OptiPrep™ Application Sheet V08

Purification of Group I (ds)DNA viruses: *Herpesviridae* and *Baculoviridae* in a self-generated gradient

- OptiPrep™ is a 60% (w/v) solution of iodixanol in water, density = 1.32 g/ml
- The **OptiPrep™ Reference List (RV01)** provides a full bibliography of papers reporting the use of iodixanol gradients for purification and analysis of **Group I viruses**; to access return to the initial list of Folders and select "**Reference List**".
- The self-generated gradient strategy described in this Application Sheet was developed for Herpes simplex 1; it can certainly be extended to other viruses of the *Herpesviridae* family but centrifugation time or iodixanol starting concentration require modulation to optimize the process. Some comments about this and the purification of other herpesviruses are given in Sections 6-9.
- Use of alternative pre-formed gradients is described in **Application Sheet V09**.
- **To access other Application Sheets** referred to in the text: return to the **2020Virapp** file and select the appropriate **V number**.

1. Background

In the following protocol, the viral particles are first concentrated on top of a dense cushion of iodixanol, in place of pelleting the virus. Subsequently, after removal of most of the supernatant, the contents of the tube are simply mixed so that the virus is suspended in 25% (w/v) iodixanol. This suspension is then centrifuged in a tube for a vertical or near-vertical rotor. The self-generated gradient that is formed is designed to band virus particles towards the bottom of gradient while allowing any contaminating membrane material to band at lower densities. The process is simple and requires many fewer manipulations than the routine techniques with sucrose and CsCl gradients. Self-generated gradients also have the merit of high reproducibility.

In all comparative studies between CsCl and iodixanol, it has been shown that the recovery of virus infectivity is much higher and the particle: infectivity ratio much lower when viruses are purified in iodixanol. Although sucrose is generally less deleterious to viral infectivity than CsCl, it can nevertheless also have serious effects on certain important aspects of viral function; in particular the loss of surface glycoproteins from retroviruses has been noted [1]. This may be related to its viscosity, which is much higher than iodixanol. Like CsCl, sucrose must be dialyzed before infectivity can be measured. In contrast, many add-on techniques can be performed and cells infected with virus, without dialysis of iodixanol.

2. Solutions required

A. OptiPrep™

B. Diluent: 0.85% (w/v) NaCl, 60 mM Hepes-NaOH, pH 7.4

C. Working solution of 50% iodixanol (ρ = 1.272 g/ml): mix 5 vol of solution A with 1 vol of solution B (see Section 5, Note 1).

D. HEPES buffered saline: 0.85% NaCl (w/v), 10 mM HEPES-NaOH, pH 7.4.

Keep Hepes (free acid) as a 200 mM stock solution at 4°C; 4.76g per 100 ml water.

Solutions B and D: Dissolve 0.85g NaCl in 50 ml water; add 30 ml or 5 ml of buffer stock solution respectively; adjust to pH 7.4 with 1 M NaOH and make up to 100 ml.

3. Ultracentrifuge rotor requirements

For concentration of the virus: a swinging-bucket rotor of suitable volume to accommodate the volume of crude virus suspension and capable of 100,00-200,000g_{av}, such as the Beckman SW28 or Beckman SW28.1 or equivalent rotors.

For gradient purification: any vertical or near-vertical rotor with tube capacity of approx 12 ml and capable of approx 350,000g. The sedimentation path length of the rotor should be 17-25 mm.

Separations described in this Application Sheet were obtained with a Beckman VTi65.1 vertical rotor, NVT65 near-vertical rotor or NVT65.2 near-vertical rotor. High performance fixed-angle rotors may only be used for the rapid formation of self-generated gradients if the tube volume is relatively small (<6 ml). **For a summary of the range of density profiles achievable with OptiPrep™ see Application Sheet V03.**

4. Protocol

1. Clarify the virus suspension by centrifugation at 1000 *g* for 10 min.

2. Transfer a known volume of the supernatant to suitable tubes for a swinging-bucket rotor and underlay with a small volume (2-4 ml) of Solution C (see Section 5, Note 2).

3. Centrifuge at 160,000 g_{av} for 1 h to band the virus sharply at the working solution interface.

4. Remove all of the supernatant except for a volume equal to the volume of cushion and mix the residual contents of the tubes. This will produce a concentrated virus suspension in 25% (w/v) iodixanol.

5. Transfer the suspension to tubes suitable for a vertical or near-vertical rotor to band the virus in a self-generated gradient of iodixanol.

6. Any tubes that are not filled should be topped up and mixed with 25% (w/v) iodixanol (mix equal volumes of Solutions C and D).

7. Centrifuge at 350,000 g_{av} for 2.5h and at the end of the centrifugation use either a controlled deceleration programme or turn off the brake below 2000 rpm.

8. Either harvest the virus band with a syringe and metal cannula or unload the entire gradient by tube puncture, or other suitable method. Under the centrifugation conditions described the Herpes virus will band in the bottom third of the tube.

