OptiPrep™ Application Sheet V19

Purification of Group IV ((+)ss) RNA viruses: *Flaviviridae* family and hepatitis E in pre-formed gradients

- OptiPrep™ is a 60% (w/v) solution of iodixanol in water, density = 1.32 g/ml
- This Optiprep™ Application Sheet is concerned principally with the following genera
  - ***Hepacivirus*:** hepatitis C virus and hepatitis C virus-like particles (Sections 2-7)
  - ***Flavivirus*:** Dengue virus, West Nile virus and yellow fever virus (Section 8b)
  - ***Pestivirus***: bovine viral diarrhea virus (Section 8a)
  - ***Hepevirus***: hepatitis E (Section 8c)
- The **Axis-Shield Mini-Review (MV06)** "Purification and analysis of hepatitis C virus" summarizes the published methods and provides a full bibliography of all published papers reporting the use of iodixanol gradients for the purification of this virus; to access return to the initial list of Folders and select "**Mini-Reviews**".
- To access other Application Sheets referred to in the text return to the Virus Index; key Ctrl "F" and type the V-Number in the Find Box.
- Sections 2 and 5b address the problems associated with larger volumes of virus suspension.

1. Background

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. Most iodixanol gradients can also be made isoosmotic over the entire density range. 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. Thus iodixanol is being increasingly used for hepatitis C virus particle purification from lysed cultured cells, conditioned culture medium or plasma samples from patients.

2. Concentration of virus particles

After clarification of the virus-containing fluid by centrifugation at 3000-4000 *g* for 10 min and/or filtration through a 0.2 or 0.45 µm filter it is often necessary to concentrate the virus before applying to a gradient. Direct pelleting from culture the suspension, e.g. at 125,000 *g* for 4 h, may lead to loss of infectivity. One useful alternative is to use a centrifugal ultrafiltration, for example the Centricon PBHK Centrifugal Plus-20 filter unit with an Ultracel PL membrane (100 kDa cut off) as described by Yi et al [2,3]. Bartolomé et al [4] centrifuged viral particles from sera over 10% sucrose at 100,000 g_{av} for 17 h. Virus can also be precipitated from culture fluid by incubating it with 0.25 vol. of 40% (w/v) polyethylene glycol 8000 in PBS at 4°C overnight [5,6]. After collection of the virus by centrifugation it was suspended in 0.85% (w/v) NaCl, 0.02% bovine serum albumin, 10 mM HEPES-NaOH, pH 7.55; and pelleted through a 20% (w/v) iodixanol cushion by centrifugation for 6 h at 190,000 g_{av}.

Use of an iodixanol cushion to concentrate the virus was also used by Elmowalid et al [7], but in this case the iodixanol concentration was 40% (w/v). Banding of the virus at an interface is probably the gentlest method of concentration and is ideal if the virus is subsequently used to bottom load the gradient [7]. If the gradient is to be top-loaded however, virus concentration on to a dense cushion may be problematical. A top-loading strategy means that the virus suspension must contain <10% iodixanol and great care must be exercised in recovering as little of the cushion as possible when the virus is harvested. In Beckman ***k***onical tubes the volume of the dense cushion can be very small (e.g. in the

Beckman SW41Ti, approx 0.5-1.0 ml) and if most of this cushion is aspirated (using fine bore Teflon tubing attached to a syringe) before the virus band is harvested, it is possible to achieve a suitably low iodixanol concentration in the harvest. For more information on density cushions see Section 5b.

For more information on handling large volumes of virus see **Application Sheet V06** and Section 5b of this Application Sheet.

3. Gradient solution preparation

3a. NaCl as osmotic balancer (see Box 1, from ref 8)

A. OptiPrep™

B. Buffer: 120 mM HEPES-NaOH, pH 7.6, 0.12% (w/v) bovine serum albumin (BSA)

C. Salt solution: 1 M NaCl

Box 1
Keep HEPES (free acid) as a 200 mM stock solution at 4°C; 4.76 g per 100 ml water.

Solution B: Adjust 60 ml of HEPES stock solution to pH 7.55 with 1 M NaOH; dissolve 0.12g BSA (check pH) and make up to 100 ml. Make up fresh or keep frozen.

Solution C: Dissolve 58.5 g NaCl in 50 ml water and then make up to 100 ml.

