OptiPrep™ Application Sheet S10

Isolation of nuclei from animal tissues and cultured cells

- OptiPrep™ is a 60% (w/v) solution of iodixanol in water: density = 1.32 g/ml
- **OptiPrep™ Mini-Review MS01 "Purification of nuclei from tissues and cells in isoosmotic iodixanol gradients – a methodological review"** lists many of the protocol variations
- **OptiPrep™ Mini-Review MS02 "Purification of nuclei from tissues and cells in isoosmotic iodixanol gradients – a bibliography"** lists all the published papers. The methodology has been adapted various **plant and invertebrate sources**
- To access MS01 and MS02 return to the initial list of Folders and select "**Mini-Reviews**".
- To access other Application Sheets referred to in the text return to the Subcellular Membranes Index; key Ctrl "F" and type the S-Number in the Find Box.
- **This Application Sheet describes a widely used discontinuous iodixanol gradient but simpler density barrier methods have also been used (see Section 4)**

1. Background

Earlier methods for purifying nuclei involve pelleting through a 60% sucrose density barrier at 100,000 *g* for 1-2 h. Not only is the sedimentation of the particles very slow because of the high viscosity of the sucrose barrier, the nuclei become severely dehydrated because of loss of water from their internal space due to the osmotic gradient across the membrane. This process may disrupt the macromolecular structures, which are normally highly hydrated. In 1984 the introduction of Nycodenz®, whose solutions have a much lower viscosity and osmolality than those of sucrose, permitted for the first time, the banding of nuclei at an interface using only 13,000 *g* for 1 h. A commonly used method is to layer the crude nuclei in 35% Nycodenz® between layers of 20% and 40%, with a 50% cushion; the nuclei sediment to the 40%/50% Nycodenz® interface (the 40% and 50% Nycodenz® solutions are not however isoosmotic). The method, originally worked out for mouse liver [1-3] has been used for gut mucosa [3], HeLa cells [4], ovarian cells [5] and testicular cells [6,7].

Using iodixanol gradients nuclei can now be isolated by isopycnic banding in an iso-osmotic environment [8]. Because the nuclei retain their normal hydration their density is much lower than that in sucrose (1.20-1.22 against >1.32 g/ml) and slightly lower than in Nycodenz®. Buoyant density banding in iodixanol thus requires only 10,000 *g* for 20 min. The homogenate is adjusted to approx ρ = 1.14 g/ml (25% iodixanol); layered over two solutions of 30% and 35% iodixanol (ρ = 1.175 g/ml and 1.20 g/ml) and centrifuged at 10,000g to band the nuclei at the lower interface. This protocol, designed for mammalian liver, has been applied to many tissue types and to cultured cells; only in a few cases were small modifications required. It has also been applied to plant tissue **(see Section 3).**

2. Mammalian tissues and cultured cells

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

A. OptiPrep™

B. Diluent: 150 mM KCl, 30 mM $MgCl_2$, 120 mM Tricine-KOH, pH 7.8

C. Working solution containing 50% (w/v) iodixanol: mix 5 vol. of solution A with 1 vol. of solution B

D. Homogenization Medium: 0.25M Sucrose, 25 mM KCl, 5 mM $MgCl_2$, 20 mM Tricine-KOH, pH 7.8

E. Gradient solutions: Prepare two gradient solutions of 30% and 35% (w/v) iodixanol by diluting solution C with solution D (6 vol. + 4 vol. and 7 vol. + 3 vol. respectively)

Keep the following stock solutions at 4°C:
500 mM Tricine; 8.96 g per 100 ml water
1 M KCl; 7.45 g per 100 ml water
1 M $MgCl_2 \bullet 6H_2O$; 20.3 g per 100 ml water

Solution B: To 50 ml water add 24 ml, 15 ml and 3 ml respectively of the Tricine, KCl and $MgCl_2 \bullet 6H_2O$ stock solutions; adjust to pH 7.8 with 1 M KOH and make up to 100 ml.

Solution D: Dissolve 8.5 g of sucrose in 50 ml of water; add 4 ml, 2.5 ml and 0.5 ml respectively of the Tricine, KCl and $MgCl_2 \bullet 6H_2O$ stock solutions; adjust to pH 7.8 with 1 M KOH and make up to 100 ml.

2b. Protocol

Carry out all operations at 0-4°C

1. Produce an homogenate of the tissue or cell using solution D and use this for Step 2. Alternatively produce a crude nuclear pellet by centrifugation at 1000 *g* for 10 min; resuspend the pellet in solution D and use this for Step 2.

