OptiPrep™ Application Sheet S63

Mammalian cell exosomes and other microvesicles from conditioned medium

1. Introduction

♦ 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 **2020SMemapp** file and select the appropriate S-number.

♦ **OptiPrep™ Reference List (RS10)** "**Mammalian (including all chordata) cell exosomes and other microvesicles from tissues, cells and conditioned medium**" provides a full reference list: to access return to the initial list of Folders and select "**Reference List**".

♦ There is a companion to **Application Sheet (S62)** on purification of bacterial and fungal extracellular vesicles from culture medium and a corresponding **OptiPrep™ Reference List (RS11)**.

♦ The culture medium is often purified before contact with the cells under study (see Section 2).

♦ After the period of culture, the medium usually undergoes pre-gradient treatment for the removal of larger particles and reduction in sample volume; these are considered in Sections 3a-3d.

2. Preparation of the culture medium

To minimize contamination of the exosomes expressed into the culture medium, the latter is often, but not always, centrifuged at approx 100,000 g for 1-16 h and/or filtered (usually a 0.2-0.22 µm filter) to remove any particulate material, before it is put in contact with the cell monolayer. Sometimes it is just the foetal bovine serum, used as a supplement to the culture medium, that is clarified using the same ultracentrifugation and/or filtration. Occasionally serum-free medium is used.

Once the culture medium has been in contact with the cell monolayer for the requisite length of time, the conditioned medium (CM) is harvested and then variously clarified, partially purified and/or concentrated prior to iodixanol gradient purification.

3 Pre-iodixanol gradient treatment
3a. Differential centrifugation (low and high-speed)

Sometimes a single low-speed centrifugation is used to remove cells and other large particles such as apoptotic bodies, e.g. 1000-2000 g for 10 min [1]; more often a more extensive differential centrifugation scheme is used prior to the final recovery of exosomes (and other microparticles) by ultracentrifugation. Although it might be tempting to delete the first low-speed centrifugation, failure to include this step will probably lead to the entrapment and loss of small vesicles into aggregates of rapidly-sedimenting larger particles at a higher g-force. Some examples are given in Table 1.

Table 1 CM differential centrifugation schemes

Centrifugation schedule	Ref	Centrifugation schedule	Ref
480 g/5 min; 1,900 g/10 min	2	500 g/15 min; 10,000 g/30 min	7
300 g/10 min; 5,000 g/10 min	3	300 g/10 min; 2,000 g/20 min; 20,000 g/70 min	8[1]
500 g/10 min; 800 g/15 min (2x)	4	300 g/10 min; 1,000 g/ 10 min; 20,000 g/25 min	9[1]
300 g/10 min; 2,000 g/10 min; 10,000 g/20 min	5	800 g/10 min/10,000 g/10 min	10
480 g/5 min; 2,000 g/10 min	6	500 g/10 min/10,000 g/30 min	11

[1] The centrifugation of CM from a muscle cell culture 20,000 g was carried out to pellet microparticles (MPs), which are distinct from the more slowly-sedimenting exosomes [8]. This 20,000 g pellet was identified as containing microvesicles and was used for subsequent iodixanol gradient purification of vexosomes, membrane-bound particles containing rAAV capsids that are efficient delivery vehicles for vectors in cell transduction [9].

♦ Microvesicles have also been isolated from human plasma [12] and have been identified as agents in the transport of miRNAs between cells. The plasma was treated to a similar sequence of differential centrifugation steps of 300 g, 1200 g, and 10,000 g

♦ See Section 3c for details of the final centrifugation steps to concentrate the exosomes prior to iodixanol gradient purification.

3b. Filtration

Removal of larger contaminants is commonly performed using a 0.20 or 0.22 µm syringe filter, occasionally with a 0.1µm [2,6] or 0.45µm [13] filter. The filtration may be carried out before [3], or more frequently after the first low-speed centrifugation [1,2,11,14]; or occasionally after a second higher speed centrifugation [6]. Occasionally filtration is the only pre-gradient treatment [13,15,16,17].

3c. Concentration of exosomes and other vesicles by pelleting

Virtually all of the published methods involve a final pelleting of the exosomes and other microvesicles at 100-150,000 g for 1-2 h before resuspending in a suitable buffered medium for application to the iodixanol gradient. The exceptions to the use of these g-forces are the methods that use 20,000 g for muscle cell microparticles [8] and vexosomes [9] - see Table 1.

