OptiPrep™ Application Sheet V06

Purification of viruses and viral vectors using OptiPrep™

- **This OptiPrep™ Application Sheet summarizes the range of methods, using OptiPrep™, for the purification of viruses and the principal viral vectors involved in studies on the transduction of cells and tissues**

1. Comparison with other density gradient media

Compared to CsCl and sucrose there are procedural advantages to the use of OptiPrep™:

- OptiPrep™ is a sterile solution of 60% (w/v) iodixanol; it is simply diluted with saline to prepare sterile gradient solutions. It is the only gradient medium manufactured under strict FDA and EU cGMP compliance.
- CsCl and sucrose are both toxic to cells.
- Iodixanol is non-toxic to cells; it has very low endotoxin levels (<1 EU/ml); measured levels on each batch are usually <0.13 EU/ml.
- CsCl must be removed prior to HPLC or gel electrophoresis; iodixanol rarely needs removing prior to further processing, except maybe for some electron microscopy studies.
- CsCl gradients lead to major reductions in viral infectivity. Virus from iodixanol gradients shows a higher % recovery of infectivity and much lower average particle/infectivity ratios compared to that from CsCl gradients.
- 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 enveloped viruses [1].

2. Practical considerations in the selection of a gradient technology

2a. Pre-gradient procedures

2a-1. Pelleting

The choice of a gradient method must take into consideration the often large volumes of culture fluid that require processing. This is particularly the case of large-scale viral vector production. After clarifying the suspension (removal of cells and large cellular fragments) using low speed centrifugation (2,000-4,000 *g* for 15-20 min) or passage through a suitable filter (pore size 0.2-0.45 µm), the simple practice of pelleting the virus at 50,000-150,000 *g* for 1-4 h, can often lead to a serious loss of infectivity. Nevertheless, this remains a common approach using either sucrose or CsCl gradients, prior to loading the resuspended virus pellet on top of the gradient. Further loss of infectivity may be caused by the liquid shearing forces required to resuspend the virus pellet; this can however be reduced by allowing the pellet to disperse in a suitable medium overnight.

If the virus-containing suspension is a cell lysate containing subcellular organelles, the initial clarification step will remove large debris and nuclei. A 0.2 µm filter will remove many of the larger organelles (mitochondria, lysosomes, peroxisomes etc). The subsequent virus pelleting conditions will co-sediment most of the microsomal membrane vesicles, except perhaps at lower g-forces and times (e.g. 50,000 *g* for 1.5 h), which may be insufficient to pellet the smallest vesicles.

It is not entirely clear whether the loss of infectivity that occurs during pelleting is due to the aggregation of the viral particles, the high hydrostatic pressure at the bottom of the tube or the shearing forces that are necessary to disperse the virus pellet after the centrifugation, or a combination of all three effects.

- In order to overcome the shearing force problems some workers have allowed pelleted Semliki Forest virus to disperse itself in a buffered saline solution at 4°C overnight.

2a-2. Low-density barriers

A commonly used alternative to direct pelleting from the virus-containing fluid is the use of a low-density barrier through which the virus is pelleted. A variety of types of barrier have been used, which traditionally were 15-20% (w/v) sucrose or, occasionally, 30%. More recently, these have been replaced with 5-15% (w/v) iodixanol barriers and since an iodixanol gradient is used in the subsequent purification, then it makes for good practice to expose the virus to just one type of gradient solute. The g-forces and centrifugation times are similar

to those used in direct pelleting, i.e. generally 50,000-160,000 *g* for 1-2 h. This barrier technique will allow some preliminary purification from soluble proteins and from small low-density vesicles. The virus pellet can be resuspended in a solution of any density for further purification.

2a-3. High-density barriers

Sedimentation of the virus on to a small volume of a dense solution (cushion) considerably reduces this loss of activity but requires careful recovery of the virus if it is to be layered on top of a subsequent gradient. These problems are abrogated if the virus is subsequently bottom-loaded rather than top-loaded. Commonly the cushion is either 50% (w/v) iodixanol or pure OptiPrep™. With sucrose and CsCl gradients: because of their high osmolality; the banding density of the virus will depend on the mode of loading.

