OptiPrep™ Application Sheet S62

Extracellular vesicles from non-mammalian sources

- 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 (RS11) "Extracellular vesicles from non-mammalian sources"** provides a full reference list: return to the initial list of Folders and select "**Reference Lists**".
- See a companion **Application Sheet (S63)** on mammalian cell exosomes and other microvesicles from conditioned medium and a corresponding **OptiPrep™ Reference List (RS10).**
- Samples often require pre-gradient treatment for the removal of larger particles and reduction in sample volume; these are considered in Section 2.

1. Introduction

It is widely recognized that mammalian cells, bacteria and fungi release extracellular vesicles into the surrounding medium; these vesicles are involved in communication between cells and the delivery of biologically and clinically important molecules to other cells. With regard to bacteria and fungi, the term "extracellular vesicles" (EVs) covers the outer membrane vesicles (OMVs) produced by Gram-negative bacteria and the membrane vesicles (MVs) produced by Gram-positive bacteria and other organisms such as fungi. In all cases; EVs are distinct from the intracellular vesicles present in the cytoplasm. The OMVs from Gram-negative bacteria in particular are widely researched and have been shown to be important in the transfer of virulence factors and the initiation of immune and inflammatory responses in host cells.

Gradients prepared from OptiPrep™ represent a simple and effective means for separating the EVs and MVs from any soluble proteins and the strategies are not unlike those used for the separation of mammalian cell membrane vesicles from cytosolic proteins. **They are described in Application Sheets S35 and S36**. The principle is to adjust the sample to a high density by addition of OptiPrep™ or a dense- buffered solution of 40-50% (w/v) iodixanol, prepared from OptiPrep™. This is then laid under either a continuous or discontinuous iodixanol gradient. During the centrifugation the proteins, which have a density of approx.1.26 g/ml, either remain in the load zone or sediment very slowly, while the vesicles float up to their banding position. It provides the ideal separating strategy and since the gradients are more less isoosmotic the vesicles remain fully hydrated and their density is not significantly altered by the gradient. This is unlike sucrose gradients, whose high osmolality causes any vesicles to lose water and hence raises their density.

The gradient methodology is described in Section 3. It is common to preface the iodixanol gradient purification with one or more treatments that remove larger contaminants and/or concentrate the vesicles from the often rather large volumes of starting material.

2. Pre-gradient methodology

Various forms of pre-gradient processing (including filtration, ultrafiltration, centrifugation and ultracentrifugation techniques) are employed, during which intact bacteria, aggregated material and soluble components of the culture medium are mostly removed and the EVs concentrated.

2a. Removal of cells by medium-speed centrifugation

All methods incorporate an initial clarification of the bacterial broth to remove intact cells; 10,000 *g* is the most commonly used speed, usually for 10-20 min, although there are examples both of higher *g*-forces, 13,000 *g* for 15 min [1] and 12,000 *g* for 30 min [2] and of lower *g*-forces, 8,000 *g* for 30 min [3], 6,000 *g* for 10-15 min [4-7], 5,000 *g* for 10-15 min [8,9] and 4,000 *g* for 12 min [10,11]. *Francisella novicida* bacteria [12] were removed in two steps of 5,000 *g* and 7,500 *g* (both for 30 min); this may improve yields of EVs by reducing their entrapment by large numbers of

rapidly-sedimenting intact bacteria. The choice of centrifugation conditions does not appear to be bacteria type-specific. Fungal cells (e.g. *Cryptococcus neoformans*) however appear to sediment satisfactorily at lower speeds – 2,500 *g* for 10 min [13].

2b. Concentration by volume reduction

Large volumes of clarified medium can be awkward to deal with in the final ultracentrifugation step used to pellet the EVs prior to density gradient analysis. For the purification of OMVs from *Escherichia coli* Horstman and Keuhn [14] reduced the volume to $1/25^{th}$ of the original by using a 70 kDa cut-off tangential filtration device (Pall-Gelman) after the first medium-speed centrifugation. The same device with a 100 kDa cut-off has also been used with *Pseudomonas aeruginosa* [15-17], *Francisella novicida* [12] and *Haemophilus influenzae* [18]. *S. aureus* and *B. subtilis* MVs have been concentrated in the QuixStand Benchtop System with a 100 kDa hollow-fibre membrane (Amersham Biosciences) [19]. *Cryptococcus neoformans* MVs were concentrated in an Amicon-Ultra centrifugal filter (also with a 100 kDa cut-off) [13]. In most cases the volume reduction is applied to the supernatant from the first medium-speed centrifugation step (see Section 2a) prior to any filtration steps to remove residual bacteria (see Section 2c). Sometimes ultrafiltration is performed after an initial passage through a 0.45 μm filter [19]. Occasionally a subsequent repeat of the initial medium-speed centrifugation is carried out [17].

