OptiPrep™ Application Sheet V21

Purification of Group IV ((+)ss) RNA viruses: *Nidovirales*: *Coronaviridae and Arteriviridae*

- OptiPrep™ is a 60% (w/v) solution of iodixanol in water, density = 1.32 g/ml
- Whether the protocols described in this Application Sheet can be applied to other members of the *Nidovirales* order, such as those viruses of the *Roniviridae* can only be determined by experimentation.
- **To access other Application Sheets** referred to in the text: return to the **2020Virapp** file and select the appropriate **V number.**

1. Background

There are now many published papers that report the use of iodixanol gradients not only to purify viruses but also to investigate their assembly. In all comparative studies between CsCl and iodixanol, 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, in solutions of the same density, is much higher than that of iodixanol.

Like CsCl, sucrose must be dialyzed before infectivity can be measured. In contrast both infectivity measurements using cultured cells and many add-on techniques can be performed without dialysis of iodixanol. Combined with the availability of OptiPrep™ as a sterile solution, this makes the use of OptiPrep™ for virus purification and assembly analysis much more convenient than the use of either CsCl or sucrose.

Clarified suspensions of murine coronavirus [2] and later human coronavirus [3] were first concentrated in PEG 8000 (containing 0.5 M NaCl) and subsequently purified in Nycodenz® gradients. Initially the virus was concentrated at the boundary of a 10% and 50% (w/v) Nycodenz® discontinuous gradient (83,000 *g* for 3.5 h) and then it was further purified in a continuous 10-50% Nycodenz® gradient (83,000 *g* for 16 h). The Nycodenz® solutions were made up in 0.1 M NaCl, 1 mM EDTA, 100 mM Tris-maleate, pH 6.2.

However, because of the relative ease of using OptiPrep™, this Application sheet will focus on the use of this medium for the purification of the severe acute respiratory syndrome coronavirus (SARS-CoV) (*Coronaviridae*) and the porcine reproductive and respiratory syndrome virus (PRRSV), which is an *Arteriviridae* virus. SARS-CoV has been purified principally on the basis of buoyant density in either a self-generated gradient [4-8] or a pre-formed continuous gradient [9,10], while for PRRSV both self-generated [11-13] and sedimentation velocity gradients have been used [14]. All three options are described in this Application Sheet in Sections 2-4 respectively.

- **Note that methods from some of the more recent papers are briefly discussed in Section 5.**

2. Self-generated gradient (adapted from refs 4-8)

2a. Solutions required

A. OptiPrep™

B. Phosphate buffered saline (PBS) (**see Section 2d, Note 1**)

2b. Rotor requirements

For virus concentration: swinging-bucket rotors with approx 17 or 38 ml tubes (e.g. Beckman SW28 or SW28.1)

For self-generated gradient: near-vertical rotor with tube capacity of 5-13 ml (e.g. Beckman NVT65, NVT65.2, NVT90 or similar rotor.

2c. Protocol

1. Freeze-thaw the cells three times in Solution B.

2. Clarify the cell lysate by centrifugation at 3,000 *g* for 20 min at 4°C.

3. Prepare a 50% (w/v) iodixanol solution by diluting 5 vol. of OptiPrep™ with 1 vol. of PBS (**see Section 2d, Note 2**).

4. Concentrate the virus by sedimentation on to a small cushion of the 50% iodixanol by centrifugation at 50,000 *g* for 1.5 h in the chosen swinging-bucket rotor. In 38 ml tubes use 3-4 ml of cushion, in 17 ml tubes use 1-2 ml. Make sure that the volume of virus-containing liquid in each tube is known, this is important for Step 5 (**see Section 2d, Note 3**).

5. Carefully aspirate all of the supernatant except for a small volume equivalent to that of the cushion (**see Section 2d, Notes 4 and 5**).

6. Mix the contents of the tubes very well and then transfer them to sealed tubes for the chosen near-vertical rotor (**see Section 2d, Note 6**).

7. Centrifuge at 350-400,000 *g* for approx. 3.5 h at 4°C and allow the rotor to decelerate using a slow deceleration program below 4000 rpm or turn off the brake below 4000 rpm (**see Section 2d, Note 6**).

