OptiPrep™ Application Sheet V24

Purification of Group V ((-)ss) RNA viruses: *Bunyaviridae – Hantavirus, Orthobunyavirus and Phlebovirus*

- **OptiPrep™** is a 60% (w/v) solution of iodixanol in water, density = 1.32 g/ml
- This Application Sheet covers Bunyamwera virus (Section 2), Hantavirus (Section 3), Rift Valley fever virus (Section 2), Hazara virus (Section 4).
- **To access other Application Sheets** referred to in the text: return to the **2020Virapp** file and select the appropriate **V number.**
- For purification and analysis of other Group V ((–)ss) RNA viruses see **OptiPrep™ Application Sheets V23 and V25-V28**.

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.

Bunyamwera virus and Rift Valley fever virus (Section 2) have been purified in continuous gradients of iodixanol; Sin Nombre hantavirus (Section 3) in self-generated and pre-formed discontinuous iodixanol gradients. Whether a particular method can be applied to any –ve sense RNA virus can only be determined experimentally.

2. Analysis of Bunyamwera virus

The protocol is adapted from refs 2 and 3. **See Section 2d, Notes 1 and 6 re Rift Valley virus**.

2a. Solutions required (see box ⇒)

A. OptiPrep™

B. OptiPrep™ Diluent: 0.2 M NaCl, 2 mM EDTA, 0.02 M Tris-HCl, pH 7.4

C. Working solution (30% w/v iodixanol): Mix equal volumes of OptiPrep™ and Solution B.

D. Buffer: 0.1 M NaCl, 1 mM EDTA, 0.01 M Tris-HCl, pH 7.4

Include protease inhibitors in Solutions B and D as required.

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: To 30 ml of water; add 20 ml, 20 ml and 2 ml respectively of the Tris, NaCl and EDTA stock solutions; adjust to pH 7.4 with HCl and make up to 100 ml.

Solution C: Dilute Solution B with an equal volume of water.

2b. Rotor requirements

Swinging-bucket rotors with approx 13 ml tubes (e.g. Beckman SW41Ti) and approx 38 ml (e.g. Beckman SW28)

2c. Protocol

1. Clarify the culture medium or the cell lysate by centrifugation at 3,700 *g* for 20 min at 4°C (**see Section 2d, Note 2**).

2. Using a swinging-bucket rotor concentrate and partially purify the virus by sedimentation through a 20% (w/v) iodixanol cushion (dilute Solution C with Solution D) at 67,000 *g* for 2.5 h (**see Section 2d, Note 3**).

3. During step 2 prepare two iodixanol solutions of 13% and 22% (w/v) by diluting Solution C with Solution D (**see Section 2d, Note 4**).

4. In tubes for the 13 ml swinging-bucket rotor use either a two-chamber gradient maker or a Gradient Master™ to prepare approx 12 ml continuous gradients from equal volumes the 13% and 22% iodixanol solutions (**see Section 2d, Note 4**).

5. Resuspend the virus pellet from step 2 in Solution D and apply approx 0.5 ml to each gradient.

6. Centrifuge the gradients at 250,000 *g* for 1.5 h. Use a slow deceleration program or turn off the brake during deceleration from 3000 rpm.

7. Collect the gradient by aspiration from the meniscus, upward displacement with a dense medium or tube puncture (for more information on harvesting gradients **see Application Sheet V04**) and analyze the fractions (**see Section 2d, Note 5**).

2d. Notes

1. Rift Valley virus was concentrated by ultrafiltration using a centrifugal filter device, rather than by sedimentation on to a dense cushion of iodixanol (see Step 2 in Protocol 2c). The virus was then purified on a 10-30% (w/v) iodixanol gradient [4,5]; the 10% and 30% iodixanol solutions should be prepared from OptiPrep™ using the same dilution procedure, with the same NaCl/EDTA/Tris solutions as described above. After forming the gradient in 14 ml tubes from equal volumes of the 10 and 30% iodixanol solutions, the gradient was top-loaded with the concentrated virus and the centrifugation was carried out at 210,000 *g* for 1.5 h at 4°C. The virus banded approx. half-way down the gradient.
2. Intracellular forms of the virus can be released by three rounds of freeze-thawing [2].
3. Novoa et al [2] pelleted the virus through a 30% (w/v) sucrose cushion; to maintain an isoosmotic environment for the virus, the 30% sucrose has been replaced by 20% (w/v) iodixanol. 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 <13% (w/v) to permit loading on to 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 prior to purification **see Application Sheet V06**.
4. Novoa et al [2] prepared nine solutions between 13% and 22% iodixanol (in 1% steps) and layered equal volumes (dense end first) in a centrifuge tube, snap-freezing each layer in dry ice before applying the next. The tubes were kept frozen until required and continuous gradients were formed by allowing the tubes to thaw at room temperature overnight. For more information on preparing continuous gradients **see Application Sheet V02**.
5. Virus particles of increasing density can be harvested from the gradient: Type I has an annular structure (immature precursors); Type II is an intermediate dense form (these are both intracellular forms) and finally a dense extracellular form, distinguished morphologically from the Type II form [2].
6. Ariza et al [6] studied the nucleocapsid protein structure from Bunyamwera virus using the iodixanol gradient method of Novoa et al [2]. Working with Rift Valley fever virus Weingart et al [7] first concentrated the virus by pelleting through a 20% (w/v) iodixanol cushion (115,500 g for