2. Mix equal volumes of the sample (homogenate or resuspended nuclear pellet) and Solution C and transfer 10-15 ml to a suitable centrifuge tube (40-50 ml) for a swinging-bucket rotor of a high-speed centrifuge **(see Section 2c, Note 2).**

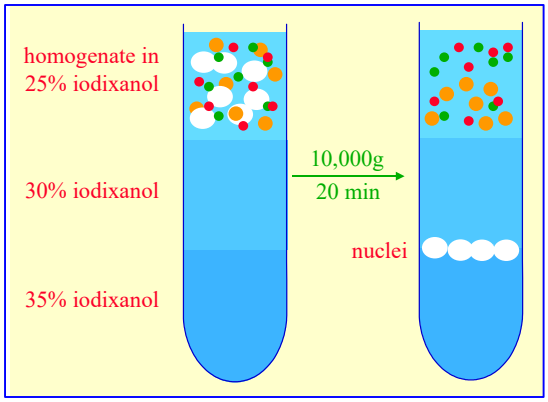

Figure 1 Isolation of mammalian nuclei in iodixanol gradient

3. Underlayer the sample with 10 ml of the 30% iodixanol and 5-10 ml of the 35% iodixanol; for more information on creating discontinuous gradients **see Application Sheet S03**.

4. Centrifuge at 10,000 g_{av} for 20 min **(see Section 2c, Notes 3-5)**.

5. Collect the band of nuclei at the 30%-35% iodixanol interface (see Figure 1).

♦ The 30-35% interface material contains >90% of the total DNA. Phase contrast microscopy shows no discernible contaminants and 95% of the succinate dehydrogenase is recovered in the top layer.

2c. Notes

1. Protease inhibitors (PMSF, leupeptin, antipain, aprotinin etc) may be included in any or all of the media at the operator's discretion. The preparation of a working solution of 40% or 50% (w/v) iodixanol as described ensures that the ionic concentration is constant through the gradient. If this is considered unnecessary, the gradient solutions may be prepared simply from OptiPrep™.
2. With a crude nuclear (1000 *g*) pellet, the protocol can be simplified by resuspending the pellet in homogenization medium and adjusting it to 30% iodixanol. After centrifugation the nuclei will form a pellet and contaminating membranes will float to the top. If this approach is used with an homogenate, the pellet of nuclei will be tend to be contaminated by peroxisomes.
3. The rapid sedimentation rate of nuclei, compared to that of other particles present, ensures that only the nuclei are able to sediment through the 30% iodixanol layer at the time and rotor speed used. Other particles remain in the sample or at the sample/ 30% iodixanol interface (see Figure 1)
4. With mammalian liver, the protocol can be carried out at g-forces as low as 5000 *g* (for 20 min) without any significant reduction in recovery of nuclei.
5. The density and/or the rate of sedimentation of nuclei from other tissues and from cultured mammalian cells may be different to those from mammalian liver. However, the described protocol seems to have a quite wide application; according to the published papers rather few modifications have been required for the satisfactory purification of nuclei from a range of cells and tissues. It may however be necessary to modulate either the centrifugation time or the density of the layers. Some variations are:
   - ♦ CHO cell nuclei banded at a 25%/30% interface, centrifugation time 40 min [9].
   - ♦ Crude nuclear pellet suspended in median 30% iodixanol layer, HeLa [10] and Caco2 [11] cells
   - ♦ Invertebrates: 15-20% (w/v) iodixanol continuous gradient, 100,000g for 2h [12-14]
   - ♦ Mouse liver: 12,000 *g* for 2 h [15]
   - ♦ Squirrel/hamster liver: 0-35% (w/v) iodixanol continuous gradient [16,17]

3. Plant protoplasts

3a. Background

Xiong et al [18] and Lannoo et al [19] used a slightly modified iodixanol gradient to prepare nuclei from cultured BY2 tobacco cells, but the method almost certainly has a wider application to any lysed plant protoplast preparation. This Application Sheet is not primarily concerned with a detailed description of the method for protoplast preparation, which will probably vary from source to source. A method for the purification of protoplasts from green leaf tissue by flotation through a low-density iodixanol barrier is given in **Application Sheet C18**. The protocol for protoplast preparation from BY2 cells is clearly detailed by Xiong et al [18]. Once the plant cells had been digested in a mixture of pectolyase, cellulose and driselase and washed, they were isolated by flotation through a 20%, 10%, 0% (w/v) Ficoll 400 gradient.

