OptiPrep™ Application Sheet M13

Fractionation of nucleic acids and nucleic acid-protein complexes

- 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 Complex Index; key Ctrl "F" and type the M-Number in the Find Box**

1. Introduction

Sucrose sedimentation velocity gradients are widely used in the analysis of polysomes and their component ribosomal subunits, of messenger ribonucleoproteins (mRNPs) and of the nuclear heterogeneous ribonucleoproteins (hnRNP). Separation of mRNP from ribosomal subunits is however difficult by this method. An alternative is to fractionate the particles according to density, but to provide the necessary high density it has been necessary to use heavy metal salts (e.g. CsCl). Such high ionic strength gradients however have serious shortcomings and to prevent dissociation of the macromolecular components particles are fixed in formaldehyde or glutaraldehyde.

Ford and Rickwood [1] and Houssais [2] developed the use of gradients of the non-ionic gradient solute Nycodenz®. Not only is it unnecessary to prior fix the particles, the high water activity (low osmolality) of Nycodenz® solutions compared to those of CsCl means that the RNA molecules retain their normal levels of hydration. Ford and Rickwood [1] showed that in Nycodenz® 40 molecules of water were bound to each mole of nucleotide, while in CsCl this figure dropped to 3. Consequently the density of RNA in Nycodenz® is much lower than that in CsCl, 1.184 g/ml against >1.9 g/ml. Nycodenz® gradients therefore offer great advantages over those of CsCl and other heavy metal salts.

It is likely (but untested) that iodixanol could substitute for Nycodenz® and since iodixanol solutions are prepared much more easily from OptiPrep™, some suggestions are given in Section 3. Published OptiPrep™-based methods primarily on DNA- and RNA-complexes are given in Section 4.

2. Nycodenz®-based methodology

2.1 Gradient solution preparation

Nycodenz® gradients have been used for the analysis of RNA-containing complexes from *Xenopus* oocytes or embryos and from mammalian cells. In both cases the gradients cover a broad range of densities, commonly from approx 1.05 or 1.10 g/ml to 1.32 g/ml (equivalent to 10-20% Nycodenz® to 60% (w/v) Nycodenz®).

Xenopus

Tafuri and Wolffe [3] used a 20-60% (w/v) Nycodenz® gradient containing 0.3 M KCl, 2 mM $MgCl_2$, 20 mM HEPES, pH 7.4 containing 0.1% diethylpyrocarbonate and 0.5% NP40

Keep the following stock solutions at 4°C:

400 mM HEPES (free acid): 9.52 g per 100 ml water
1 M KCl: 7.45 g per 100 ml water
100 mM $MgCl_2 \bullet 6H_2O$; 2.03 g per 100 ml water

Solution A: Mix 10 ml, 60 ml and 4 ml respectively of HEPES, KCl and $MgCl_2$ stock solutions; adjust pH to 7.4 with 1 M KOH and make up to 100 ml with water

Solution B: Mix 5 ml, 30 ml and 2 ml respectively of HEPES, KCl and $MgCl_2$ stock solutions; dissolve 0.1 g diethylpyrocarbonate and 0.5 g of NP40 in the liquid; adjust pH to 7.4 with 1 M KOH and make up to 100 ml with water

To make up a stock solution of 60% (w/v) Nycodenz® place 50 ml of Solution A (see box) in a 150 ml beaker on a heated magnetic stirrer set at approx. 50°C and add 60 g of Nycodenz® powder in small amounts until dissolved. Allow the solution to cool to room temperature, dissolve 0.1 g diethylpyrocarbonate and 0.5 g of NP40 and make up to 100 ml with water. Filter sterilize if required. Make up solutions of lower density by dilution of the 60% Nycodenz® stock with Solution B. This strategy for making up the gradient solutions maintains the concentrations of

KCl, HEPES and $MgCl_2$ in all solutions (and the gradient) constant. If this is deemed unnecessary then make up the Nycodenz® 60% (w/v) stock solution as above using Solution B instead of Solution A and water.