8. Collect the gradient by aspiration from the meniscus, upward displacement with a dense medium or tube puncture and analyze the fractions. For more information on harvesting gradients **see Application Sheet V04**. The banding density of coronavirus is approx 1.2 g/ml according to Huang et al [6].

2d. Notes

1. The PBS may be replaced by any buffered saline solution.
2. When OptiPrep™ is diluted with PBS, the final solution will be approximately isoosmotic, but if it is considered that the ionic strength of the final solution is not sufficiently high, then mix the OptiPrep™ instead with 0.6 vol. of 10xPBS and 0.4 vol. of water.
3. The use of Beckman "*k*onical" tubes permits the volume of cushion to be reduced; this might be convenient with large volumes of virus-containing fluid. For more information on concentrating virus **see Application Sheet V06**.
4. Note that Berry et al [4] removed a volume of supernatant equivalent to 1.5x that of the cushion, so that when the residual material was mixed, the final concentration of iodixanol was 20%; while Huang et al [6] removed a volume equivalent to two thirds of that of the cushion, so that when the residual material was mixed, the final concentration of iodixanol was 30%. In the mid-course approach adopted here, mixing the cushion with the same volume of supernatant will reduce the iodixanol concentration to 25% (w/v). All three approaches probably work equally well, but because density profile of the self-generated gradient will be different in each case, the position of the virus in the tube will also be different.
5. In their purification strategy for PRRSV, Li and Murtaugh [11-13] collected the pelleted virus from two rounds of 0.5 M sucrose barrier sedimentation and then suspended the virus in 20% (w/v) iodixanol and used a self-generated gradient created at a lower centrifugation speed of 250,000 *g* for a longer time – 9 h.
6. Vertical rotors of the same capacity are permissible and the gradient that is generated will be more or less identical, but a small cushion of 0.5 ml of 40% iodixanol should be included to stop any dense material from reaching the tube wall.

3. Pre-formed gradients (adapted from ref 9; but see also Section 3d Notes)

3a. Solutions required

A. OptiPrep™

B. Phosphate buffered saline (PBS)

3b. Rotor requirements

For virus concentration: swinging-bucket rotor with approx 13 ml tubes (e.g. Beckman SW41Ti)

3c. Protocol

1. Concentrate and partially purify the virus by sedimentation through a 15% (w/v) iodixanol cushion (dilute 1.5 vol. of OptiPrep™ with 4.5 vol. of Solution B) at 67,000 *g* for 2.5 h (**see Section 3d**).

2. Resuspend the virus in 1-2 ml of PBS.

3. Prepare solutions of 12% and 30% (w/v) iodixanol by diluting 1.2 vol. and 1 vol. of OptiPrep™ with 4.8 vol. and 1 vol. of PBS respectively (**see Section 3d**).

4. In tubes for the swinging-bucket rotor prepare a linear gradient from equal volumes (5.5-6.0 ml) of the 12% and 30% iodixanol solutions using a two chamber gradient maker or a Gradient Master™. Alternatively make a discontinuous gradient from 12%, 18%, 24% and 30% (w/v) iodixanol and allow the gradient to diffuse. For more information on making continuous gradients **see Application Sheet V02**.

5. Layer 1-2 ml of the virus suspension on top of the gradient and centrifuge at 260,000 *g* for 2.5 h. Allow the rotor to decelerate using a slow deceleration program or turn off the brake at 2000 rpm.

6. Collect the gradient in 0.7 ml fractions low-density end first and analyze them for the virus; for more information on gradient unloading **see Application Sheet V04**.

3d. Notes

Virus concentration: Beniac et al [9] concentrated the virus by pelleting at approx 140,000 *g* for 90 min. The recommended method of pelleting through a cushion at a lower *g*-force for a longer time is rather more gentle, but the best method is to sediment the virus on to a dense cushion. The latter does however pose a problem for subsequent layering of the virus on top of a gradient starting at 12% iodixanol – for a more complete discussion of the problems and possible solutions **see Application Sheet V06**.

