

OptiPrep™ Mini-Review MS01

Purification of nuclei from tissues and cells in isoosmotic iodixanol gradients – a methodological review

- ◆ **Mini-Review MS18** provides a full bibliography of all papers reporting the use of iodixanol gradients for isolation of nuclei; references are indexed according cell or tissue type and, where relevant, according to research topic.

1. Sucrose-barrier and detergent methods

The low-speed pellet produced by the centrifugation of tissues or cell homogenates at 600-1000 g for 10 min will contain 90-95% of the total nuclei, but it will be contaminated by significant amounts of mitochondria, large fragments of membrane, intact or partially broken cells and other organelles trapped by these rapidly sedimenting particles. Although the contamination may be partially reduced by repeated washing of the pellet, this procedure risks progressive damage to the nuclei by the shearing forces used to disperse the pellet. In the nineteen-sixties two influential papers [1,2] were published both of which reported the pelleting of nuclei through high-density sucrose solutions to remove the contamination effectively. In the method of Widnell and Tata [1] a crude nuclear pellet (homogenate centrifuged at 600 g for 10 min) was washed once in the homogenization medium; suspended in 2.2 M sucrose, 1 mM MgCl₂, 10 mM Tris-HCl, pH 7.4 and then the nuclei pelleted at 60-80,000 g for 80 min at 4°C. The method of Blobel and Potter [2] omitted the low-speed pelleting of the nuclei directly from the homogenate: the sucrose concentration in the filtered homogenate was adjusted to 1.6 M by addition of 2.3 M sucrose, 50 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM MgCl₂, and then layered over this 2.3 M sucrose solution. The nuclei were pelleted at 130,000 g for 30 min at 4°C.

The major disadvantages of these high-density sucrose barriers are:

- ◆ Difficulty in preparing and handling high concentration sucrose solutions that are close to saturation.
- ◆ The high viscosity of the solutions requires the use of an ultracentrifuge to pellet the nuclei.
- ◆ Solutions are grossly hyperosmotic (approx. 2,400 mOsm; the cytosol is approx. 290 mOsm). Nuclei lose water and become highly condensed; loss of bound water from nucleoproteins may affect their stability.

2. Homogenization media

2a. Mammalian tissues

One aspect of the sucrose barrier methods that has been retained in many subsequent techniques is the nature of the homogenization medium; **approximately isoosmotic** solutions containing K⁺ and Mg²⁺ ions generally promote the retention of nuclear structure and function during their isolation. The marginally hyperosmotic **Blobel and Potter medium**, 0.25 M sucrose containing 25 mM KCl, 5 mM MgCl₂ buffered originally with 50 mM Tris-HCl, pH 7.4-7.8 [2], is widely used for rodent liver with some variation in the buffer, which has often been changed to the more organelle-friendly HEPES-KOH or Tricine-KOH and whose concentration is frequently reduced to 10 or 20 mM [3-9]. Provost et al [4] added 1 mM EDTA to the medium and Pyhtila et al [7] further added 1 mM DTT. The Blobel and Potter medium is also used for rat and sheep kidney [10-13], rodent brain [9,14,15], spleen [6], thymus [16] and testis [6,16]. In the case of frozen human brain tissue specimens the medium is supplemented with Brij [17,18]. Occasionally the medium is a general-purpose buffered 0.25 M sucrose solution containing EDTA [19] or it may be supplemented with Ca²⁺, for example 0.25 M sucrose, 20 mM HEPES-KOH, pH 7.4, 200 μM CaCl₂ [20].

More rarely, the medium is **significantly hyperosmotic**: frozen hamster and squirrel livers have been homogenized in 25 mM KCl, 1 mM EDTA, 10 mM HEPES-KOH, pH 7.6 containing 40% glycerol [21,22]. Spermine and spermidine were also included to inhibit nitric oxide synthase. **Hypoosmotic** solutions are more often used for cultured cells, but they are occasionally used for tissues: rat brain tissue has been homogenized in 25 mM KCl, 2 mM MgCl₂, 10 mM HEPES-KOH, pH 7.5 [23] and spleen in 20 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1mM DTT, 1% NP40 [24].

2b. Cultured mammalian cells

Although there are examples of the use a standard, more or less isoosmotic, **Blobel and Potter medium** for carcinoma cells [25-27], hepatoma cells [28], epithelial cells [29], fibroblasts [30-32] and smooth muscle cells [33], efficient homogenization of cultured cells may require reductions in overall solution osmolality and/or

divalent cation concentration and/or inclusion of chelating agent(s). Sometimes low concentrations of a non-ionic detergent, to which nuclei are more resistant than the plasma membrane, are included in the medium. Some of the solutions are given in Table 1. Note that a diverse range of protease inhibitors is routinely included in all of these media.