♦ Duelli et al [14] described a method in which the CM was initially centrifuged at 500 g and filtered, before being centrifuged sequentially at 12,000 g, 70,000 g and 110,000 g. In the differential centrifugation fractions, exosomal fusogenic activity was heavily concentrated in the 70,000 g pellet, with rather little in the 110,000 g pellet and it was the former that was further purified in the iodixanol density gradient. The lower g-force of 70,000 g was also reported in [11].

Some protocols, particularly those that are applied to large volumes of CM, include a preliminary concentration using centrifugal ultrafiltration, to reduce the total volume prior to this final pelleting at 100-150,000 g. This conveniently avoids the use of large volume ultracentrifuge rotors. These ultrafilters have cut-offs from 5 kDa [2,6] to 100 kDa [4,17].

3d. Sucrose gradients

As part of the pre-iodixanol gradient purification and concentration procedure a discontinuous sucrose gradient is sometimes included. The combination of multiple two-layer sucrose density gradients and iodixanol gradient separations was investigated by Choi et al in their studies on exosomes from colon carcinoma cells [18]. The exosomes were concentrated at the interface of 0.8 and 2.7 M sucrose layers after centrifugation at 100,000 g for 4 h. Microvesicles from mesenchymal stem cells were interface-concentrated in the same two-layer gradient at 100,000 g for 2 h [4].

4. Iodixanol gradient methodology
4a. Gradient solution preparation

A. OptiPrep™

B. 0.85% (w/v) NaCl, 60 mM HEPES (or Tricine) -NaOH, pH 7.4

C. 0.85% (w/v) NaCl, 10 mM HEPES (or Tricine) -NaOH, pH 7.4

When OptiPrep™ is diluted with an isoosmotic solution such as a buffered saline the gradients produced from such a solution will be positive with respect to iodixanol and negative with respect to all the diluent components. If it is considered advantageous to maintain the buffer composition constant then make up the solutions described in Box 1 and produce solutions B and C. Dilute 5 vol. of OptiPrep™ with 1 vol. of Solution B to produce

> **Box 1**
> Keep HEPES (free acid) or Tricine as a 100 mM stock solution at 4°C; Hepes (2.38g) or Tricine (1.79g) per 100 ml water.
>
> Solution B: Dissolve 0.85g NaCl in 30 ml water; add 60 ml of HEPES or Tricine stock solution; adjust to pH 7.4 with 1 M NaOH and make up to 100 ml.
>
> Solution C: Dissolve 0.85g NaCl in 30 ml water; add 10 ml of HEPES or Tricine stock solution; adjust to pH 7.4 with 1 M NaOH and make up to 100 ml.

a working stock of 50% (w/v) iodixanol containing 10 mM buffer (ρ = 1.268 g/m l). When this is subsequently diluted with Solution C all solutions will contain 10 mM buffer.

The same strategy can be applied to other isoosmotic media such as a typical homogenisation medium such as 0.25 M sucrose, 1 mM EDTA, 20 mM HEPES-NaOH, pH 7.4. Solutions B and C are prepared as in Box 2 and handled in the same manner as described for the NaCl based media; the concentrations of EDTA and buffer will be constant in all solutions.

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Box 2
Keep the following stock solutions at 4°C:
500 mM Hepes (free acid), 11.9 g per 100 ml water.
100 mM EDTA (Na$_2$•2H$_2$O), 3.72 g per 100 ml water

Solution B: Dissolve 17 g sucrose in 100 ml water; add 2.0 ml and 8.0 ml respectively of EDTA and Hepes stocks; adjust to pH 7.4 with 1 M NaOH and make up to 200 ml.

Solution C: Dissolve 8.5 g sucrose in 50 ml water; add 6 ml of EDTA stock and 24 ml of Hepes stock; adjust to pH 7.4 with 1 M NaOH; make up to 100 ml.

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An additional sophistication was described in ref 13. A 6% (w/v) iodixanol solution was made up in 215 mM sucrose and a 65.4% (w/v) iodixanol solution in 5 mM sucrose, both solutions containing 2 mM EDTA, 10 mM Tris-HCl (pH 8.0).