3b. Solutions required (see Section 3d, Notes 1-2)

A. OptiPrep™

B. Lysis buffer: 0.4 M sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 0.1 mM dithiothreitol (DTT), 5 mM EDTA, 10 mM MES-KOH, pH 5.3

C. OptiPrep™ diluent: 30 mM NaCl, 15 mM $MgCl_2$, 0.3 mM dithiothreitol (DTT), 15 mM EDTA, 30 mM MES-KOH, pH 5.3

D. Iodixanol (40% w/v) Working Solution (WS): mix 4 vol. of OptiPrep™ with 2 vol. of Solution C

Keep the following stock solutions at 4°C:
1 M MES; purchased ready-made solution
1 M NaCl; 5.84 g per 100 ml water
100 mM $MgCl_2 \bullet 6H_2O$; 2.03 g per 100 ml water
10 mM DTT; 0.154 g per 100 ml water
100 mM EDTA ($Na_2 \bullet 2H_2O$) 3.72 g per 100 ml water

Solution B: Dissolve 13.7 g of sucrose in 50 ml of water; add 1 ml of MES, 1 ml of NaCl, 5 ml of $MgCl_2$, 5 ml of EDTA and 1 ml of DTT stock solutions. Adjust to pH 5.3 with 1 M KOH and make up to 100 ml.

Solution C: Mix 3 ml of MES, 3 ml of NaCl, 15 ml of $MgCl_2$, 15 ml of EDTA and 3 ml of DTT stock solutions. Adjust to pH 5.3 with 1 M KOH and make up to 100 ml.

3c. Protocol (adapted from ref 18)

1. Wash the protoplasts in Solution B using 160 *g* for 5 min to pellet the protoplasts; suspend in Solution B and disrupt by eight passages through a 26G syringe needle.

2. Prepare solutions of 10%, 25%, 30% and 36% (w/v) iodixanol by diluting Solution D with Solution B (1:3, 2.5:1.5, 3:1 and 3.6:0.4 v/v respectively).

3. Prepare a discontinuous iodixanol gradient from 2 ml of each gradient solution; for more information on preparing gradients **see Application Sheet S03 (see Notes 3-6)**.

4. Layer the lysed protoplast suspension on top ($2x10^6$ protoplasts per 8 ml gradient) and centrifuge the gradients at 3000 *g* for 30 min. Harvest the nuclei from the 30%/36% iodixanol interface.

3d. Notes

1. Protease inhibitors may be included in any or all of the media at the operator's discretion. The preparation of a working solution of 40% or 50% (w/v) iodixanol as described ensures that the concentration of ions, EDTA etc is constant through the gradient.
2. Because of the variable hydration of MES powder, use of a commercial MES stock is preferred.
3. Nuclei from wheat-germ have been isolated using 0.4 M sucrose, 25 mM KCl, 5 mM $MgCl_2$, 10 mM MES, pH 6.2 as a lysis medium [20]. Gradient solutions of 1.167 and 1.234 g/ml were used (OptiPrep™: lysis medium of 8.5:11.5 and 13.5:6.5 respectively). The osmolality of these solutions is 480-500 mOsm. A crude nuclear suspension (20 ml) was layered over 5 ml each of the gradient solutions. After centrifugation at 5,600 *g* for 30 min the nuclei band at the lower interface, other organelles at the top interface and the starch granules pellet.
4. As with animal tissues and cells, the density of the gradient layers (and the centrifugation conditions) may also require modulation to optimize the purification for specific types of plant tissue. Nuclei have been isolated from *Sorghum bicolor* leaves (in this study the authors preferred iodixanol to Percoll®) [21] and cow-pea leaves [22]. Preliminary indications suggest that for yeast nuclei it is necessary to increase the density of the bottom layer to at least 1.26 g/ml.

5. For Xanthi protoplasts a simplified gradient of 25% and 36% (w/v) iodixanol was used [23] under the same centrifugation conditions.
6. Liu et al [24] separated a cytoplasmic and nuclear fraction from *Arabidopsis thaliana* by loading an homogenate on top of a two layer gradient of 15% and 45% (w/v) iodixanol. Using 1,500 *g* for 15 min resolved a cytoplasmic fraction (sample zone) and purified nuclei at the interface of the two iodixanol solutions.

4. Density barrier methods

There are a few examples of the use of a simple density barrier, first reported for HEK cells [25] in which a crude pelleted nuclear fraction was suspended in 30% (w/v) iodixanol and centrifuged at 10,000 *g* for 10 min; subsequently the pellet was resuspended in the same medium and the centrifugation repeated. The method has been applied with minor variations) to neural progenitor cells [26] carcinoma cells [27] and *Xenopus* embryo [28]. In the latter case the *g*-force used was only 1000 *g*. Rat brain nuclei have been pelleted through a 20% iodixanol barrier [29] and those from endothelial cells and fibroblasts have been pelleted through 6% iodixanol at 20,000 *g* for 30 sec [30].

5. References

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