Application Sheet S52

Endocytosis in rat liver: analysis of lysosome and late endosome events using a two-phase Nycodenz®-polysucrose gradient

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1. Background

Branch et al [1] compared the efficacy of short-spin (1 h) continuous polysucrose and Nycodenz® gradients in the analysis of the membrane compartments in rat liver during the transcytosis of polymeric IgA and endocytosis of asialofetuin. In both instances a Beckman VTi50, vertical rotor was used at 206,000 *g*. The authors concluded that while polysucrose gradients were superior for resolving light (early) and dense (late) endosomes, Nycodenz® gradients provided far greater discrimination between late endosomes and lysosomes; moreover discrimination was achieved between lysosomes and very dense endosomes [2]. This led to the use of hybrid polysucrose-Nycodenz® gradients for the simultaneous isolation of early and late endosomes and lysosomes [3-6]; also, a simplified discontinuous gradient for the separation of lysosomes, very dense endosomes and other less dense endosomes was developed. The latter is described in Section 2 of this Application Sheet.

A more recent development has been the use of *in vitro* systems to study the transfer of molecules between endosomes and lysosomes that occurs during fusion between these two compartments. The use of such a system allows detailed study of this process in isolation from other events. Hybrid polysucrose-Nycodenz® gradients were also used to analyze the result and have identified and permitted the isolation of endosome-lysosome hybrids subsequent to incubation of previously purified late endosomes and lysosomes [3-6].

2. Discontinuous polysucrose-Nycodenz® gradients (adapted from refs 3-7)

This methodology applies to rat liver and although in principle it may be applied to other tissues and cultured cells, but in view of the functional uniqueness of liver, it is likely that some optimization of the gradient and centrifugation conditions may be necessary.

2a. Solutions required (see Section 2d, Note 1)

A. Homogenization medium: 0.25 M sucrose, 1 mM $MgCl_2$, 10 mM TES-NaOH, pH 7.4

B. Nycodenz® buffer: 10 mM EDTA, 100 mM TES-NaOH, pH 7.4

C. Gradient diluent: 0.25 M sucrose, 1 mM EDTA, 10 mM TES-NaOH, pH 7.4

Add protease inhibitors as required to Solutions A-C. For polysucrose solution see Section 2c, Step 3.

2b. Ultracentrifuge rotor requirements

Beckman VTi50 rotor (or equivalent) with a tube volume of approx 36 ml (**see Section 2d, Note 2**)

Keep the following stock solutions at 4°C:
200 mM TES (free acid), 9.16 g per 200 ml water
100 mM $MgCl_2 \bullet 6H_2O$), 2.03 g per 100 ml water
100 mM EDTA ($Na_2 \bullet 2H_2O$) 3.72 g per 100 ml water

Solution A: Dissolve 8.5 g sucrose in 50 ml water; add 1 ml of $MgCl_2$ stock and 5 ml of TES stock; adjust to pH 7.4 with 1 M NaOH and make up to 100ml

Solution B: Mix 50 ml of TES stock with 10 ml of EDTA stock; adjust to pH 7.4 with 1 M NaOH and make up to 100ml

Solution C: Dissolve 8.5 g sucrose in 50 ml water; add 1 ml of EDTA stock and 5 ml of TES stock; adjust to pH 7.4 with 1 M NaOH and make up to 100ml

2c. Protocol

1. Prepare a Nycodenz® stock solution (45%, w/v): into approx. 50 ml of water at approx. 50°C in a 150 ml beaker on a heated magnetic stirrer add slowly 45 g of Nycodenz®. When all the Nycodenz® has dissolved allow the solution to cool to room temperature; add 10 ml of Solution B and make up to 100 ml with water. This may be filter sterilized and stored at 4°C.

2. Make up a 20% (w/v) Nycodenz®: mix 2 vol. of the 45% Nycodenz® solution prepared in Step 1 with 2.5 vol. of Solution C.

3. Make up a 20% (w/v) polysucrose in Solution C (**see Section 2d, Notes 3 and 4**).

4. Keep the solutions prepared in Steps 1-3 on ice and carry out the following at 4°C.

5. After perfusing the liver with an appropriate ligand, perfuse with Solution A until the lobes are well blanched (**see Section 2d, Note 5**)

6. Excise the liver into a beaker on ice and chop finely with scissors.

7. Stir the liver mince in approx. 10 ml of Solution A, then decant after allowing the mince to settle.

8. Transfer about half the mince to a Potter-Elvehjem homogenizer in approx 20 ml of Solution A and homogenize using 3-5 strokes of the pestle rotating at approx. 2000 rpm (**see Section 2d, Note 6**).