The crude vesicle fraction and the diluent for the OptiPrep™ are quite frequently a cell buffer such as phosphate-buffered saline [e.g. refs 1,3,11,14 and15], occasionally supplemented with 2.5 mM MgCl$_2$ [9]. Rather than a phosphate buffer the saline may contain organic buffer such as 20 mM HEPES-NaOH, pH 7.2 [4,5,18]. Dilutions with 0.25 M sucrose, 10 mM Tris-HCl pH 7.5 are also common [2,6,8]. In some instances the exosomes are suspended in, and the gradient solutions prepared in, a simple buffer such as 20 mM HEPES-NaOH, pH 7.4 [10,17]. A 20 mM HEPES-buffered solution of 100 mM KCl and 2 mM MgCl$_2$ was used in ref 12 and in the case of the self-generated gradient method [16], the layers were simply produced by adjusting the density of the crude exosome suspension with OptiPrep™.

4b. Purification and analysis in a top-loaded sedimentation velocity (short spin) gradient

Dettenhoffer and Yu [19] developed a sedimentation velocity iodixanol gradient to purify HIV-1 virions without affecting the infectivity of the virus. The authors noted that in buoyant density sucrose gradients the extracellular Vif gene always co-purifies with the virus and the latter is also contaminated with cell-derived microvesicles. In rate-zonal iodixanol gradients on the other hand the HIV-1 was effectively separated both from Vif and from the microvesicles. Another important point about sucrose gradients is that although sucrose is generally less deleterious to viral infectivity than CsCl, it can nevertheless have serious effects on viral structure; in particular the loss of surface glycoproteins from retroviruses has been noted [20]. This may be related to its viscosity, which is much higher than that of iodixanol. The loss of surface glycoproteins may not be restricted to the virus; it is feasible that similar losses may occur with membrane-bound vesicles.

The sedimentation velocity gradient originally developed by Dettenhoffer and Yu [19], for the purification of HIV particles, comprises a 6-18% (w/v) iodixanol gradient and is usually run in a 12-13 ml swinging-bucket rotor, such as a Beckman SW41. It was constructed as a discontinuous gradient from multiple solutions; the concentration interval from step to step was only 1.2% (w/v) iodixanol. This method was also described in Table 2, lines 6, 7 and 11. It was adapted to a near-vertical (Beckman NVT65) rotor, with tubes of approx. the same volume by Cantin et al [15] and Park and He [21] see Table 2 lines 10 and 12. Because of the much reduced radial distance occupied by the sample, vertical or near-vertical rotors are regarded as the ideal ones for sedimentation velocity. Taking into account the much reduced path length of the near-vertical rotor, compared to the swinging-bucket rotor, it is perhaps surprising that the *g*-forces and centrifugation times used with these two types of rotor were so similar.

♦ Because of the small iodixanol concentration interval between steps of the Dettenhoffer and Yu gradient [19] and the small volume of each step, it is highly likely that the gradient will rapidly become more or less continuous during the preparation and run times. An easier gradient

preparation alternative therefore may be to create a continuous 6-18% (w/v) iodixanol at the outset.

- ♦ **See OptiPrep™ Application Sheet V34 (virus index) for practical details on this method.**
- ♦ **See OptiPrep™ Application Sheet S03 for more information on the construction of gradients**

Table 2 Purification of particles from conditioned medium; gradient and centrifugation conditions