Mammalian cells and tissues

The solutions used for the gradient reflect the different solutions selected for homogenizing the cells. Nycodenz® solutions for analyzing cytoplasmic extracts have been prepared in the following: 150 mM KCl, 3 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.6 containing 1 mM DTT, together with protease inhibitors and rRNasin [4] or 115 mM KCl, 20 mM HEPES-KOH, pH 7.3 [5], while for nuclear extracts Ladomery et al [6] used 2mM $MgCl_2$, 1 mM EDTA, 20 mM Tris-HCl, pH 7.5.

The same principles regarding solution preparation apply as described in section 2a. If it is important that the reagent concentration should be constant in the gradient make up the 60% (w/v) Nycodenz® solution initially using double concentration buffer to dissolve the Nycodenz® or if this is not important use the regular buffer for all solution preparation.

2.2. Ultracentrifuge rotor requirements

In most cases a 4-5 ml gradient is used, so Beckman rotors such as the SW55Ti or SW50.1, or Sorvall rotors such as the AH650 or TH660 are suitable. Sometimes the gradient volume is smaller, e.g. a 1.5 ml gradient in a Beckman TLS-55 [3].

2.3. Gradient preparation

Gradients for *Xenopus* material are usually 20-60% (w/v) Nycodenz® [3,7] for cultured mammalian cells 10-60% [4,5,8,9] or 20-60% [6,10,11]. For mouse testis 20-60% is the chosen gradient type [12,13] Sample volumes are routinely small (approx. 0.2 ml, but may be as large as 1 ml) so the total gradient volume needs to be adjusted accordingly to take account of the sample load so that the tubes are filled according to the manufacturer's instructions. Prepare continuous gradients from equal volumes of the densest and lightest solution in either a two-chamber gradient maker or a Gradient Master™. Alternatively prepare a discontinuous gradient from equal volumes of e.g. 10%, 22.5%, 35%, 47.5% and 60% (w/v) Nycodenz® and allow to diffuse for 3-4 h at room temperature. Bring all gradients to 4°C before use. For more information on making these gradients see Application Sheet M02.

2.4. Sample loading and centrifugation

Carry out all operations on at 4°C. Centrifuge the cell lysate at approx. 800 *g* for 8 min; the 800 *g* supernatant at 12-15,000 *g* and then load this supernatant on to the gradient. The most commonly used centrifugation condition for the gradient is 150,000 *g* for 16-22 h at 4°C, although 44 h at 75,000 *g* has also bee reported [4,8]. Allow the rotor to decelerate from 3000 rpm without the brake or use a slow deceleration program.

2.5. Gradient harvesting

The gradients are routinely harvested in small equal volume fractions (approx 75-125 µl). Low-density end first is recommended because of the viscosity of the densest fractions. Upward displacement with a dense medium or aspiration from the meniscus are recommended. For more information on unloading gradients see **Application Sheet M04**.

2.6. Analysis

Analysis of the fractions from *Xenopus* oocytes showed that 42S RNPs (distinguished by the presence of 4-5S RNA) banded in the top third of the gradient; RNPs containing mRNA in the middle third and ribosomes in the bottom third [3]. Mammalian cells demonstrated a similar distribution; free proteins were observed in fractions 4-10, overlapping the 5S RNA, mRNPs banded in the middle (fractions 14-17) and polysomes in fractions 18-23 [10]. The gradient was used to show that an IGF-II-mRNA binding protein localizes not to the endoplasmic reticulum, which banded around 1.09-1.16

g/ml but to RNPs around 1.23 g/ml [5]. Fractionation of material from mouse testis revealed that the majority of the mRNA was associated with non-polysomal mRNP fractions, distinct from monosomes and polysomes [12]. Nuclear extracts analyzed on these (and metrizamide gradients) showed that cytoplasmic messenger RNP peaked around 1.21 g/ml while pre-mRNP banded around 1.31 g/ml and at 1.18 g/ml [14].