2c. Removal of residual bacteria by filtration

Following the first centrifugation step, residual bacteria and other larger contaminants are usually removed by vacuum filtration through either a 0.45 (most common) or a 0.22 μm filter; sometimes the filtration through a 0.22 μm filter is immediately repeated [10,11]; sometimes the 0.45 and 0.22 μm filters are used sequentially [5,6]. It is also quite common to repeat a filtration after the membrane vesicles have been pelleted (see Section 2d) and resuspended in a suitable medium prior to density gradient loading [14,15,20-23] or after an ultrafiltration concentration step [19].

2d.Sedimentation of membrane vesicles

Prior to gradient fractionation MVs are routinely pelleted by ultracentrifugation (in a fixed-angle rotor); the centrifugation conditions are surprisingly variable. Most papers report the use of 34-40,000 *g* for 1 h [14-16, 18,20-26] but there are examples of higher *g*-forces: 85,000 *g* for 1-2 h [2,3], 100,000 *g* for 1 h [9-13] and 140-150,000 *g* for 2-3 h [4-6,10,19].

2e. Precipitation with ammonium sulphate

In a few cases the MVs were harvested from the clarified fluid by precipitation with ammonium sulphate. This was first reported by Ferndandez-Moreira et al [27] for *Legionella pneumophila* OMVs. After removing the bacteria by medium-speed centrifugation and filtration of the culture fluid through a 0.45 μm filter, the OMVs were precipitated in 70% $(NH_4)_2SO_4$ at 4°C for 30 min and centrifugation at 10,000 *g* for 15 min. The pellet was resuspended in PBS and dialyzed against this medium overnight. Concentration was carried out by centrifugal ultrafiltration (>100 kDa cut-off) – see Section 2b. Bauman and Kuehn [15] subsequently increased the $(NH_4)_2SO_4$ concentration (71 or 75%) and the precipitation time to >3h; the resuspended pellet was dialyzed against HEPES buffer and concentrated by >50kDa cut-off ultrafiltration. The $(NH_4)_2SO_4$ precipitation time was increased to overnight by Ellis et al [17] and Nieves et al [7].

3. Gradient methodology

3a. 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

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.

Prepare a 50% (w/v) iodixanol stock solution (approx. ρ = 1.268 g/ml) by mixing 5 vol. of OptiPrep™ with 1 vol. of Solution B and then to make lower density solutions dilute this stock with Solution C. This ensures that the buffer concentration and osmolality is more or less constant throughout the gradient. If this is deemed unnecessary then simply dilute the OptiPrep™ with Solution C. For more information on the preparation of gradient solutions using NaCl as an osmotic balancer see **Application Sheet C01 (Cell index)**; for use of sucrose as an osmotic balancer see **Application Sheet S01**.

Horstman and Kuehn [14] who published the first paper on the use of this strategy for OMVs from *Escherichia coli*, suspended the OMVs in 50 mM HEPES-NaOH pH 6.8 buffer (occasionally 20 mM HEPES is used, see ref 12). This simple buffer is still widely used at pHs from 6.8 to 7.5. It is however hypoosmotic. In a later paper Kesty and Kuehn [24] included NaCl in the same buffer at a lower concentration (0.85% w/v NaCl, 10 mM HEPES-NaOH, pH 6.8). Sometimes a routine phosphate-buffered saline has been used [27,28].

- ♦ Returning the vesicle suspension to an isoosmotic NaCl-containing medium before adjusting the suspension to a high density is probably the most convenient method for preparing the sample for the density gradient.

3b. Gradient preparation (general comments)

The most widely-used iodixanol concentration for the sample is 45% (w/v). This was first used by Horstman and Kuehn [14] and has been widely adopted. Occasionally higher 50% (w/v) [5,19,24] or lower 40% (w/v) [10,12] or 35% (w/v) [13] concentrations have been used.

Without exception all of the gradients are discontinuous, the layers being deposited on the dense sample. However, those gradients that are centrifuged overnight (16-18 h) will become more or less continuous (but not necessarily linear) owing to diffusion of the solute from high to low concentration solutions. If the gradient solutions are prepared by diluting OptiPrep™ with the same buffered 0.85% (w/v) NaCl solution used for the EV suspension, then the entire gradient will be approximately isoosmotic.

- ♦ Although layering of discontinuous gradients is most often executed by overlayering (i.e. starting with the most dense layer first), underlayering starting with the least dense solution first is often more easily carried out, using a syringe and wide-bore (approx. 0.8 mm internal diameter) metal filling cannula. For more information see **OptiPrep™ Application Sheet S03**.