Source of conditioned medium	Gradient (% w/v) iodixanol[1]	Centrifugation conditions	Ref
1 Breast adenocarcinoma cells (MCF-7)	6-40% cont **60**%	140,000*g*/16h	7
2 Colon carcinoma cells (human)	**0**,5,10,20,40% disc	100,000*g*/16h	2
3 Colon carcinoma cells (human)	5,20,**30**% disc	200,000*g*/3h	18
4 Colon carcinoma cells (human)	**0**,5,10,20,40% disc	100,000*g*/18h	6
5 HEK cells (Epstein-Barr virus proteins)	5-30% cont. **35**%	Not given	3
6 HEK cells (AAV producer cells) – vexosomes (large-scale)	**0**,6-18% disc	124,000*g*/2h	9
7 HEK cells (HIV-infected)	**0**,6-18% disc	200,000*g*/1.5h	11
8 Human plasma	**0**,5,10,20,40% disc	200,000*g*/21h	12
9 Lung fibroblasts (human embryo) MRC5	**0**, 6-56.4% cont	110,000*g*/15 h	13
10 Lymphocytes (T cells), dendritic cells and macrophages): HIV-1 infected cells, resolution of HIV and exosomes in gradient	**0**,6-18% disc/near-vertical rotor	200,000*g*/1.25h	15
11 Lymphocytes (T cells) HIV Nef secretion in exosomes	**0**,6-18% cont	250,000*g*/1.5 h	1
12 Lymphocytes (T cells CD+) HIV resolution from exosomes	**0**,6-18% disc/near-vertical rotor	200,000*g*/1.25h	21
13 Mason-Pfizer monkey virus, induced cell fusion (human lung embryonic cells, human skin fibroblasts, COS-1 and CMMT cells)	**0**,0.6-30% cont	Not given	14
14 MDCK cells (on permeable tissue culture inserts) – filtrates from apical and basolateral compartments	**10,20,30**% self-generated/vertical rotor	350,000*g*/3h	16
15 Medulloblastoma cells (D283MED)	**0**,6,12,18,24,30,36% disc	100,000*g*/18h	17
16 Melanoma cells (B16BL6)	5,20,**30**% disc	200,000*g*/2h	5
17 Mesenchymal stem cells (human)	5,20,**30**% disc	200,000*g*/3h	4
18 Mouse brain tumour cell lines	**0**,6,12,18,24,30,36% disc	100,000*g*/18h	10
19 Mouse tumour tissue-derived nanovesicles[2]	5,20,**30**% disc	200,000*g*/2h	5
20 Muscle cells (differentiated human neonatal myoblasts)	**0**,5,10,20,40% disc	100,000*g*/16h	8

[1] Disc = discontinuous; cont = continuous. The loading position of the sample on top of, beneath or throughout the gradient is given in **red text**
[2] These nanovesicles, although not strictly exosomes, nevertheless share some properties of the latter.

4c. Purification and analysis in a short-spin, bottom-loaded gradients

Although the gradients described in Section 4b were all continuous (or close to continuous) top-loaded gradients, those in this section are all discontinuous with centrifugation times of 2-3 h. Because they were all bottom-loaded it is likely that the separation is based on density but some contribution from the rate of flotation cannot be ruled out. A two-layered gradient of 5 and 20% (w/v) iodixanol under-layered by the crude sample adjusted to 30% (w/v) iodixanol and centrifugation at 200,000 *g* is a common format (see lines 3, 16, 17 and 19 in Table 2).

4d. Purification and analysis in buoyant density gradients

Some of these long-spin (16-21 h) gradients are set up as pre-formed continuous gradients (for example see lines 1 and 9 in Table 2). In line 1, the gradient is bottom loaded, in line 9 it is top-loaded; the advantage of bottom loading is that any residual soluble proteins will remain at the bottom of the gradient. If the gradient is top loaded soluble proteins will sediment through the gradient at a rate proportional to their molecular mass. All other examples (lines 2, 4, 8, 15, 18 and 20 in Table 2) feature discontinuous gradients, but these will become more or less continuous by diffusion during the centrifugation. The *g*-forces are generally 100-150,000 *g* but may be as high as 200,000 *g*.

4e. Purification and analysis in self-generated density gradients

The final variation (line 14 in Table 2) relies on the ability of iodixanol to form a self-generated gradient in a vertical (or near-vertical rotor); self-generated gradient formation is discussed in **OptiPrep™ Application Sheet S04**. An advantage of the use of self-generated gradients is that the sample can be simply adjusted to a single iodixanol concentration (for example 20% w/v iodixanol) and then centrifuged at approx 350,000 *g* for 2-3 h. The gradients produced by this method are often not completely linear (**see OptiPrep™ Application Sheet S04**), but there are three big advantages to the use of such gradients:

♦ The gradient profiles are very reproducible
♦ The tube set-up is very simple
♦ The particles in the starting solution are very dilute and do not encounter any interfaces during their sedimentation or flotation (compared to bottom- or top-loading): thus aggregation between particles is minimal.

In the example in Table 2 the set-up of the three-layer format (sample contained in each layer) is a little more time-consuming; it's big advantage however is that an almost completely linear gradient is achieved in a very short time.

4f. Density barrier

Occasionally it may be sufficient to use a simple density barrier to concentrate the exosomes, rather than use a more sophisticated gradient to provide the additional resolution from other membrane bound particles is no required. Hasegawa et al [22] layered conditioned medium from an epithelial cell line over layers of 2% and 50% (w/v) iodixanol and centrifuged at 100,000 *g* for 1 h. The soluble proteins remained in the sample layer and the exosomes banded at the 2%-50% iodixanol interface.