3. Using OptiPrep™ as a replacement for Nycodenz®

There is no obvious reason why iodixanol cannot be substituted for Nycodenz®. Its availability as a sterile 60% (w/v) solution (OptiPrep™) generally makes solution preparation much more easy (see **Application Sheet M01**), the most convenient way of preparing solutions containing 0.3 M KCl, 2 mM $MgCl_2$, 20 mM HEPES-KOH, pH 7.4 would be to dilute 5 vol. of OptiPrep™ with 1 vol. of 1.8 M KCl, 12 mM $MgCl_2$, 120 mM HEPES-KOH, pH 7.4, so the highest achievable iodixanol concentration would be 50% (w/v).

4 OptiPrep™-based methods

4.1 Ribonucleoproteins

Apcher et al [15] studied the capacity of the glycine-alanine repeat (GAr) sequence of the Epstein-Barr virus-encoded EBNA-1 to suppress mRNA translation in *cis*. Iodixanol gradients (not defined) centrifuged at 155,000 *g* for 2 h, were able to identify 40S ribosomes, 60S ribosomes and 80S ribosomes + polysomes. See also Section 4.7.

4.2 Hepatitis C virus RNA

Hepatitis C virus (HCV) released from infected human hepatoma cells has been analyzed in iodixanol gradients. The RNA-containing material was first concentrated on a cushion of 40% (w/v) iodixanol in Tris-buffered 0.85% NaCl at 52,000 *g* for 6 h. After discarding the supernatant the interfacial material was mixed into the cushion; overlaid by a 0-28% iodixanol gradient and centrifuged at 110,000 *g* for 16 h. The characteristic lipid rich RNA containing particles banded at 1.04-1/06 g/ml, while residual RNA banded at 1.13 g/ml [16]. A broadly similar dichotomy of low density lipid-associated RNA and high density RNA from HCV-infected cells has been observed using self-generated iodixanol gradients [17-21]. In this case the cell homogenate was simply adjusted to approx. 25% (w/v) iodixanol, 0.084 M sucrose, 4.8 mM EDTA, 20 mM Tris-HCl, pH 7.4 and centrifuged at 165,000 *g* for 24 h.

4.3 Hepatitis B nucleocapsid

Wang et al [22] analyzed DDX3, HBV Pol, and the core protein from lysed transfected HEK293 cells in 2 ml 10-50% (w/v) iodixanol gradients in 1% NP-40, 150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, pH 7.4 (plus protease inhibitors); the gradients were centrifuged at approx. 200,000 g_{av} for 45 min at 20°C. Each protein had a distinct density distribution profile and the DDX3 protein was shifter to lower densities after RNase A treatment and the iodixanol gradient showed that it is this protein that is incorporated into nucleocapsids.

Kim et al [23] also used small volume sedimentation velocity gradients of the same range, again made up by dilution of OptiPrep™ with cell lysis buffer and centrifuged under the same conditions. The gradient showed that unlike wild-type Pol, mutant Pols were not incorporated into capsid particles. This gradient demonstrated that core proteins of the nucleocapsid banded about two-thirds of the way down the gradient, Pol on the other hand, while overlapping the core proteins, showed a broader distribution towards lower densities [23]. The eIF4E (a eukaryotic translation initiation factor) showed a biphasic distribution, predominating in the lower density fractions (maybe polysomes). Proteinase K and RNase treatment caused a loss of the less dense banding of both eIF4E and Pol, which were detected only in the denser nucleocpasid region of the gradient [24].

4.4 Micro RNA

Detzer et al [25] analyzed siRNA and RNAi-associated proteins from normal and $NaAsO_2$-stressed ECV 304 cells; post-nuclear supernatants were layered on top of 10-25% (w/v) iodixanol gradients in

250mM sucrose, 140mM NaCl, 1mM EDTA, 20mM Tris–HCl, pH 8.0, 2mM DTT and centrifuged at 48,000 *g* for 18 h.

