# Virus purification

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Virus purification is the physical separation of virus in a concentrated form from the host cell milieu in which it has grown. Viruses need to be purified for many studies in which properties or structure of the virus must be distinguished from those of the host cells or culture medium, such as analyses of structure of viral polypeptides, function of membrane glycoproteins, etc.

In this review, we will describe proven methods of purifying enveloped versus nonenveloped viruses, and labile versus stable viruses, as these properties constitute important differences in the methods. We will also discuss those criteria used by various laboratories for determining the degree of purity achieved in virus preparations.

# Principles of ultracentrifugation

Ultracentrifugation is the usual technique of choice for the purification of particles of defined size (i.e. virions) from their contaminating materials. In any suspension of particles their rate of sedimentation depends not only on the size, density and morphology of the particles but also on the nature of the medium in which they are suspended and the force applied to the particles during centrifugation.

One important contributory factor to consider in the separation and ultimate purification of virions from contaminating materials is therefore the viscosity of the medium in which they are centrifuged and this chapter addresses such an issue later.

## Types of centrifugal separations

It is of course theoretically possible to separate particles solely on the basis of *differential pelleting*, where asymmetrical particles will sediment slower than spherical particles of the same mass and density. By increasing the time of centrifugation or the centrifugation speed smaller particles will also pellet. Differential centrifugation also separates particles on the basis of their density. Whilst the procedure can be successful in achieving good separation of a variety of particles as long as there are large differences in their mass and/or densities, the yield of such a procedure is likely to be low and the purity of the particles questionable. The technique is however often used as a starting point designed so as to enrich populations of particles before further purification. Virus purification is more likely to be achieved by the technique of density gradient centrifugation, where separation of the components is achieved by sedimentation through a density gradient, (i.e. a solution which increases in density with increasing distance down the centrifuge tube).

Two types of approach are routinely used – rate-zonal centrifugation and isopycnic centrifugation.

In rate-zonal centrifugation the problem of co-sedimentation of particles is overcome by layering the sample in the form of a narrow layer on top of a density gradient. During centrifugation the sample particles separate as a series of bands or zones, each with its own characteristic sedimentation rate. Centrifugation is stopped before the particles pellet and the separated components are collected by fractionation of the gradient. The rate at which the particles sediment depends on their size, shape, density, the centrifugal force, and the density and viscosity profile of the gradient.

Isopycnic centrifugation separates particles solely on the basis of their different densities. The sample is loaded directly on to a preformed density gradient and then centrifuged or is mixed with the gradient medium to give a solution of uniform density and the density gradient forms during centrifugation (e.g. Dea and Tijssen 1988).

In rate-zonal centrifugation the density of the gradient must not exceed that of the particles being separated whereas in isopycnic centrifugation the maximum density must always exceed the density of the particles.

The above procedures are carried out in *preparative* ultracentrifuges which are usually grouped on the basis of their speed. Routinely one would wish for a machine capable of $40–80 \times 10^3$ rpm and $600 \times 10^3$ $g$.

Centrifuge rotors come in various sizes but would routinely be swing-out rotors for rate-zonal centrifugation and fixed angle or vertical rotors for isopycnic centrifugation.

# PEG precipitation

Concentration of viral suspensions by precipitation techniques is a useful starting point for virus purification.

## Advantages over other concentration methods

Precipitation of macromolecular proteins such as viruses by high molecular weight polyethylene glycol–6000 (PEG), pioneered by Yamamoto et al (1970) for bacteriophages, is an effective concentration method because the viruses are slowly precipitated in a cold, high-salt environment which protects them from chemical and physical denaturation.

PEG precipitation is more gentle than physical concentration by ultracentrifugation or molecular sieve filtration. These are also done in the cold, but ulltracentrifugation often packs the virions so tightly, even atop sucrose cushions, that they cannot be resuspended without significant loss of virus, and ultrafiltration requires magnetic mixing to keep the filter cleared and loses a great deal of virus trapped in the filter itself. PEG is also more effective, in our hands, than ammonium sulfate precipitation, although the latter has been used with good results for astroviruses, caliciviruses, coronaviruses, picornaviruses and many others (Ashley and Caul 1982; Tannock 1973; Wadey and Westaway 1981; Minor 1985). The PEG precipitation procedure outlined below has been performed with good results for coronaviruses (Hierholzer 1976; Lanser and Howard 1980), rhabdoviruses (Obijeski et al 1974), parainfluenzaviruses (Hierholzer et al 1993), respiratory syncytial virus (Anderson et al 1984; Cash et al 1977; Hierholzer et al 1994), rubella virus (Fuccillo and Sever 1989), and picornaviruses (Hasegawa and Inouye 1983; Hierholzer et al 1984).