An alternative top-loaded 10-40% (w/v) iodixanol gradient was used by Tseng et al [10] for SARS-coronavirus; it was centrifuged in 5 ml tubes at approx. 175,000 *g* for 16 h. The gradient solutions were again made up by dilution of OptiPrep™ with PBS. The gradient was initially formed from 1.25 ml each of 10, 20, 20 and 40% (w/v) iodixanol, but during the centrifugation it will become continuous and more or less linear. Tseng et al [10] compared the particles expressed from cultured cells transfected with either the M protein or M plus the N nucleocapsid proteins. Interestingly the viral-like particles expressed from the cells with M protein banded at a slightly lower density (1.13 g/ml) than those with M+N proteins (1.14 g/ml). The gradient thus exhibits a high resolving power.

Human coronavirus has also been isolated on a shallow 10-20% iodixanol gradient, centrifuged at 175,000 *g* for 18 h [15,16].

4. Sedimentation-velocity gradient (adapted from ref 14)

4a. Solutions required

A. OptiPrep™

B. Buffered saline: 150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, pH 7.4

C. Gradient solutions: 10%, 12.5%, 15%, 17.5% and 20% (w/v) iodixanol, dilute OptiPrep™ with Solution B at the following volume ratios respectively, 1:5, 1.25:4.75, 1.5:4.5, 1.75:4.25 and 2:4

Prepare 100 ml of each of the following stock solutions and keep at 4°C:

100 mM Tris (free base)	1.21 g
1 M NaCl	5.84 g
100 mM EDTA (Na_2•$2H_2O$)	3.72 g

Solution B: Mix 50 ml, 15 ml and 1 ml respectively of the Tris, NaCl and EDTA stock solutions; adjust to pH 7.4 with HCl and make up to 100 ml.

4b. Rotor requirements

For virus concentration: swinging-bucket rotors with approx 17 or 38 ml tubes (e.g. Beckman SW28 or SW28.1)
For sedimentation-velocity gradient: swinging-bucket rotor with tube capacity of approx.13 ml (e.g. Beckman 41Ti or similar rotor)

4c. Protocol

1. Concentrate and partially purify the virus by sedimentation through a 15% (w/v) iodixanol cushion (dilute 1.5 vol. of OptiPrep™ with 4.5 vol. of Solution B) at 67,000 *g* for 2.5 h (**see Section 4d**).

2. Towards the end of step 1 make a discontinuous gradient from 2.4 ml each of 10%, 12.5%, 15%, 17.5% and 20% (w/v) iodixanol by underlayering or overlayering. **For more information on making gradients see Application Sheet V02**.

3. Resuspend the viral pellet from Step 1 in Solution B and layer 1 ml on top of each gradient.

4. Centrifuge at 41,000 rpm (200,000 g_{av}) for 2 h at 4°C.

5. Collect the gradient by aspiration from the meniscus, upward displacement with a dense medium or tube puncture and analyze the fractions for virus. For more information on gradient unloading **see Application Sheet V04**.

4d. Notes

Use whichever rotor is more suitable for the volume of virus fluid. Only 1-2 ml of cushion is required in a 17 ml tube or 3-4 ml in a 38 ml tube. The ideal way of concentrating the virus is sedimentation on to a dense cushion of iodixanol, rather than pelleting. This however may be less convenient when, as in this case, the concentration of iodixanol in the viral suspension needs to be <10% (w/v) to permit loading on the gradient. When recovering the band of virus as little as possible of the cushion must be aspirated. For more information on concentration of virus **see Application Sheet V06**.

5. Recent publications

De Wit et al [17] have studied Middle East respiratory syndrome coronavirus (MERS-CoV), pelleting the virus through a simple 15% (w/v) iodixanol cushion OptiPrep™. Tseng et al [18] investigated the conditions that promote the formation of virus-like SARS-CoV particles, in which the virus was purified in a 5 ml 10-40% (w/v) iodixanol gradient at approv. 150,000 *g* for 16 h. SARS-Corona virions have also been purified in shallow 20-30 % (w/v) iodixanol gradients at 111,000 *g* for 18 h [19]

Giles et al [20,21] used a self-generated gradient method very similar to that described **in Section 2** to purify the nidovirus associated with wobbly possum disease; the concentrated virus was adjusted to 25% (w/v) iodixanol and centrifuges in a small volume (approx 4 ml tubes) vertical rotor at 200,000 *g* for 6.5 h.

6. References

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