OptiPrep™ Application Sheet V12

Purification of Group I (ds)DNA viruses: *Poxviridae*

- OptiPrep™ is a 60% (w/v) solution of iodixanol in water, density = 1.32 g/ml
- This Application Sheet primarily covers the purification and analysis of modified Vaccinia virus (Ankara), but Section 6 also briefly covers *Molluscipoxvirus*
- **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 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.

Section 4a in this OptiPrep™ Application Sheet [2] describes the purification of modified vaccinia virus Anakara (MVA), released from infected cells by sonication, by banding at an interface between two solutions of iodixanol (22% and 32%).

Section 4b in this OptiPrep™ Application Sheet describes a slightly more sophisticated technique in which the intracellular mature virus (IMV) was processed from a post-nuclear supernatant of homogenized infected cells [3]. The iodixanol gradient used to purify the IMV was continuous rather than discontinuous [4]. This same gradient was also used to purify the extracellular enveloped virus (EEV) form of the virus (from the culture medium) that is formed as the IMV is exocytosed at the cell surface [4]. More recently this continuous gradient has been used to separate IMV and EEV particles from MVA preparations [5] and the purification of a recombinant vaccinia virus (VV-Osp-A) [6].

- **The notes referred to in the following methods can be found in Section 5**

2. Solutions required (see Note 1)

A. OptiPrep™
B. OptiPrep™ diluent: 60 mM Tris-HCl, pH 9.0 (see Note 1)
C. 50% (w/v) Iodixanol Working Solution: mix 5 vol. Solution A with 1 vol. of Solution B
D. Diluent: 10 mM Tris-HCl, pH 9.0
E. Phosphate-buffered saline or cell culture medium
F. Barrier (for Protocol B only): 36% (w/v) sucrose in 10 mM Tris-HCl, pH 9.0 (**see Step 6 of Protocol B**)

Keep the following stock solution at 4°C:
1 M Tris (free base), 12.1 g per 100 ml

Solution B: To 50 ml water, add 6 ml of Tris stock solution; adjust to pH 9.0 with 1 M HCl; make up to 100 ml.

Solution D: To 50 ml water, add 1 ml of Tris stock solution; adjust to pH 9.0 with 1 M HCl; make up to 100 ml.

Solution F: Dissolve 36 g sucrose in 50 ml water, add 1 ml of Tris stock solution; adjust to pH 9.0 with 1 M HCl; make up to 100 ml.

3. Rotor requirements

Protocol 4a: Swinging-bucket rotors for 39 ml or 17 ml gradients, such as the Beckman SW28, Beckman SW28.1 or Sorvall AH629; for 5 ml gradients, rotors such as the Beckman SW55Ti, MLS50 or Sorvall AH650

Protocol 4b: Swinging-bucket rotor for 17 ml gradients, such as the Beckman SW28, Beckman SW28.1 or Sorvall AH629

4A. Protocol (adapted from ref 2)

Carry out all operations at 4°C

1. Wash the infected cells in Solution E; resuspend in the same medium and freeze-thaw the cells.
2. Release the virus from the cells by mild sonication.
3. Clarify the suspension by low speed centrifugation (approx 1000 *g* for 15 min) to remove cellular debris.
4. To concentrate the virus transfer the suspension to 17 ml or 39 ml tubes for the swinging-bucket rotor and underlay with a cushion of 2-5 ml of Solution C using a syringe and metal cannula (**see Note 2**)
5. Centrifuge at 80,000 g_{av} for 3 h
6. Using a syringe and metal cannula remove as much of the cushion as possible (below the virus band) then recover the virus band from each gradient in 2-3 ml of the supernatant (**see Notes 3 and 4**).
7. If necessary these concentration steps can be repeated in tubes for the 5 ml swinging-bucket rotor using just 1 ml of 50% iodixanol cushion and centrifuging at 128,000 *g* for 2 h (**see Note 5**).
8. During the final concentration centrifugation, mix Solution C with Solution D to make solutions of 22% and 32% (w/v) iodixanol (**see Note 6**).
9. Layer the concentrated virus suspension over equal volumes of the two iodixanol solutions and centrifuge at 50,000 g_{av} for 3 h. In a 5 ml tube use 0.5-1.0 ml of virus suspension over approx. 2 ml of each iodixanol solution; in a 17 ml tube use 2-3 ml of virus over approx. 7 ml of each iodixanol solution (**see Notes 7 and 8**).
10. Harvest the purified virus from the interface between the two iodixanol solutions.