- Prepare 10% (w/v) iodixanol by diluting 1 vol. of OptiPrep™ with 1 vol. of Solution B, 0.75 vol. of Solution C and 3.25 vol. of water
- Prepare 40% (w/v) iodixanol by diluting 4 vol. of OptiPrep™ with 1 vol. of Solution B, 0.3 vol. of Solution C and 0.7 vol. of water.

3b. Sucrose as osmotic balancer (see Box 2, from ref 9)

A. OptiPrep™

B. Diluent 1: 0.25 M sucrose, 12 mM EDTA, 60 mM Tris-HCl 5 pH 8.0

C. Diluent 2: 0.25 M sucrose, 2 mM EDTA, 10 mM Tris-HCl 5 pH 8.0

Box 2
Keep the following stock solutions at 4°C:
100 mM Tris (free base): 1.21 g per 100 ml water
100 mM EDTA (Na_2•$2H_2O$): 3.72 g per 100 ml water

Solution B: Dissolve 8.5 g sucrose in 60 ml of Tris stock; add 12 ml of EDTA stock; adjust to pH 8.0 with 1 M HCl and make up to 100 ml

Solution C: Dissolve 8.5 g sucrose in 60 ml of water; add 10 ml and 2 ml respectively of Tris and EDTA stocks; adjust to pH 8.0 with 1 M HCl and make up to 100 ml

- Prepare 50% (w/v) iodixanol by mixing 5 vol. of OptiPrep™ with 1 vol. of Solution A.
- Dilute the 50% iodixanol with Solution C to give lower concentrations of iodixanol.

3c. Notes

The two solutions described in Section 3a provide a positive gradient of iodixanol (10-40%) and a negative gradient of NaCl (0.125-0.05M) and are designed to maintain isoosmotic conditions. However when OptiPrep™ is simply diluted with any buffered isoosmotic solution, the resulting solution should also be approx. isoosmotic, see **Application Sheet V01**. Thus it may be sufficient simply to dilute the OptiPrep™ with 0.85% (w/v) NaCl, 0.02% bovine serum albumin, 20 (or 10) mM HEPES-NaOH, pH 7.6. The recipe in Section 3b maintains constant concentrations of EDTA and Tris in all solutions, with sucrose as the osmotic balancer [9]. A similar strategy may be used with NaCl as osmotic balancer [10]. Other methods use Hank's balanced salt solution [2,3] to dilute the OptiPrep™ or the cell lysis medium containing 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM PMSF, 10 mM Tris-HCl, pH 7.4 [7].

4. Sedimentation in pre-formed gradients (adapted from ref 8)

4a. Protocol

1. Using a two-chamber gradient maker or a Gradient Master™ prepare an approx. 12 ml gradient in tubes (for a swinging-bucket rotor with approx 14 ml tubes, e.g. Beckman SW41Ti) from equal volumes of the two iodixanol solutions described in Section 3a (see Section 4b, Notes 1-3).

2. Layer 0.5-1.0 ml of the crude virus suspension on top and centrifuge at 197,000 g_{av} for at least 6 h at 4°C (see Section 4b, Notes 1-3).

3. Collect the gradient in 0.5-1.0 ml fractions either by tube puncture or aspiration from the meniscus or if the band of virus is sufficiently distinct retrieve it using a syringe. For more information on harvesting gradients see **Application Sheet V04**.

4b. Notes

1. Lindenbach et al [8] was the first group to use this 10-40% iodixanol gradient and this technique is probably the most widely used. Although 10-40% iodixanol is a common range for the gradient, others are equally successful: 10-50% [10], 6-56% [9] and 20-50% for purifying hepatitis C virus-like particles released from insect cells infected by recombinant baculoviruses [6].
2. If a mechanical device for creating a continuous gradient is not available, then make up a discontinuous gradient from equal volumes of 10%, 20%, 30% and 40%. Because gradients are centrifuged for at least 6 h these discontinuous gradients will become more or less linear (depending on the volume of the gradient steps) by diffusion during centrifugation. For more information on preparing continuous gradients see **Application Sheet V02**.
3. Occasionally discontinuous gradients from 10-50% (or 10-40%) iodixanol are produced using only a 5% step interval. Often they may be allowed to diffuse at 4°C for 4-24 h, prior to loading the sample or the sample may be loaded without any diffusion time. Centrifugation at *g*-forces between 90,000 *g* and 250,000 *g* has been reported. Moreover the centrifugation forces and times do not necessarily relate to the size of the gradient: examples are 230,000 g_{av} for 5 h (4 ml gradient) to 90,000 g_{av} for 24 h or 190,000 g_{av} for 6-16 h (12-13 ml gradient). Just a few of the variations in gradient construction and *g*-force are given in Table 1.