If the virus is subsequently to be loaded on top of a pre-formed continuous or discontinuous gradient then there are some limitations to the cushion-banding technique that need to be considered, if for example the iodixanol concentration at the top of the subsequent gradient is, for example 6% (w/v). Even with just 0.5 ml of a 50% (w/v) iodixanol cushion, mixing the residual contents of the tube after removal of the majority of the supernatant would require at least 5 ml of supernatant to reduce the iodixanol concentration to <5% iodixanol. In this situation therefore it is important to remove as much as the cushion as possible before harvesting the virus band.

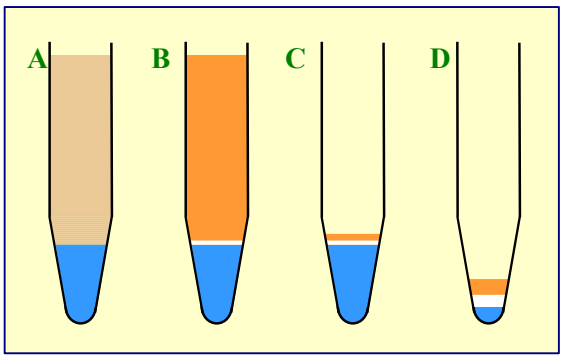


Figure 1: Virus banding on dense iodixanol cushion in a Beckman ***k***onical tube. A: Before centrifugation; B: After centrifugation; C: After removal of most of supernatant; D: After removal of most of cushion

Conical tubes facilitate this process, and Beckman manufacture ***k***onical™ tubes for all their swinging-bucket rotors. Small volumes of cushion occupy a greater linear height in a conical tube than in the traditional round-bottomed ultracentrifuge tube and most of this cushion can also be more easily removed prior to harvesting the virus band in a small volume of supernatant. A thin metal cannula or length of Teflon tubing, attached to a syringe may be used to remove as much of the cushion as possible after centrifugation. Figure 1 summarizes the sequence of events. It should then be possible to remove the virus band in the residual supernatant, whilst aspirating as little of the cushion as possible.

Coleman et al [2] only used 0.22 ml of cushion and removed all of the supernatant (except for the last 0.22 ml) and then harvested all of the remaining liquid (including the cushion) and diluted the suspension 2.5x with buffer before centrifuging it at 6000 *g* for 24 h at 4°C to pellet HIV-1. Since the final volume of suspension was very small, it was possible to pellet the virus efficiently at this very gentle g-force. This would not have been feasible with a large volume of virus suspension, because of the longer sedimentation path length and low g-force at the top of the sample.

If the concentrated virus is to be loaded in a dense solution beneath a pre-formed gradient for a separation on the basis of its buoyant density then the need to eliminate as much of the cushion as possible does not apply.

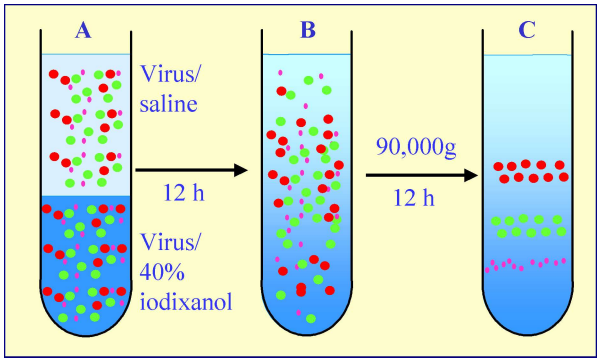
Because iodixanol gradients can be made more or less isoosmotic over the entire density range (unless the ionic strength of the gradient, or part of the gradient is deliberately raised) the banding density is considerably less dependent on the mode of loading. Bottom loading of the virus sample has one particular advantage: any soluble proteins remaining in the virus suspension will remain in the load zone at the bottom of the tube rather than sediment in the same direction as the virus in the top-loaded version

3. Modern gradient loading alternatives

Some of the modern gradient loading alternatives with OptiPrep™ are shown in Figures 2-4. Figure 2 (see next page) describes a method devised by Adair et al [3] for the purification of hepatitis C virus in which a suspension of virus in saline was layered over virus in 40% (w/v) iodixanol (A). After allowing the iodixanol to diffuse for 12 h at 4°C; the end-result was a 0-40% (w/v) linear gradient of iodixanol in which the virus was dispersed (B); this was then centrifuged for 12 h to band the virus isopynically (C). The lack of any interfaces and the initial low concentration of the virus minimises any potential interaction between the virus particles and contaminants.