4.5 Mitochondrial nucleic acids and nucleoproteins

He et al [26] and Di Re et al [27] described the use of iodixanol gradients for the analysis of mitochondrial nucleoproteins. A hypotonic DDM-containing medium was used to lyse trypsinized mitochondria; then after a low-speed centrifugation (1000 *g* for 10 min) the lysate was loaded on to a 20–45% (w/v) iodixanol gradient (in 20 mM HEPES-NaOH pH 7.6, 1mM EDTA, 25mM NaCl, 1mM DTT, 0.1% DDM) and centrifuged at 100,000 *g* for 12 h. For more details see refs 26 and 27. The mtDNA bands sharply in the gradient at approx. 30-32.5% (w/v) iodixanol and it binds proteins involved in mitochondrial nucleoid organization [26] and DNA polymerase γ (POLGβ) [27]. More recently the same group has reported the binding of β-actin, myosin and TFAM [28]

A slightly shallower gradient of 20–42.5% (w/v) iodixanol gradient run for 14 h was used by Sharma et al [29]. The distribution of t-RNA, m-RNA and DNA in the gradient is shown diagrammatically in Figure 1. This gradient showed that mtDNA associated with (a) the telomerase reverse transcriptase [29] (b) PABPC5 (a member of the cytosolic poly(A) binding protein family) [30], (c) recombinant C4orf14 [31]. In spite of the proximity of m-RNA and mtDNA in the gradient the careful fractionation used by He et al [32] in their studies on nucleoid interacting proteins and mitochondrial protein synthesis, clearly demonstrated that some proteins were primarily bound to m-RNA and others to mtDNA. Kazak et al [33] showed that an isoform of replication protein Flap endonuclease 1 interacts with RNA/DNA hybrids in mtDNA.

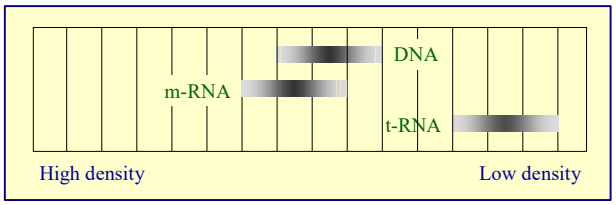

Figure 1: Distribution of nucleic acids in 20-42.5% iodixanol gradient; adapted from ref 29; for details of gradient see text.

A gradient of 10-50% (w/v) iodixanol centrifuged at 85,000 *g* for 15 h was used in studies of the involvement of specific m-RNAs in circadian clock negative feedback [34].

Detergent-solubilized mitochondria from HEK cells, when analyzed by a top-loaded 20-42.5% (w/v) iodixanol gradient (100,000 *g* for 14h) showed that PrimPol, a DNA primase and polymerase, was resolved from mtDNA and DNA maintenance proteins. However formaldehyde cross-linking prior to detergent lysis revealed a transient cross-linking of PrimPol with mtDNA [35]. Identical gradient and centrifugation conditions were used by Rosa et al [36] to show that the protein MPV171.2 co-fractionated with mitochondrial nucleoids.

A similar gradient, in which the sample was bottom-loaded was used by Rajala et al [37] to study the association of replication factors with mtDNA. Briefly, purified mitochondria were either lysed in Triton X-100 with or without a preliminary pelleting of the membranes with digitonin. The material was adjusted to 42.5% (w/v) iodixanol and overlaid with a discontinuous 0, 20% and 25-40% iodixanol gradient (in 2.5% steps), containing buffered saline and 1% T-X100. The mtDNA + nucleoid-associated protein banded at approx 25-27% iodixanol, while larger ribosomal subunit proteins banded at approx. 37.5% iodixanol after centrifugation at 100,000 *g* for 14 h.

Studies of mtDNA nucleoids by Lee et al [38] and Bogenhagen [39] use a double gradient approach which used a sedimentation velocity glycerol gradient followed by a buoyant density iodixanol gradient. Briefly, mitochondria were lysed in Triton X100 and the clarified material was loaded on to a 15-40% glycerol gradient (over a cushion of 30% (w/v) iodixanol/20% glycerol) and centrifuged at 210,000 *g* for 4 h. The nucleoids (which banded close to the bottom of the gradient) and the mitochondrial ribosomes (peak banding approx. 23% glycerol) were harvested and re-run through a 20-40% (w/v) iodixanol gradient, centrifuged at 140,000 *g* for 12-14 h. In this gradient mtDNA and the DNA-binding protein (TFAM) banded around 33% iodixanol, while ribosomal complexes banded at

approx 26% iodixanol. A detailed account of the double-gradient procedures for resolution of these mitochondrial nucleoids can be found in ref 40.