1 h); then suspended the virus in a buffered saline (containing 1 mM EDTA) and then banded the virus in a discontinuous gradient of 10, 15, 20, 25 and 30% (w/v) iodixanol (210,000 *g* 1.5 hr).

3. Analysis of Hantavirus (self-generated gradient)
The protocol is adapted from refs 8-11 (**see also Section 3d, Notes 1 and 2**).

3a. Solutions required
A. OptiPrep™
B. OptiPrep™ Diluent: Any suitable iso-osmotic balanced salt solution

3b. Rotor requirements
Swinging-bucket rotor with approx. 13 ml tubes (e.g. Beckman SW41Ti)
Near-vertical rotor with approx. 5 ml tubes (e.g. Beckman NVT65.2)

3c. Protocol
1. Dilute 5 vol. of OptiPrep™ with 1 vol. of Solution B to produce a 50% (w/v) iodixanol solution.

2. In tubes for the swinging-bucket rotor underlayer approx. 10 ml of the crude virus suspension with 2 ml of the 50% iodixanol solution using a syringe and metal cannula (**see Section 3d, Note 3**).

3. Centrifuge at approx 190,000 *g* for 3 h at 4°C.

4. Using a syringe and metal cannula collect 4 ml of liquid from the bottom of the tube i.e. the cushion + the banded virus + 2 ml of the balanced salt solution).

5. Mix the suspension with an extra 1 ml of the 50% iodixanol and transfer to an Optiseal™ for the near-vertical rotor (**see Section 3d, Notes 3-5**).

6. Centrifuge at approx 350,000 g_{av} for 5 h and allow the rotor to decelerate from 2000 to 0 rpm using a controlled deceleration program (over at least 5 min) or turn off the brake at 2000 rpm.

7. Collect the gradient by tube puncture or any other method; the virus will band in the bottom half of the gradient. For more information on harvesting gradients **see Application Sheet V04**.

3d. Notes
1. An alternative method in which the concentrated virus was layered over pre-formed continuous gradient formed by diffusion overnight at 4°C from 1.5 ml of 50%, 42%, 35%, 28%, 21%, 14% and 7% (w/v) iodixanol (OptiPrep™ diluted with HEPES-buffered 135 mM NaCl) centrifuged at 25,000 *g* for 15 h was devised by Huiskonen et al [12]. Although it may be acceptable to use a higher *g*-force for a shorter time, there is little doubt that the use of relatively low *g*-forces for longer times will provide the best resolution of any biological particles; moreover during the 15 h centrifugation the gradient will certainly become continuous and at the low speed of 25,000 *g* there will be little or no sedimentation of the iodixanol molecules themselves. A similar gradient was used by Li et al [13] at approx/ 110,000 *g* for 3 h.
2. More recently a 5-25% (w/v) iodixanol gradient was generated from a discontinuous one (5% step interval); after layering each step the gradient was frozen and after the final freezing the gradient was top-loaded with sample and centrifuged at 28,000 *g* for 1.5 h [14].
3. For larger volumes of virus use for example a Beckman SW28 for the virus concentration step and an NVT65 for the self-generated gradient and scale up all the volumes proportionately to fill the tubes. Although the larger volume swinging-bucket rotor cannot be run at 190,000 *g*, it is probably not necessary to increase the centrifugation time in step 3 to band the virus (although this should be checked out).

4. 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 OptiPrep™ should be included to stop any dense material from reaching the tube wall.
5. The final concentration of iodixanol is 30% (w/v).

4. Hazara virus/Crimean-Congo hemorrhagic fever virus

Hazara virus and Crimean-Congo hemorrhagic fever virus are members of the *Nairovirus* genus; Surtees et al [15] purified Hazara virus after precipitation in polyethylene glycol at 4000 *g* for 30 min. The pellet was resuspended in a buffered saline and transferred to the top of a 5-25% (w/v) iodixanol gradient and the virus was banded sharply in the gradient after centrifugation at 250,000 *g* for 2.5 h.

Crimean-Congo hemorrhagic fever virus was purified by Wang et al [16] using a modification of the methodology used by Ariza et al [6] for orthobunyaviruses. The gradient covered the same density range as described above for Hazara virus, the gradient centrifugation conditions were however modified to 28,000 *g* for 1.5 h.

5. References

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