OptiPrep™ Application Sheet M11

Analysis of protein size in pre-formed 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.

1. Background

Compared to nucleic acids proteins are much less highly hydrated, consequently the water activity of the density gradient medium has rather little effect on their density. Thus in iodinated density gradient media most proteins have a density very similar to that in either CsCl or sucrose, i.e. approx 1.26 g/ml [1,2]. In metrizamide gradients proteins did not form single discrete bands; the protein profile always demonstrated a significant shoulder on the high-density side of the peak. This effect was due to a weak irreversible interaction between metrizamide and the protein molecules [1]. A similar effect is seen with the banding of proteins in Nycodenz® gradients, but the effect is less marked [1] suggesting that Nycodenz® interacts much less than metrizamide.

The density of proteins in iodixanol is similar to that in Nycodenz®, but there is no obvious high-density shoulder on the protein profile, suggesting that iodixanol interacts with proteins even less than does Nycodenz®. However OptiPrep™ provides, for the first time, an opportunity for density banding of proteins under isoosmotic conditions (see Section 3). In all of the other commonly used media (sucrose and inorganic salts), proteins band at densities that are grossly hyperosmotic; even in Nycodenz® a density of 1.26 g/ml is slightly hyperosmotic. Iodixanol solutions on the other hand can be isoosmotic up to a density of 1.32 g/ml. Although the use of an isoosmotic gradient may not have any significant benefit for protein banding, it is now recognized that a hyperosmotic environment may be deleterious to the integrity of macromolecules such as proteins. In the native state, water molecules associated with various residues on the polypeptide chain are important for maintaining its stability and their removal can lead to instability and changes in the propensity of the protein molecules to aggregate [3]. Thus iodixanol gradients are particularly important for the study of protein complex formation **see Application Sheet M09**.

There are also secondary advantages to the use of an iodinated density gradient medium. Because of the very low density of most nucleic acids in these media (DNA has a density of approx 1.1 g/ml in iodixanol) the study of protein-nucleic acid complexes is facilitated [1,2]. In addition, because of the inert non-ionic nature of the medium, proteins harvested from iodixanol gradients can be run on polyacrylamide gels directly without the need to remove the medium. A disadvantage of sucrose is its viscosity; protein banding thus requires long centrifugation times in this medium.

2. Use of linear Nycodenz® gradients

Linear Nycodenz® gradients have recently been reported for analysis of the oligomerization of the Ebola virus VP30 protein [4], the gradients were also very effective in the resolution of protein markers covering a wide molecular mass range.

2a. Solutions required

A. 45% (w/v) Nycodenz® stock solution (see Step 1 of Protocol – Section 2c)

B. Nycodenz® diluent: 1 M NaCl, 10 mM EDTA, 100 mM Tris-HCl, pH 7.5

C. Buffered saline: 0.1 M NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5

Keep the following stock solutions at 4°C:
1 M Tris (free base): 12.1 g per 100 ml water
100 mM EDTA ($Na_2 \bullet 2H_2O$): 3.72 g per 100 ml water

Solution B: Dissolve 5.9 g NaCl in 50 ml water, add 10 ml of each of the stock solutions; adjust pH to 7.5 with 1 M HCl and make up to 100 ml with water

Solution C: Dissolve 0.59 g NaCl in 50 ml water; add 1 ml of each of the stock solutions; adjust to pH 7.5 with 1 M HCl and make up to 100 ml with water

2b. Ultracentrifuge rotor requirements

Any suitable swinging-bucket; the following protocol has been devised for the Beckman SW60Ti rotor, which accommodates 4 ml tubes (see Note 1).

2c. Protocol

1. To make up the 45% Nycodenz® stock solution place 50 ml of water in a 150 ml beaker on a heated magnetic stirrer set at approx. 50°C and add 45 g of powder in small amounts until dissolved. Allow the solution to cool to room temperature and then add 10 ml of Solution B and make up to 100 ml with water (see Note 2). Filter sterilize if required.

2. Make a 10% (w/v) solution of Nycodenz® by diluting 1 vol. of the 45% stock solution with 3.5 vol. of Solution C. iodixanol (ρ = 1.16 g/ml) by diluting OptiPrep™ with an equal volume of Solution of B.

3. Prepare a linear gradient from equal volumes of the two Nycodenz® solutions using a Gradient Master™ or a two chamber gradient maker or by allowing a discontinuous gradient to diffuse (see Note 3). In a 4 ml tube use a 3.6 ml gradient (see Note 4).