Gerhold et al [41] used a flotation strategy to show that the helicase Twinkle, which is required for mtDNA replication in nucleoids is associated with a detergent-resistant membrane domain that was resolved by floatation through an iodixanol gradient. The discontinuous gradient, which was formed from 40, 37.5, 35, 32.5, 30, 27.5, 25, 20 and 0% iodixanol solutions containing 1% Triton X-100 was under-layered by the detergent-treated membrane sample and centrifuged at 100000 *g* for 14 h. During the centrifugation the gradient would have become continuous; Twinkle and DNA co-banded towards the top of the gradient.

4.6. Analysis of Tacaribe virus nucleoproteins

In a study of RNA replication in Tacaribe virus Baird et al [42] developed a very useful iodixanol gradient for the analysis of replication transcription complexes (RTCs). The virus-containing cells are lysed in a medium containing K-aspartate, K-glutamate and K-gluconate (all 38 mM), !0 mM $KHCO_3$, 2 mM $MgCl_2$ (or 5 mM EDTA), 2mM DTT, 10μM $ZnCl_2$ and 20 mM MOPS pH 7.1 (plus protease inhibitors), either by Dounce homogenization or addition of a detergent (2% NP40). A 15-48% (w/v) iodixanol gradient (containing the same reagents as the lysis medium) is formed in 5 ml tubes for a swinging-bucket rotor; either using a gradient former or by allowing a discontinuous gradient (equal volumes of 15%, 26%, 37% and 48% iodixanol to diffuse. After adjusting the sample to 50% (w/v) iodixanol it is layered beneath the continuous gradient and centrifuged at 100,000 *g* for 20 h. **For more information on gradient formation and the underlayering of samples see Application Sheet M02**. As with all flotation gradients, soluble proteins remain in the load zone, allowing the other macromolecules and macromolecular complexes to float into the gradient. Most of the Tacaribe virus nucleoprotein banded at a density that confirmed its association with the virus membrane [42]. Importantly the gradient was also able to distinguish the full length RNAs (which co-banded with the nucleoprotein) and denser mRNA nucleoprotein. Baird et al [42] also observed that the gradient was able to resolve other novel RNA species. For more information on the analysis see ref 42.

4.7 RNA granules

Fritzsche et al [43] designed a gradient system to purify the neuronal granules that transport RNAs to dendrites. A soluble fraction (20,000 *g*/15 min supernatant) was layered over a 15-30% iodixanol gradient and centrifuged at 280,000 *g* for 2 h. The granules banded in the middle of the gradient and were well separated from the soluble proteins, which remained near the top of the gradient. This methodology was also used by Donlin-Asp et al [44] for the study of fibroblastic ribonucleoproteins from patients with spinal muscular atrophy [44].

4.8 siRNA carriers

Separation of excess nanogel surfactant from the surfactant-coated siRNA was achieved by sedimenting the particles through 7.5% OptiPrep at 30,000 *g* for 30 min [45].

4.9 Separation of ribosomsal and non-ribosomal RNA

In a study of the stress complexes from kidney proximal tubule cells, the cell extract (adjusted to 2.5% iodixanol) was analyzed on a gradient of 5, 10, 15, 20, 25, 50 and 54% iodixanol (155,000 g_{av} for 3 h). Cytoplasmic proteins were found at the top of the gradient, non-ribosomal RNA in the 2.5-15%, while ribosomal RNA banded in the 20-54% region [46].

4.10 DNA nanotechnology

New methods of encapsulating DNA may use iodixanol gradients for purification (see refs 47-49).

5. References

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