9. Repeat the procedure with the other half of the mince.

10. Centrifuge the homogenate at 2000 *g* for 10 min to sediment cell debris, nuclei and most of the heavy mitochondria.

11. Transfer 12.5 ml of the 20% polysucrose to 36 ml Optiseal tubes for the VTi50 rotor, then using a syringe and metal cannula underlayer with 12.5 ml of 20% Nycodenz® and 4 ml of 45% Nycodenz® solution (**see Notes Section 2d, 7 and 8**).

12. Layer the 2000 *g* supernatant on top, to fill the tube, as specified by the manufacturer.

13. Centrifuge at 200,000 *g* for 1 h, using a slow acceleration and deceleration programs up to and below 2000 rpm (alternatively turn off the brake below 2000 rpm).

14. Harvest the gradient in a series of equal volume fractions prior to analysis (**see Notes Section 2d, 9-11**).

2d. Notes

1. It is not known if iodixanol can be substituted for Nycodenz® in this application. Certainly the availability of iodixanol as a 60% (w/v) solution (OptiPrep™) makes gradient solution preparation much easier than is the case with Nycodenz®. Iodixanol and Nycodenz® solutions of the same % (w/v) concentration have almost identical densities, but solutions of Nycodenz® are hyperosmotic above 1.15 g/ml, in contrast to those of iodixanol which can be made isoosmotic at all densities. Whether the osmolality of Nycodenz® solutions plays an important role in achieving the separations described in this Application Sheet is not known. Comparisons can only be made empirically. For the preparation of gradient solutions **see Application Sheet S01**.
2. If a vertical rotor is unavailable, either a fixed-angle or a swinging-bucket rotor may be used, but the longer sedimentation path length of these rotors will require longer centrifugation times.
3. Although Ficoll was used in the original method, the Axis-Shield product polysucrose, which has an almost identical molecular weight, may be substituted. When making up solutions of these high molecular weight sucrose polymers, it is better to add small aliquots (2-3 ml) of the solvent to the weighed-out powder, using a glass rod to mix well after each addition.
4. Ellis et al [7] made up a much more concentrated Ficoll solution in water (1 ml per g) before dialyzing it for 2 h against a large volume of water and adjusting it to the appropriate concentration in 0.25 M sucrose, 1 mM EDTA, 10 mM TES-NaOH, pH 7.4. It is probably easier to make up a 25% (w/v) polysucrose in 0.25 M sucrose, 1 mM EDTA, 10 mM TES-NaOH, pH 7.4 and to dialyze it against the same medium, before checking and adjusting the volume to make it 20% with respect to polysucrose.
5. See ref 7 for more information about liver perfusion.

6. **See Application Sheets S05 and S06** respectively for more information on homogenization of tissues and cells.
7. A simplified method for separating lysosomes and endosomes in which the 20% polysucrose layer was omitted has also been used [4].
8. More detailed analysis of the light and dense endosomes may be carried out on continuous 1-22% polysucrose gradients (with a 45% Nycodenz® cushion) using approx. the same gradient volume, rotor and centrifugation conditions. The material containing lysosomes and dense endosomes at the cushion interface may be reanalyzed in a 0-35% or 0-45% Nycodenz® gradient, see refs 3-7 for more details.
9. Collect the gradient in approx 1 ml fractions either by tube puncture, upward displacement with Maxidens®, or aspiration from the meniscus. **For more information on gradient harvesting see Application Sheet S08**.
10. Lysosomes band at the 45%/20% Nycodenz® interface, very dense endosomes at the 20% polysucrose/20% Nycodenz®; all other endosomes band at the sample/20% polysucrose interface.
11. The methodology has been widely used [8-10] to study the subcellular distributions of sterols and oxysterols in lipid-loaded macrophages (foam cells); their ability to oxidise LDL [11] and the mobilization of free and esterified cholesterol in response to cyclodextrins [12]. It has also been used to study the role of lysosomal membrane proteins in intracellular lactosylceramide traffic [13].

3. References

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