4B. Protocol (adapted from refs 3 and 4)

Carry out all operations at 4°C

For pre-gradient processing of IMV follow Steps 1-7

For pre-gradient processing of EEV follow Steps 8-9

For purifying IMV and EEV follow Steps 10-13

1. Scrape the infected cells from the dish and pellet them at approx. 600g for 10 min.
2. Suspend the cells in Solution D.
3. To release the IMV homogenize the cells using 10-12 strokes of a tightly fitting pestle (Wheaton Type A) of a Dounce homogenizer (**see Note 9**).
4. Centrifuge the homogenate at approx 1000 *g* for 5 min to pellet the nuclei and carefully aspirate the supernatant.
5. Repeat Step 4 using the post-nuclear supernatant in order to maximize the removal of the nuclei.
6. Concentrate and partially purify the virus by centrifuging it in 17 ml tubes through Solution F at 70,000 g_{av} for 30 min (**see Note 10**).
7. Remove all the supernatant and suspend the viral pellet in 2 ml of Solution D.
8. Harvest the culture medium from the cells and clarify it at 2000 *g* for 10 min.
9. Pellet the virus at 75,000 g_{av} for 30 min and resuspend in 2 ml of Solution D.
10. Sonicate the viral suspension (water bath sonicator) for 1 min.
11. Mix Solution C with Solution D to make solutions of 22% and 32% (w/v) iodixanol and use equal volumes (7.5 ml) of the two iodixanol solutions in a two-chamber gradient maker or a Gradient Master™ to make a continuous gradient in a tube for the 17 ml swinging-bucket rotor (**see Note 11**).

12. Layer the viral suspension over the iodixanol gradient and centrifuge at 75,000 g_{av} for 45 min (**see Note 12**).
13. Collect the banded IMV (higher density) and/or EEV (lower density). Each band may be collected separately using a syringe or the whole gradient may be unloaded by upward displacement, aspiration from the meniscus or by tube puncture in 0.5-1.0 ml fractions (**see Notes 13 and 14**).

5. Notes

1. Any low-density solution compatible with the virus and/or with the subsequent analysis can be used to dilute the OptiPrep™ or a Working Solution produced from OptiPrep™. More details on the making up of gradient solutions may be found in **Application Sheet V01**.
2. Underlayering the small volume of dense cushion is certainly much easier than overlayering the large volume of virus-containing fluid.
3. It is important to avoid contaminating the recovered concentrated virus with too much of the dense cushion since the virus suspension will subsequently be layered on top of a 22% iodixanol solution. For more information on the concentration of virus **see Application Sheet V06**.
4. The use of an interface to concentrate the virus, rather than pelleting, may make the production of a non-aggregated suspension of the virus easier and also avoids the loss of infectivity that often accompanies pellet formation.
5. A second round of concentration may be even more useful when larger volumes of virus suspension are used in the first concentration step.
6. Sandgren et al [7] used a discontinuous gradient of 16/32% (w/v) iodixanol (140,000 *g* for 1h) and then, after dilution of the virus collected from the interface, it was concentrated on to a 50% iodixanol cushion at 24,000 *g* for 45 min in a 14 ml tube of a swinging-bucket rotor.
7. Make sure that the virus suspension has a sufficiently low density to permit layering on the 22% iodixanol.
8. It may be permissible to use larger volumes of virus and smaller volumes of the gradient solutions; this will need to be confirmed by recovery and purity data.
9. There are a variety of methods for homogenizing cells. The hypoosmotic Solution D would facilitate the breakage by causing cell swelling, but this may not be optimal for the preservation of nuclear integrity. Since the nuclei are subsequently removed from the homogenate by centrifugation (Step 4) this may be a point worth investigating further. For more information on cell disruption see ref 8 and **Application Sheet S06 (see Subcellular membrane index)**.
10. Since Sancho et al [5] recognized that the final iodixanol gradient was more efficient than the original sucrose gradient [3] in the recovery of infective EEVs, replacement of the sucrose barrier by an iodixanol one might merit consideration. It is difficult to predict what might be a suitable solution because of the big difference between the osmolality of sucrose and iodixanol solutions, but a 20% (w/v) solution might be a good starting point. This will need to be confirmed by recovery and purity data.
11. A continuous gradient can alternatively be constructed by allowing a discontinuous gradient (22%, 25%, 28% and 32% iodixanol layers) to diffuse. For more information on making gradients **see Application Sheet V02**.
12. The sample loaded on to the 22-32% (w/v) iodixanol gradient could also be an MVA suspension [5].
13. For more information on harvesting gradients **see Application Sheet V04**.
14. The denser band is essentially pure IMVs, while the lighter EEV band contains some IMV contamination.

6. *Molluscipoxvirus*

Bugert [9] purified this virus from skin lesions using iodixanol gradients and obtained excellent structural analysis data from the isolate, but did not provide a detailed methodology.

7. References

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7. Acknowledgements

We would like to thank Jacomine Krijnse-Locker, European Molecular Biology Laboratory, Heidelberg for valuable information in the preparation of this Application Sheet.

OptiPrep™ Application Sheet V12; 8th edition, January 2020