♦ **See Section 6 for more information on use of other rotors and centrifugation conditions.**

5. Notes

1. Strategies for preparing working solutions are given in **Application Sheet V01**.
2. The actual volumes will depend on the total volume of virus fluid and the volume of the tubes used. For example: for approx. 15 ml supernatant per tube use 1-2 ml cushion solution, for approx. 35 ml use 2-4 ml.
3. Since the virus bands close to the bottom of the gradient and contaminating membranes are lighter, collection from the bottom is the method of choice. For more information on harvesting gradients **see Application Sheet V04**.
4. The separations shown in Figure 1 were obtained with a Beckman VTi65.1 vertical rotor. After approx 2.5-3.5 h the gradient that is generated is relative shallow towards the top and becomes progressively steeper in the denser regions. It is very effective for banding the virus sharply near the bottom of the tube (see Figure 1A) while any membranous contamination bands at lower densities. At shorter times (e.g. 1.5 h) the gradient is more S-shaped and shallower in the virus-banding area; this permits possible

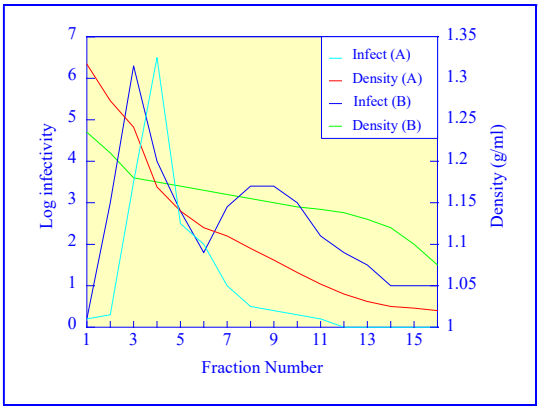

Figure 1: Purification of Herpes virus in a self-generated iodixanol gradient. Infectivity and density profiles after centrifugation in 25% iodixanol at 350,000g in a Beckman VTi65; (A) 2.5 h; (B) 1.5 h

subfractionation of the virus (Figure 1B). In Figure 1A the shoulder seen in the infectivity profile becomes more clearly resolved at the shorter time (Figure 1B). The significance of this subfractionation has not been investigated.

5. Herpes virus (and astrovirus) harvested from iodixanol gradients have been used to re-infect cells without removal of the medium. Preliminary analysis shows that recoveries of more than 90% are achieved.
6. The method can be scaled up for the use of larger vertical rotors such as the Beckman VTi50, but the longer sedimentation path length and lower maximum RCF means that longer centrifugation times will be necessary.

6. Brief review of other published methods reporting the use of self-generated gradients

The methodology as described above has been extensively used and reported by a group working on, amongst other topic areas, $CD4^+$ and $CD8^+$ T cell and dendritic cell interactions and inflammatory reactions in Herpes stromal keratitis, at the University of Pittsburgh School of Medicine [2-7]. For a more extensive listing of publications from this group see **Reference List RV03**.

Published papers also a reveal some variations in the centrifugation conditions used for the self-generated iodixanol gradient banding of the virus; some are relatively trivial; some are significant. Most *g*-forces are in the range 330,000-402,000 *g* [8-12] and centrifugation times in the range 3-4.5 h [8-12] in Beckman vertical or near-vertical rotors (tube volumes approx. 5-11 ml). Some notable exceptions to this are 58,000 *g* for 3.5 h [13] and 70,000 *g* for 3.5 h [14]. Gradient formation at these lower *g*-forces has not been investigated in any detail; the gradients are more likely to be shallow in the middle and steep in the lower and higher density sections. It is not known if this has any beneficial effects regarding the purification of the virus. Small changes to the iodixanol starting concentration have also been made: 22% [9] and 20% [11]. In a very detailed account of the methodology [15] an 11 ml tube was charged with 9.2 ml of the virus in 24% (w/v) iodixanol, overlaid with 1.5 ml of 22 % iodixanol. The gradient was generated at approx 300,000 *g* and the time varied from 4-15 h; during this time period the density profile of the gradient will change and approach equilibrium: longer times may permit the resolution of subpopulations of the virus.

7. Epstein-Barr virus

The same strategy of virus concentration on a 50% (w/v) iodixanol barrier, followed by a self-generated gradient formed from 25% iodixanol (350,000 *g* for 2.4h) has been used by a group at the University of Birmingham (UK) for the purification and analysis of Epstein-Barr virus [16-24]. Although vertical or near-vertical rotors are the recommended rotors for self-generated gradients, small-volume, high-performance swinging-bucket rotors are a possible alternative, especially if they are used in conjunction with small volume tubes with so-called *g-Max* adaptors (Beckman), which reduce the path length of the tube rather than its diameter. Even some high-performance swinging bucket rotors, similarly adapted, may be an option.

Using a flotation separation, Ruiss et al commented that the banding of Epstein-Barr virus at 1.03-1.08 g/ml in iodixanol gradients was much lower than that in hyperosmotic sucrose gradients (1.13-1.18 g/ml) [25].

8. Cytomegalovirus

Murine cytomegalovirus has been purified under similar conditions to those described in Sections 2-4 [26]. The gradient has also been self-generated using rather lower *g*-forces and longer centrifugation times - 144,000 *g* for 16 h [27].

9. Baculovirus

After centrifuging the virus on to a 50% (w/v) iodixanol cushion (80,000 *g* for 1 h, the fluid above the virus band was removed and the virus harvested in the residual cushion and the iodixanol concentration adjusted to 25% (w/v) and the virus banded in self-generated gradient in a Beckman NVT65 (near-vertical rotor) at 350,000 *g* for 3 h [28].

Nucleocapsids from a 1% NP-40 (in a buffered saline containing 2 mM EDTA) treated baculovirus suspension (6 ml) can be separated on a two-layer iodixanol gradient (3 ml each of 25% and 50% w/v) centrifuged at 16,000 *g* for 3 h. The capsids were concentrated just above the barrier of the two iodixanol solutions [29].

- **Segura et al [30] commented in a review of gene therapy viral vectors that iodixanol's cell- and virus-friendly properties made it much more useful in gradient purification than CsCl**

8. References

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