Table 1 Discontinuous gradients for top-loaded samples

Iodixanol (% w/v)	Diffuse time (h)	*g* – time (h)	Ref. #
6,10,20,30,40,50,56.4 (0.5 ml ea.)	none	90,000 - 24	4
10-50 (5% steps)	4	150,000 -16	11
10,20,30,40 (2.5 ml ea.)	18-24	151,000 - 18	12,13
10-40 (5% steps)	16	160,000 - 16	14
5-50 (five steps)	None	250,000 – 22	15

- Lindenbach et al [16] found that the level of particles in chimpanzee serum was very low; making up the 10% and 40% iodixanol solutions in the serum overcame the detection problems associated with top loading of small volumes of sample.
- In ref. 9 Nielsen et al also described a rate-zonal system comprising a 2-24% (w/v) iodixanol gradient centrifuged at 90,000 *g* for 2 or 4 h to study the homogeneity of virus samples and also for analyzing nucleocapsids.

5. Flotation in pre-formed gradients (adapted from ref 17)

5a. Protocol

1. Prepare 10%, 20%, 30% and 40% iodixanol solutions using any of the buffers described in Section 3; a Tris-buffered 0.85% NaCl solutions was described in ref 17 (see Section 5b).

2. In approx 14 ml tubes for a swinging-bucket rotor, use a syringe attached to a thin metal cannula to prepare gradients by underlayering from approx 3.0 ml of each of the iodixanol solutions and the buffered saline. For more information about the preparation of discontinuous gradients see **Application Sheet V02**.

3. Adjust the virus suspension to approx. 45% iodixanol by mixing with OptiPrep™ and underlayer each gradient with approx. 1.0 ml of the suspension (see Section 5b).

4. Centrifuge at 154,000 *g* for 16 h at 4°C (see Section 5b).

5. Collect the gradient by tube puncture or, if the band is sufficiently well defined, retrieve it using a syringe. For more information on harvesting gradients see **Application Sheet V04**.

5b. Notes

The method can be scaled down to smaller volume rotors by reducing all volumes proportionately. Smaller gradients will become more or less continuous due to diffusion during centrifugation. A pre-formed continuous gradient may also be bottom-loaded for a flotation separation, e.g. an 8-30% iodixanol gradient underlayered by the hepatitis C virus in 40% iodixanol [7]. In this case the virus had previously and very conveniently been concentrated on a 40% iodixanol cushion [7]. There are other examples of the use of flotation gradients, a selection is provided in Table 2.

Table 2 Flotation gradients protocols

Iodixanol (% w/v)	Sample in iodixanol	*g* – time (h)	Ref. #
0-30% continuous	35-40%	154,000 – 18	18-20
0-60% continuous	60%	110,000 – 18	21
8-30% continuous	40%	120,000 –16	7

A few interesting variants of these strategies, which facilitate the use of larger volumes of virus suspension are worth mentioning:

- Ref 22 described the following method: as with the routine bottom-loading, the crude virus suspension was adjusted to 40% (w/v) iodixanol, but this was overlaid by an equal volume of the suspension, to fill the tube (Fig 1a). After sealing the tube, it was gently reoriented to a horizontal position. After 24 h at 4°C, the tube was returned to the vertical. During this time, diffusion of the iodixanol will have created a linear gradient (Fig 1b) and the particles will be randomly distributed through it. This procedure is described in **Application Sheet V02**. After removing 0.5 ml from the top of the tube it was centrifuged at 90,000 *g* for 24 h. (Fig 1c) to band the particles isopynically. The big advantage of this procedure is that, rather like a self-generated gradient (see **Application Sheet V20**), there are no interfaces that may cause the build-up and aggregation of particles, in this case the gradient was able to resolve replicon RNA from HCV RNA.