Table 1 Homogenization media for mammalian tissues and cells

Tissue/cell	Homogenization medium	Ref #
Caco-2	0.25 M sucrose, 5 mM MgCl ₂ , 50 mM Tris-HCl, pH 7.4, 1% TX-100	34
Carcinoma	20 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1mM DTT, 1% NP40	35-40
	10 mM Tris, pH 7.4, 1.5 mM MgCl ₂ , 150 mM NaCl, 100 µg/ml digitonin	41
	10 mM NaCl, 10 mM Tris, pH 8.4, 5 mM MgCl ₂	42
	10 mM HEPES, pH 7.9, 1.5 mM MgCl ₂ , 10 mM KCl, 5mM DTT	43
	4 mM Tris, pH 7.8, 2.5 mM NaCl, 0.5 mM MgCl ₂	44
CHO Fibroblasts	10 mM MgCl ₂ , 1 mM KCl, 5 mM HEPES, pH 7.4	45
	10 mM HEPES, pH 7.6, 1.5 mM MgCl ₂ , 10 mM KCl	46
HEK Keratinocytes Mononuclear ¹	20 mM Tricine, pH 7.8, 250 mM KCl, 5 mM MgCl ₂ , 0.1% TX-100	47
	20 mM Tricine, pH 7.8, 0.25 M sucrose, 25 mM KCl, 5 mM MgCl ₂ , 0.1% TX-100	48
	20 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1mM DTT, 1% NP40	40,49
	4 mM Tris, pH 7.8, 2.5 mM NaCl, 0.5 mM MgCl ₂	44,50
Neural Neuroblastoma	20 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1mM DTT, 1% NP40	51-53
	20 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1mM DTT, 1% NP40	54,55
Neural Neuroblastoma	0.25 M sucrose, 1 mM EDTA, 1 mM DTT, 10 mM triethanolamine-acetic acid, pH 7.4	56
	0.25 M sucrose, 20 mM KCl, 50 mM HEPES, pH 7.8 ± 2 mM EGTA	57

¹ "Mononuclear cells" includes monocytes, lymphocytes and macrophages

2c. Invertebrates and plants

Mussel tissue is homogenized in a HEPES-buffered **Blobel and Potter medium** supplemented with 1 mM EDTA [58-60]. A commonly used solution for *Nicotiana tabacum* BY2 protoplasts and *Arabidopsis thaliana* seedlings is 0.4 M sucrose, 10 mM MES, pH 5.3, 10 mM NaCl, 5 mM MgCl₂, 5 mM EDTA, 0.1 mM DTT [61-63], which for *Arabidopsis* was supplemented with 0.1% TX-100 [63].

3. OptiPrep™ methodology

3a. Discontinuous gradient

The original methodology devised by Graham et al [3] was specifically aimed at streamlining the purification procedure, thus, as with the Blobel and Potter method [2], it is not necessary to produce an initial crude nuclear fraction from the homogenate by low-speed centrifugation. Instead the homogenate is simply adjusted to 25% (w/v) iodixanol by mixing with an equal volume 50% (w/v) iodixanol containing the same concentrations of buffer, KCl and MgCl₂ as the homogenate, and layered on top of a discontinuous iodixanol gradient as shown in Figure 1. The separation requires only a routine high-speed centrifuge, rather than an ultracentrifuge and a centrifugation time of only 20 min. A saving of at least 1 h in preparation time can be achieved over the standard sucrose gradient method.

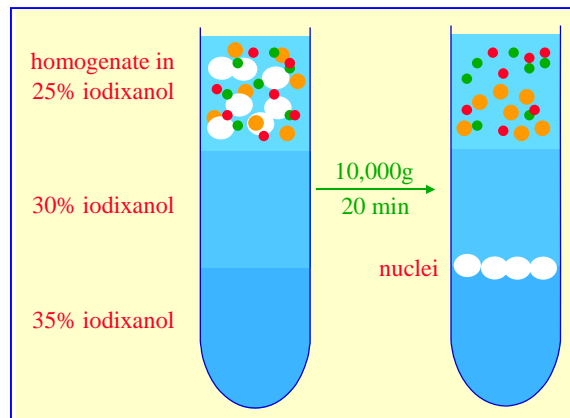


Figure 1 Isolation of mammalian nuclei in iodixanol gradient

♦ Importantly the gradients are approx. isoosmotic throughout and have a low viscosity

A detailed methodology is described in the OptiPrep™ Application Sheet S10; this can be found on the OptiPrep™ Applications flash-drive or accessed via the following website: www.axis-shield-density-gradient-media.com (click on "Methodology", then "Subcellular Membranes" and follow the links from the Index).