Figure 2: Purification of hepatitis C virus in a linear iodixanol gradient (Adair et al: see ref 3).

Figure 3 summarizes some of the options for concentration of a virus prior to using either a continuous or discontinuous iodixanol gradient for purification. In the initial centrifugation, the virus is layered over a small volume of iodixanol solution which is either less (A) or more (C) dense than the virus. After centrifugation the virus will sediment to form a pellet (B) below the less dense barrier or band at the interface of the denser barrier (D). In the first option the virus will be largely separated from soluble proteins and slowly sedimenting material, but may lose infectivity due to the pelleting. In the second option the virus will retain higher infectivity and bottom-loading of the virus under a subsequent discontinuous or continuous gradient (F,H) is the obvious method of choice for the second stage purification. Pelleting the virus (B) allows either top-loading or botttom-loading of the subsequent gradient, since the entire liquid phase above the pellet can be aspirated. To top-load the subsequent gradient (E,G) after concentrating the virus on a dense cushion (D) as much of the dense liquid as possible must be removed. A flat-tipped metal filling cannula attached to a syringe (J) is best suited to this task. The use of Beckman "*k*onical" tubes are also an advantage.



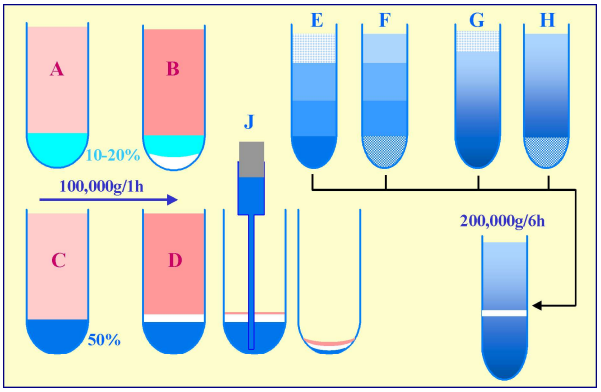


Figure 3: Options for concentrating virus prior to density gradient purification

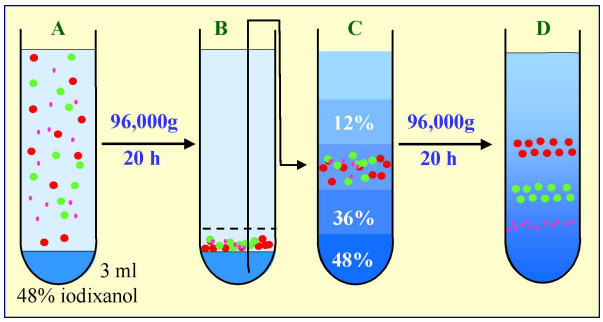


Figure 4: Purification of hepatitis C virus in a discontinuous iodixanol gradient (Merz et al: see ref 4).

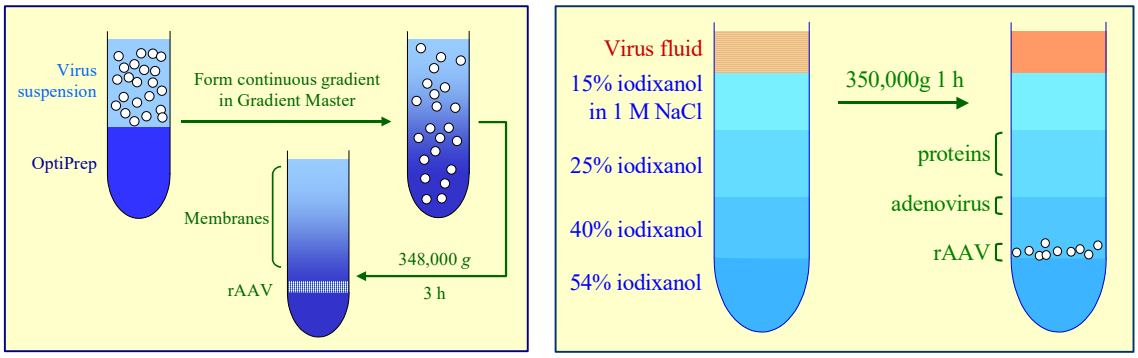
Figure 4 describes a novel way of overcoming the problems of handling virus that has been concentrated on to a dense cushion of iodixanol. It was devised by Merz et al [4] for hepatitis C virus, which is firstly sedimented from a clarified culture medium on to 3 ml of a 48% (w/v) solution of iodixanol (density 1.257 g/ml). The virus suspension was first layered over a 3 ml cushion of 48% iodixanol, then after centrifugation at 96,000 *g* overnight. The use of a relatively low *g*-force for a long time period will minimize any tendency for the virus to aggregate, but 2-3 h at 200,000 *g* would be an alternative. Then 6 ml was aspirated from the bottom of the tube (i.e. the virus was now suspended in 24% (w/v) iodixanol and this was made part of a discontinuous gradient and recentrifuged for a further 20 h to band the virus according to its density. Again the use of relatively low *g*-forces minimizes the effect on virus infectivity.