4. Bring the gradients to 4°C and then layer approx. 0.3 ml of the sample on top of the gradient so that the tube is filled to a level in accordance with the manufacturer's recommendations (see Note 4).

5. Centrifuge at approx 350,000 *g* for approx. 22 h at 4°C (use slow acceleration and deceleration programs or turn the brake off below 3000 rpm during the deceleration).

6. Collect the gradient by tube puncture or aspiration from the meniscus in approx 0.25 ml fractions (see Note 5). See Figure 1 for the approximate banding position of molecular weight markers (see Note 6).

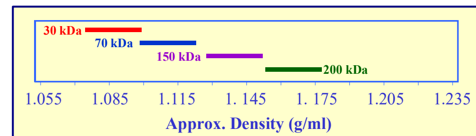

Figure 1: Approximate banding of molecular weight markers in 10-45% Nycodenz® gradient; data adapted from ref 4

2d. Notes

1. Other swinging-bucket rotors may be used; those with longer sedimentation path lengths will require longer centrifugation times, especially as the larger volume rotors may have a lower maximum *g*-force. It should be possible to adapt the method to the use of vertical rotors, in which case the centrifugation time may be considerably reduced.
2. The strategy for the preparation of the two gradient solutions, maintains the same background concentration of NaCl, EDTA and buffer throughout the gradient. If this is deemed unnecessary, the 45% (w/v) Nycodenz® solution may be simply be prepared in Solution C.
3. For more detailed information on the construction of continuous gradients **see Application Sheet M02.**
4. The ideal volume of gradient is dictated by the need to layer the sample on the gradient in a narrow band in order to maximize the resolution of the sedimentation velocity gradient. The volume of sample should not be more than approx. 7.5% of the gradient volume.
5. For more detailed information on unloading gradients **see Application Sheet M04**.
6. The gradient was used very successfully by Hartlieb et al [4] to distinguish the monomeric, dimeric and hexameric forms of VP30.

3. OptiPrep™ Applications

1. Large et al [5] used a discontinuous gradient of 0.3M, 0.5M, 0.7M sucrose and 36% (w/v) iodixanol in the fractionation of CCT type II chaperonins from *Haloferax volcanii*.
2. Salivary mucins have been size-fractionated on 10-30% (w/v) iodixanol gradients (210,000 *g* for 1 h) – three types were defined based on rate of sedimentation (at approx. 1.10, 1.13 and 1.17 g/ml). The densest granular form was studied further by Kesimer et al [6].

3. Shutova et al [7] analyzed the non-muscle myosin II (NM-II)from rat embryo fibroblasts in iodixanol gradients. After cell lysis in a buffered 150 mM KCl containing 0.5% Triton-X100, lysates were centrifuged at approx. 150,000 *g* for 15 min to remove detergent insoluble fraction. Lysates were layered on a discontinuous gradient of 50%, 25%, 12%, and 6% (w/v) iodixanol and centrifuged at 80,000 rpm for 60 min to separate monomeric and polymerized versions of this myosin. Importantly for the use of such gradients more generally for investigating the molecular mass of proteins the authors also showed that the gradient resolved M.Wt. marker proteins aldolase, catalase and ferritin (7S, 11S and 16S respectively). The same gradient was used by Lehtimäki et al [8] to study the folding of NM-II and the assembly of bipolar filaments.
4. Vijayakumar et al [9] used an iodixanol gradient of 5-40% (w/v) centrifuged at 100,000 *g* for 3 h to study the oligomerization of hensin; the following markers were used to calibrate the gradient: γ-globulin (158 kDa), catalase (232 kDa), and thyroglobulin (670 kDa).
5. Linear 1-12% (w/v) iodixanol gradients were used in studies on the oligomeric state of genetically engineered fusogenic proteins on paramyxovirus replication [10].
6. Using both culture supernatants and cell lysates, after an ultrafiltration concentration step, apolipoproteins B and E from hepatitis C virus-infected cells were purified on a 10-40% (w/v) iodixanol gradient at approx 124,000 g_{av} for 16 h [11]. Apolipoprotein(a) was shown to inhibit virus entry into hepatoma cells [12].
7. An iodixanol gradient is able to facilitate the detection of the HERV-K-ENV protein from plasma samples from individuals with an ovarian tumour or a benign tumour (or from control individuals). It was much more pronounced in the patients with ovarian tumour, while HERV-K reverse transcriptase was similarly raised in the two groups of patients [13].

4. References

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