The gradient solutions are best prepared as described in the Application Sheet, i.e. a 50% (w/v) iodixanol working solution is first prepared from 5 vol. of OptiPrep™ and 1 vol. of 150 mM KCl, 30 mM MgCl₂, 120 mM Tricine-KOH, pH 7.8 and any lower density solutions are made by dilution of the working solution with 0.25 M sucrose, 25 mM KCl, 5 mM MgCl₂, 20 mM Tricine-KOH, pH 7.8. Thus the concentrations of buffer, KCl and MgCl₂ and the osmotic pressure are approximately constant throughout the gradient.

3b. Discontinuous gradient - protocol variations

(1) If the cells have been homogenized in a hypoosmotic medium, very often the nuclei first are pelleted by low speed centrifugation and then resuspended in buffered 25% (w/v) iodixanol, 25 mM KCl, 5 mM MgCl₂. To avoid pelleting the nuclei prior to the gradient the homogenate has first been centrifuged at 300 g for 15 min over a 40% iodixanol cushion [42,63]; in the case of *Drosophila* and *Caenorhabditis elegans* the cushion was pure OptiPrep™ [64]

(2) Some gradient variations result in pelleting the nuclei rather than banding them at an interface by omission of the 35% (w/v) iodixanol layer; for example in refs 41,65, the homogenate was adjusted to 25% iodixanol and layered over 29% iodixanol.

(3) There are also some instances in which the gradient is altered, but the banding of the nuclei above a 35% (w/v) iodixanol layer is usually retained (but note #7), for example:

1. Homogenate layered over 12.5% and 35% (w/v) iodixanol [28]
2. 10%, 20% (nuclear fraction), 25%, 30% and 35% (w/v) iodixanol [10-13]
3. Homogenate layered over 30% and 35% (w/v) iodixanol [52,66,67]
4. Homogenate adjusted to 17.5% (w/v) iodixanol [20]
5. Nuclear pellet in buffered 0.32 M sucrose, layered over 25%, 30% and 35% iodixanol [55]
6. Nuclear pellet in buffered 0.25 M sucrose, layered over 15%, 20% and 35% iodixanol [31]
7. Plant nuclei were resolved by sedimenting at a 15%/45% iodixanol interface [68]

The centrifugation conditions generally deviate relatively little from the recommended *g*-force and time, with a few notable exceptions. Plant nuclei tend to be banded under more mild conditions, e.g. 3000 g for 30 min [61] and 1,500 g for 15 min [68]; lower *g*-forces (4,500 g for 30 min) have also been used for macrophage [66,67] and hepatoma [28] nuclei. Significantly higher *g*-forces are only rarely used; nuclei from mussel tissue and murine brain were banded at 100,000 g for 2 h [58-60] and 270,000 g for 3 h [20] respectively.

3c. Density barriers

A simpler variation is to layer either the homogenate or a crude nuclear fraction over a density barrier (usually 29-30% (w/v) iodixanol), through which the nuclei pellet when centrifuged at approx. 10,000 g for 10-40 min; this approach has been used with brain tissue [69,70] and maize coleoptile [71]. An even more simple option is to adjust the sample to 20-30% (w/v) iodixanol, from which the nuclei sediment while the other organelles remain in suspension (12,000 g for 4 min and 10,000 g for 10 min have been used). This approach has been used for carcinoma cells [41,72,73], HEK [73] and ovarian cells [74] and the separations, described in detail by Guilluy et al [75], are often repeated. Palmowski et al [76] described a similar system for brain nuclei in 20% (w/v) iodixanol and centrifuged for a longer time of 40 min. A more unusual variant is to pellet endothelial cell nuclei through a 6% (w/v) iodixanol barrier at 20,000 g for just 30 sec [77].

- ◆ In a study of the uptake of plasmid DNA into the nucleus Cohen et al [78] compared the widely-used detergent method for solubilizing all non-nuclear membranes and the OptiPrep™ method and found that the latter “yielded nuclei with substantially less adhering plasmids on the outside of the nuclei”.

4. References

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