3c. Gradient format

Horstman and Kuehn [14] developed a multiple-layered gradient in which the sample (in 45% w/v iodixanol) was overlaid by 35%, 30%, 25%, 20%, 15% and 10% (w/v) iodixanol, centrifuged at 180,000 *g* for 3 h. It has been adapted to a wide range of tube sizes, but the volume ratio of the different layers remained more or less constant (0.4:3:3:2:2:1:1) [4,8,9]; the volume of the two least dense layers is sometimes increased [2,3]. The same solutions have been used by other workers, but at significantly different volumes Davis et al [28] used 1 ml for all layers and included 3 ml of buffer as the topmost layer. Tashiro et al [5] used 0.5 ml of each (and added a layer of 40% w/v iodixanol). Later the same group [6] used 1.0 ml of each, except for the sample and 10% (w/v) iodixanol layers (0.5 ml).

Since the density of MVs in iodixanol gradients is generally >1.11 g/ml (many banding between 1.13 and 1.15 g/ml); it might be considered unnecessary to include layers of 15% and 10% (w/v) iodixanol which have densities of 1.085 and 1.058 g/ml respectively. Thus Kesty and Kuehn [24] introduced a discontinuous gradient of 50%, 45%, 40%, 35%, 30% and 25% (w/v) iodixanol (2 ml of each). Similar gradients (excluding the 50% layer) have been used by other groups [27], sometimes including a 20% (w/v) iodixanol layer [9,17,18,26], which may replace the 25% layer [25].

The density range of some gradients has been shifted by deleting the top 25% (w/v) iodixanol layer; in these cases the MVs band very sharply at, or towards, the top of the gradient [20,21]; the type of separation is similar to that for purifying any membrane vesicles away from denser particles and soluble proteins (**see OptiPrep™ Application Sheet S35**).

Some publications report the use of much simplified systems. MVs from the Gram +ve organisms *Staphylococcus aureus* and *Bacillus subtilis* [19] have been purified in a discontinuous gradient of 50%, 40% and 10% (w/v) iodixanol. This relatively simple gradient separated soluble proteins, protein aggregates and denatured MVs from the MVs, which had a density of 1.16-1.20 g/ml.

3d Centrifugation conditions

Although the centrifugation conditions reported in the earliest publications, which continue to be widely used for OMVs and MVs are 180,000 *g* for 3 h, they have become quite diverse; for example 100,000 *g* for 3 h [5,6,27], 111,000 *g* for 2 h [7]; 200,000 *g* for 2 h [19] and 100,000 *g* for 16-20 h [12, 16-18,20,22,23,25,26]. Long centrifugation times at reduced *g*-forces are often regarded as producing superior resolution of mammalian intracellular membranes (e.g. plasma membrane, Golgi and endoplasmic reticulum); it is not known if this has been studied rigorously for MVs.

The gradients used for the isolation of *Pseudomonas aeruginosa* OMVs have been tailored to the use of different sources [15,16] Isolation from culture and from soil: 40%, 35%, 30%, 25% and 20% (w/v) iodixanol; for cystic fibrosis isolates the 25% solution was omitted; furthermore the volumes of each layer were also optimised to the separation from soluble proteins and flagella (see refs 15 and 16 for more information.) OMVs banded very close to the top of the gradient after centrifugation at 100,000 *g* for 16 h.

- Tashiro et al [5] noted that the OMVs from *Pseudomonas aeruginosa* showed a biphasic distribution, with a major peak at 1.15 g/ml density and a minor one a 1.20 g/ml. Moreover in a timed study these workers observed a steady shift in the density of the OMVs between 4.5 h (approx. 1.12 g/ml) and 12 h (approx. 1.20 g/ml) in culture. They proposed that there was significant heterogeneity amongst the OMV population.

- Although the majority of the published papers are concerned with the analysis of OMVs from *Escherichia coli* and *Pseudomonas aeruginosa*, other sources have been investigated using similar gradients. Those from *Borrelia burgdorferi* also band at approx. 1.12 g/ml [10,11]. OMVs from *Aggregatibacter actinomycetemcomitans* [3], *Burkholderia pseudomallei* [7], *Haemophilus influenzae* [18], *Legionella pneumophila* [27], *Marinobacter guineae* [26], *Pseudoalteromonas* [26], *Psychrobacter fozii* [26], *Salmonella enterica* [1,22], *Shewanella livingstonensis* [26], *Shewanella vesiculosa* [26].

- It is also worth noting that there may be considerable heterogeneity in the physical characteristics of exosomes from different bacteria; this was emphasized by Singorenko et al [29] in their studies of *Mycobacterium smegmatis* and *Escherichia coli* and the nature of the analytical methodology.

3e Gradient harvesting

The banded vesicles may be harvested simply by aspiration using a syringe attached to a flat-tipped metal filling cannula or the entire gradient maybe unloaded either low-density or high-density end first. For more information on harvesting gradients see **Application Sheet S08**

4. Fungi

MVs from cultures of the fungus *Cryptococcus neoformans* [13] were suspended in 35% (w/v) iodixanol (3 ml) and overlaid by 3 ml of 30% and 2 ml each of 25, 20,15 and 10% iodixanol. After centrifugation at 140,000 *g* for only 15 min the MVs banded broadly approx. in the middle of the gradient..

5. References

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