5. Banding density of exosomes

The observed banding density of the exosomes in the iodixanol gradient is going to depend on a number of operational factors. In the long-spin fractionations (15-21h) the exosomes should reach their buoyant banding density, which will probably not depend on whether the gradient was originally top- or bottom-loaded (see Table 2). Differences in the observed banding density are likely to be the result of the use of different cell and tissue sources. In the case of shorter centrifugation times (1.25-3h), the exosomes may not reach their true banding density. This is particularly true of those top-loaded gradients of 6-18% (w/v) iodixanol (see Section 4b and Table 2) in which the exosomes are separated from other particles mainly on the basis of their sedimentation velocity. Other gradient systems fall in between these two extremes (see Section 4c and Table 2) and it is difficult to determine whether the exosomes have or have not reached their buoyant density. The path length of the swinging-bucket rotor tube and the distance the exosomes have either to sediment or to float to their banding density is also a consideration in deciding whether the particles have or have not reached that position in the gradient. Although the self-generated iodixanol gradient (see Section 4e and Table 2) also requires a relatively short spin-time, the much higher *g*-force and the short sedimentation path length of the vertical rotor mean that the exosomes will certainly have reached their buoyant density banding position.

- The banding position and broadness of the observed exosome band will depend on the inherent heterogeneity of the vesicles (both in terms of size and content) and all of the operational variables described above.
- Larger vesicles may have a more rapid sedimentation (or flotation) rate than smaller ones, but the latter may have a higher buoyant density
- Some of the reported banding densities of exosomes are given in Table 3.

Table 3 Reported gradient banding position of exosomes

Cell type[1]	Gradient[2]	Density (g/ml)	Cell type[1]	Gradient[2]	Density (g/ml)
1 Carcinoma (breast)	4d	1.094-1.143	10 Lymphocytes (T cells)	4b	1.05-1.069
2 Carcinoma (colon)	4d	1.10-1.12	11 Lymphocytes (T cells)	4b	1.04-1.055
3 Carcinoma (colon)	4c	1.093	14 MDCK cells	4e	1.15
4 Carcinoma (colon)	4d	1.11	15 Medulloblastoma cells	4d	1.11-1.15
5 HEK cells	4c	1.03-1.08	16 Melanoma cells	4c	1.087
6 HEK cells – vexosomes	4b	1.10	18 Mouse brain tumour cells	4d	1.13-1.15
8 Plasma	4d	1.00-1.19	19 Mouse tumour tissue	4c	1.087
9 Lung fibroblasts	4d	1.09-1.11	20 Muscle cells (myoblasts)	4d	1.11-1.14

[1] The number in this column refers back to the line number in Table 2
[2] The gradient type designation refers to their descriptions in Section 4

In Type 4b gradients (see Section 4)

In separations on the 6-18% (w/v) iodixanol sediment-velocity gradient the separation of exosomes and HIV is much better defined than on a sucrose gradient.

- Cantin et al [15] noted that the exosomes banded at 8.4–12% (w/v) iodixanol, while HIV-1 banded at 15.6%; these concentrations are equivalent to 1.050-1.069 and 1.087 g/ml respectively. Separation from denser apoptotic vesicles was also noted. Sucrose gradients are unable to provide such a satisfactory resolving power, with HIV banding at 1.16-1.18 g/ml, while the exosomes at 1.13-1.21 g/ml completely overlapped the virus.
- Lenassi et al [1] reported a density of 1.04-1.055 g/ml and 1.065-1.10 g/ml for the exosomes and HIV respectively. The virus protein p24 was absent from the exosome peak, which was rich in Nef.
- Although no specific density data was provided in ref 11, a large linear separation of acetylcholinesterase (exosome marker) in the top 1/3rd of the gradient and viral vesicles in the bottom 1/3rd was observed.

In Type 4c/4d gradients (see Section 4)

- The expected much lower density banding in iodixanol compared to sucrose gradients (1.03-1.08 and 1.13-1.18 g/ml respectively) is also observed [3].
- Duelli et al [14] isolated exosomes from conditioned culture media from a variety of cells lines; the authors commented that an iodixanol gradient was far more effective for the purpose than a sucrose gradient.

6. References

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