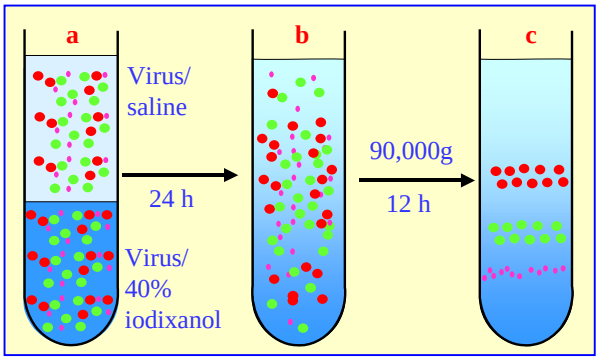

Figure 1: Purification of hepatitis C virus in a continuous iodixanol gradient (Adair et al – ref 22); see text for details

- In a similar protocol Merz et al [23] used a 3 ml 48% (w/v) iodixanol cushion initially to concentrate the HCV at 96,000 *g* for 20 h (Fig 2a-b). From the bottom of the tube 6 ml was removed (i.e. the cushion, banded virus and 3 ml of sample buffer) – so the concentrated virus was now in 24% iodixanol. This was used to construct a discontinuous gradient (Fig 2b-c) of 48%, 36%, 24%, 12% and 0% (saline), which was centrifuged at 96,000 *g* for 20 h (Fig 2d). The gradient displayed distinct banding of core protein, E2 protein and ApoE [23].

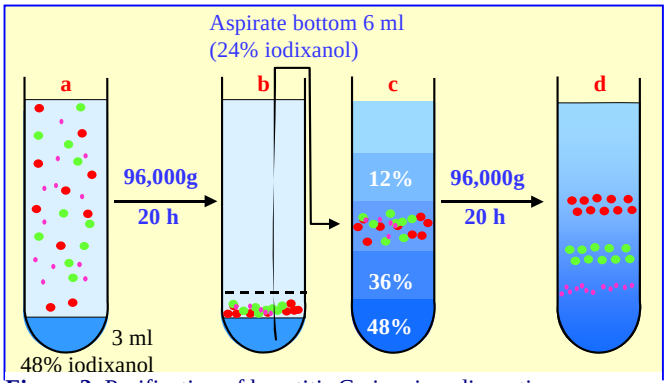

Figure 2: Purification of hepatitis C virus in a discontinuous iodixanol gradient (Merz et al, - ref 23), see text for details

6. Buoyant density of virus particles

Generally virus particles band around 1.09-1.12 g/ml [12] but the density of infectious virions was <1.093 g/ml according to ref 24. Often at least two peaks of RNA are identified in the gradient. Lindenbach et al [16] for example noted that virus from cultured cells banded principally around 1.14 g/ml, while that from animal serum was <1.10 g/ml. Yi et al [2] reported that RNA from virus containing the H77-S genotype was biphasically distributed at the same two densities; the JFH-1 strain contained mainly the denser form, while in the H77-S/ΔE1p7 mutant this form was virtually absent. Intracellular virus was identified at 1.09-1.12 g/ml while extracellular virus banded broadly from 1.03-1.12 g/ml [25]. The bimodal distribution of particles (1.086 and 1.152-1.155 g/ml) is confirmed in studies by Farquhar et al [26]. Nielsen et al [27] showed that while membrane-encapsulated particles from human liver had a density of approx 1.08 g/ml, the free nucleocapsid banded at approx 1.2 g/ml. Analysis of particles from the plasma of infected patients identified particles <1.08 g/ml that were associated with VLDL [9].

7. Sedimentation velocity gradients

Nielsen et al [9] have also used iodixanol gradients for determining the size of virus particles isolated from human plasma. The 4-24% (w/v) continuous iodixanol gradients were centrifuged at 90,000 g_{av} for 2 or 4 h. The authors compared the sedimentation coefficient ($s_{20,w}$) before and after detergent-treatment; the values were approx 215S and 180S respectively.

8. Other *Flaviviridae* viruses

8a. *Pestivirus* (Bovine diarrhea virus)

The virus was purified by flotation through a discontinuous iodixanol gradient [28]. Medium from a cell culture was clarified at 4000 *g* for 30 min and the supernatant centrifuged at 125,000 *g* for 4h to concentrate the virus as a pellet. The latter was resuspended in 0.35 ml of PBS and mixed with an equal volume of OptiPrep™ (final concentration of iodixanol = 30% w/v). Layers of 25%, 20%, 15%, 10% and 5% (w/v) iodixanol (OptiPrep™ diluted with PBS) were layered on top in tubes for a Beckman SW60Ti swinging-bucket rotor (4 ml tubes). After centrifugation at 168,000 *g* for 4 h the gradient was probably close to being a linear one; virus banded at approx 16.5% (w/v) iodixanol. Since the virus is bottom-loaded in the gradient, this is a method that would allow the first concentration step to be carried out, not by pelleting, but by banding on to a small cushion of OptiPrep™. More recently, a top loaded discontinuous gradient comprising 2 ml each of 10%, 22%, 24% and 26% (w/v) iodixanol at 150,000 *g* for 2 h was used; the virus banded at the 22%/24% interface [29-30]. More information on concentration of virus prior to gradient purification can be found in **Application Sheet V06**.

