sheet M03**.
3. The centrifugation conditions required to produce a suitable density gradient profile will vary with rotor type (see Note 2 for linked files).
4. The centrifugation conditions required will vary with the sedimentation path length of the rotor and the size of the proteins. Figure 2 and Note 6 summarize some of the data obtained by Basi and Rebois [3]. Some experimentation may be required to optimize the system to suit the operator's requirements.
5. To fractionate these small volume (<0.5 ml) gradients, Basi and Rebois [3] used a Brandel Automated Microfractionator. This device uses a fine stepper motor to advance a metal cylinder into the centrifuge tube (in 0.35 mm steps). The gradient is displaced up through a central channel in the cylinder. At the top of the cylinder a T-piece allows successive equivolume fractions to be expelled into the collection tubes. It can provide fractions of as little as 6 µl. With larger volume gradients (approx 2 ml) it may be possible to collect from the bottom of the gradient using narrow bore tubing connected to peristaltic pump. For more information on harvesting gradients **see Application Sheet M04.**

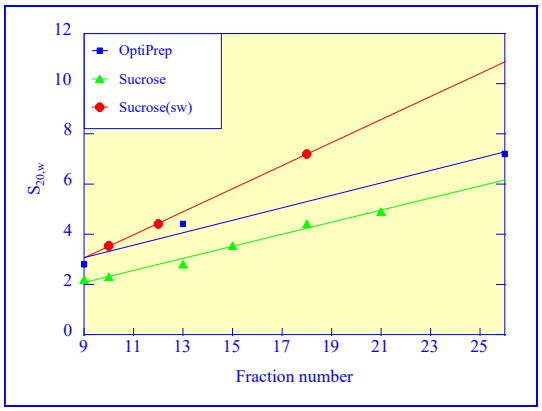

Figure 2 Sedimentation rate of protein standards in three different gradient systems. See Note 6 for details**.** Data adapted from ref 3, with kind permission of the authors and Academic Press

6. Figure 2 shows the sedimenting characteristics in iodixanol gradients of some standard proteins (lysozyme, soya bean trypsin inhibitor, carbonic anhydrase, ovalbumin, bovine serum albumin, transferrin and γ-globulin). The sedimentation coefficient of these proteins is plotted against the fraction number to obtain a straight-line graph. Three types of gradient are compared: (a) a 2 h self-generated gradient of iodixanol, recentrifuged after sample loading at 100,000 rpm for 1 h; (b) a pre-formed 5-20% (w/v) sucrose gradient (formed by diffusion of a discontinuous gradient), in the same tubes, centrifuged at 120,000 rpm for 1 h and (c) a preformed 5-20% sucrose gradient run in a standard swinging-bucket rotor (Beckman SW50.1) and centrifuged at 43,000 rpm for 17 h.

6. References

1. Marcum, J. M. and Borisy, G. G. (1978) *Sedimentation velocity analyses of the effect of hydrostatic pressure on the 30 S microtubule protein oligomer* J. Biol. Chem., **253**, 2852-2857
2. Hauge, J. G. (1971) *Pressure induced dissociation of ribosomes during ultracentrifugation* FEBS Lett., **17**, 168-172
3. Basi, N.S. and Rebois, V. (1997) *Rate zonal sedimentation of proteins in one hour or less* Anal. Biochem., **251**, 103-109
4. Lustig, A., Engel, A., Tsiotis, G., Landau, E.M. and Baschong, W. (2000) *Molecular weight determination of membrane proteins by sedimentation equilibrium at the sucrose or Nycodenz-adjusted density of the hydrated detergent micelle* Biochim. Biophys. Acta, **1464**, 199-206

7. Acknowledgements

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