3. Purification of rAAV and parvovirus vectors

There are two methods (see Figure 5) for the purification of rAAV vectors and each has its own advantages. The continuous gradient (Figure 5a) was designed for a near-vertical rotor and it has the great merit of ease of setting up, but the rAAV may be less well resolved from low MWt soluble proteins. The lack of any interfaces will however minimise any particulate aggregation. Hermens et al [5] compared iodixanol and CsCl gradients for rAAV purification and found that both % recovery and infectivity were considerably better with an iodixanol gradient. The more widely-used discontinuous gradient (Figure 4b) developed by Zolotukhin et al [6] was designed for a 39 ml fixed-angle rotor. Large-volume swinging-bucket rotors are not normally capable of achieving 350,000 *g*, with such a rotor the centrifugation time would need to be increased. Any soluble proteins band well away from the rAAV [6]. The NaCl in the 15% (w/v) iodixanol minimises any association between the rAAV and these proteins. Zolotukhin et al [6] observed that OptiPrep™ routinely produced more than 50%

recovery of rAAV, which was more than 99% pure. Moreover the rAAV product had particle-to-infectivity ratios of less than 100 - significantly better than conventional methods. Both methods also permit complete purification of rAAV in 1 working day.

Figure 5: Purification of rAAV: (a – left) in a continuous iodixanol gradient, see ref 4; (b – right) in a discontinuous iodixanol gradient, see ref 5.



- Parvovirus vectors have also generally been purified using the discontinuous iodixanol gradient method.

4. Purification of adenovirus vectors

In 2005 the discontinuous iodixanol gradient described in Figure 5b was first reported for the purification of adenovirus vectors by Manninen et al [7]. The gradients are centrifuged in an approx. 12 ml swinging-bucket rotor at 100,000 *g* for 6 h. Later Arpiainen et al [8] used a modified gradient of 15%, 30% and 40% (w/v) iodixanol with centrifugation at 100,000 *g* for 14-16 h.

5. Self-generated iodixanol gradients

The ability of iodixanol to form self-generated gradients (see **OptiPrep™ Application Sheet V03** for more details) considerably simplifies the process of purifying viruses. Herpes simplex virus (HSV) was the first virus to be purified in self-generated gradients of iodixanol and it is now a very widely used method for HSV vectors and other viruses. Self-generated gradients make sample handling very easy; the virus is first sedimented on to a 50% (w/v) iodixanol cushion (1-2 in Fig. 6). The *g*-force will depend on the virus. All of the supernatant is discarded (3) except for a volume equal to that of the cushion. After mixing the remaining liquids – the virus suspension (now in 25% iodixanol) is transferred to a tube for a vertical or near-vertical rotor and centrifuged at approx. 350,000 *g* for 1.5-2.5 h (4-5). During this time the gradient forms and the virus moves to its banding density. As with the continuous gradient method for rAAV (see Section 3, above) there are no interfaces to produce particulate aggregation. The shape of the density profile changes gradually from 1 to approx. 4.5 h, after which the profile is more or less stable. Figure 7 compares infectivity and density profiles after centrifugation of a suspension of HSV in 25% (w/v) iodixanol in a Beckman VTi65.1 vertical rotor after centrifugation at 350,000 *g* for 1.5 and 2.5 h. The shallower S-shaped gradient formed after 1.5 h allows a better separation of the lighter immature virus.