8b. *Flavivirus*

8b-1 Dengue virus

Virus from a cell culture supernatant was concentrated by pelleting through 20% (w/v) sucrose at 72,000 *g* for 5 h. The resuspended pellet was layered on a 10-40% (w/v) iodixanol gradient and centrifuged at 164,000 *g* for 2 h. Virus was recovered from gradient fractions (after dilution with buffer) by pelleting at 72,000 *g* for 5 h [31-33]. Other workers have used the same concentration technique, with a marginally shallower gradient (9-36% iodixanol) and much milder centrifugation conditions – 30,000 *g* for 2.5 h [34,35]. In a simple two-layer 20/55% (w/v) iodixanol discontinuous gradient (210,000 *g* for 2 h) the virus bands at the interface [36,37].

Smith et al [38] and Vancini et al [39] used two rounds of centrifugation, also in a top-loaded two-layer gradient of 12% and 35% (w/v) iodixanol; the first centrifugation was executed at approx. 105,000 *g* for 8-16 h and the second at approx. 150,000 *g* for 3 h. An important aim of the centrifugation was to concentrate the virus and to separate the virus from the bovine serum albumin in the virus preparation. The authors [38,39] stressed the importance of using a gradient solution that was less viscous than the traditional sucrose and importantly non-toxic to cells so that vaccine strains could be administered to African green monkeys in the gradient harvest containing 33% (w/v) iodixanol. It was also observed that other gradient purification and concentration reagents such as PEG may induce fusion events between viral particles [39]. Essentially the same two-layer gradient has also been used in a single overnight centrifugation [40] and for a shorter time (2.75 h) [41].

Multi-layer discontinuous gradients have also been used. A post-nuclear supernatant from infected cultured cells was fractionated on a discontinuous gradient of 6%, 18%, 30% and 48% (w/v) iodixanol centrifuged overnight at approx. 100,000 g_{av} in a study of Dengue virus replication complexes; the gradient showed that the increased fatty acid synthesis that occurs in infected cells is associated with a membrane compartment that also contains most of the Dengue RNA [42]. Zaitseva et al [43] used a 15%, 20%, 25%, 40% (w/v) iodixanol gradient, centrifuged at approx. 340,000 *g* for 1.5 h to separate a Dengue virus fraction loaded with a lipophilic fluorescent protein (which banded at the 20-25% interface) from any unincorporated protein. Virus in 5% (w/v) iodixanol has been layered over 10%, 20%, 25% and 35% (w/v) iodixanol centrifuged at 240,000 *g* for 1.5 h (the virus banding at the 20-25% interface [44]. A similar gradient is reported in ref 45.

8b-2 West Nile virus

The virus was concentrated by pelleting at 48,000 *g*, for 15 h and then the pellet resuspended in buffered saline (or culture medium) and layered on a 15-55% (w/v) iodixanol gradient and centrifuged at 100,000 *g* for 18 h [46,47]. Whether the relatively low *g*-forces used reflect the particular sensitivity of this virus to centrifugal forces is not clear. Vancini et al [39] also used their 12%/35% (w/v) iodixanol gradient (see Section 8b-1) for West Nile virus purification.

8b-3. Yellow fever virus

Yellow fever virus was purified and analyzed on a 5-40% (w/v) iodixanol gradient centrifuged at approx. 175,000 g_{av} for 7 h. The gradient was described as "rate-zonal" but under these centrifugation conditions, it is more likely to be a buoyant density separation. Whatever the nature of the separation, a very interesting observation made by Patkar et al [48] was that some amino acid deletion sequences made to the capsid protein caused a significant shift in the banding position compared to that of the wild-type. The gradient is capable of very high discriminatory powers.

8c. Hepatitis E virus

Continuous iodixanol gradients of 8-40% (w/v) [49], 6-56% (w/v) [50] and 0-40% (w/v) [51] have been used to compare the properties of virus that is released into the culture medium from infected cell monolayers versus that from cell lysates [49] or that which is present in blood serum or in fecal matter from infected animals [50,51]. The iodixanol gradients clearly showed that the virus in plasma or culture medium was enveloped and had a density in the range 1.08-1.11 g/ml while that from cell lysates and fecal matter was much higher (1.20-1.25 g/ml), characteristic of a non-enveloped form.

9. References

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