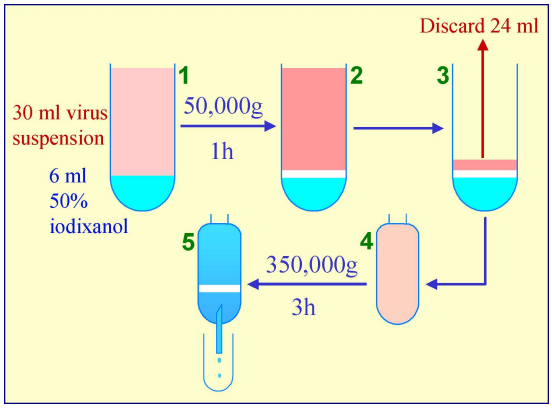


Figure 6 Purification of virus by sedimentation on to an iodixanol cushion followed by banding in a self-generated iodixanol density gradient

- Some short path-length small-volume fixed-angle rotors can replace the vertical or near-vertical rotors that are usually used for the construction of self-generated gradients.

6. Sedimentation velocity gradients

The final type of iodixanol gradient that was developed primarily for HIV by Dettenhoffer and Yu [9] comprises an essentially linear gradient, usually covering the range 6-18% (w/v) iodixanol, in which the sample is top-loaded. It is a sedimentation velocity gradient, which removes the more slowly sedimenting Vif gene,

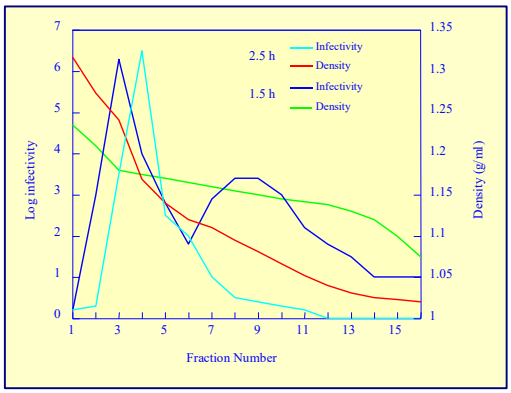


Figure 7. Purification of Herpes simplex virus in a self-generated iodixanol gradient. Effect of time of centrifugation at 350,000*g* in a vertical rotor; iodixanol starting concentration = 25% (w/v).

soluble proteins and microvesicles. Importantly the latter includes the exosomes that are often essential to resolve from the intact virus. Its use has been extended to several other viruses of similar structure.

7. Comments from the lierature

Lock et al [10] noted that yields are routinely greater than $1x10^{14}$ genome copies per run, with capsid protein purity exceeding 90%. Improved transduction both *in vitro* and *in vivo* was observed when compared with small-scale, CsCl gradient-purified vectors. Moreover the OptiPrep™ method effectively separates infectious particles from empty capsids.

Segura et al [11] observed that the low viscosity and isoosmotic nature of iodixanol solutions helps preserve virus particle integrity and functionality. The authors stated that the low toxicity compared to CsCl allows for direct in vitro or in vivo experimentation directly without the need to remove the iodixanol.

Buclez et al [12], by modifying the standard discontinuous iodixanol gradient for the purification of rAAV, noted that by combining this with an earlier tangential flow filtration, "it was possible to purify several litres of crude lysate produced by baculovirus expression vector system in only one working day".

Ammersbach, M. and Bienzle, D [13] noted that the iodixanol sedimentation velocity gradient, first introduced by Detenhoffer and Yu [9] for HIV purification, had the advantages of being both isoosmotic and low viscosity. It was less damaging to viruses and cells, required shorter centrifugation times and subsequent electrophoresis or HPLC could be executed without dialysis. Kol et al [14] also noted that the morphological and mechanical properties are far better preserved in iodixanol than in sucrose gradients.

8. OptiPrep™ Application Sheets/Reference Lists

To access other Application Sheets return to the **2020Virapp** file and select the appropriate V-number. Comprehensive lists of references describing the use of **OptiPrep™ methods** are listed under "**Reference Lists**".

9. References

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