## Protocol and follow-up

1. It is best to start with a large-volume virus culture in which calf serum and other protein additives have been withheld from the maintenance medium.
2. At complete CPE, the cells and medium are harvested by scraping with a rubber policeman. (Multiple cycles of freeze–thawing can effectively break up the cells if the virus is stable to such treatment.) The pooled harvest is clarified by large-volume, low-speed centrifugation, such as in a Beckman JA-14 rotor in a J2-21 centrifuge at 10,000 rpm (15,300 $g$) or a J-21 rotor in an L8-70 centrifuge at 12,000 rpm (15,000 $g$), for 20 min at 3°C.
3. Transfer the supernatant to a large beaker in an ice bath on a magnetic stirrer.
4. *Slowly* add NaCl to a final concentration of 2.3%, with constant but gentle stirring.
5. *Slowly* add PEG-6000 to a final concentration of 7.0%, also with constant and gentle stirring. Cover the beaker and stir for about 1 h more to ensure complete solubilization of the PEG. (Others have used higher salt [to 2.7%] and lower and higher PEG (6.0–10.0%) with good results (Fuccillo and Sever 1989; Hasegawa and Inouye 1983; Hierholzer et al 1984; Lanser and Howard 1980; Yamamoto et al 1970).)
6. Transfer the beaker and ice bath to a refrigerator, and allow the virus (and other proteins) to precipitate overnight at 4°C.
7. Collect the precipitate by the same

centrifugation method used for clarification (step 2). Aspirate or drain the centrifuge bottles thoroughly to remove as much PEG as possible.

8. Resuspend the precipitate in a small volume of TES buffer (0.01 M Tris-HCl, pH 7.2, 0.002 M EDTA, 0.15 M NaCl). The buffer should be added at about 2 ml per centrifuge bottle and aspirated thoroughly with a syringe and 22-gauge needle. The suspension is then transferred to a clean tube, and each bottle is rinsed with an additional 1 ml of buffer which is added to the pooled suspension.

9. Finally, the PEG is removed (pelleted) by centrifugation of this pooled suspension at 13,000 $g$ for 4 min at 23°C in a Beckman Microfuge or similar device. The supernatant now contains approximately 100–fold concentrated virus in isotonic TES buffer; the virus preparation may be considered enriched, but not purified.

# Sucrose gradients

Sucrose is suitable for most rate-zonal centrifugation procedures and is often the ideal method for the formation of sharp, easily isolated bands of pure virus. Sucrose is however very viscous at densities greater than 30% w/v and some laboratories use Ficoll or Dextran (Pharmacia Fine Chemicals) as an alternative. The procedures for gradient formation are, however, the same. The gradient solution is usually made up in an aqueous buffer at neutral pH; borate buffer should never be used. The gradients are usually of the continuous type, i.e. the density of the sucrose solution increases smoothly with increasing distance from the axis of rotation. In designing gradients it is essential that the top of the gradient is of sufficient density to support the virus sample whereas the density at the bottom must exceed that of the virus particles. Step gradients (e.g. 5–30%, 10–40% or 15–45%) should be used whenever possible as they result in better separation of virus particles from contaminating materials. Gradients should also never be overloaded with sample, and whilst this procedure is of course a 'learning curve' for different viruses Hull (1985) suggests that SW27 (large swing out rotors) should never be loaded with more than 5 mg virus whereas the smaller tube (SW50) should contain 1 mg of virus or less.

Gradients are either produced by the diffusion method, where a series of differing sucrose concentrations are layered one on top of the other and allowed to diffuse, or by the use of gradient makers, which range from home-made devices put together by the local glass-blower to sophisticated commercially acquired apparatus.

The diffusion method relies on the aliquoting, by underlaying or overlaying, of identical volumes of a series of sucrose solutions to produce a step gradient. Once prepared the tube is stood either vertically or horizontally and the sucrose allowed to diffuse to form the gradient. If left vertically the tubes should be allowed to stand overnight at 4°C.

Typical volume examples for preparation of a variety of different sized gradients are shown below (reproduced from Hull, 1985).

Gradients made by means of a gradient maker can be used immediately. The gradient maker consists of two vessels of equal cross-sectional area joined by a connecting channel which is opened or closed by means of a stopcock. The outlet or mixing chamber has a fine tube exit which, via a piece of flexible tubing, leads to the centrifuge tube. Vessels are constructed for various volume gradients. Whilst sucrose will flow by gravity many workers prefer to use a peristaltic pump between the glass exit tube and the centrifuge tube (see Fig. 4.1)

The methods for gradient preparation are well documented by Hames (1984) but a brief description of the 'dense end first' method is given here.

1. Always use clean dry vessels and tubing.

**Table 4.1**

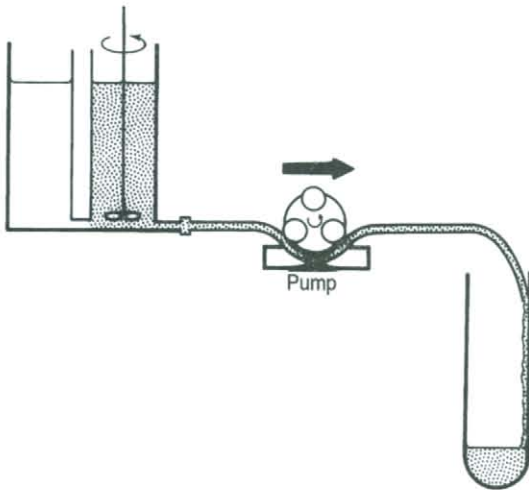| Tube size (inches) | Rotor | ml of sucrose | | | |
| | | 40%* | 30% | 20% | 10% |
|---|---|---|---|---|---|
| 3 × 1 | SW25 | 7 | 7 | 7 | 4 |
| 3.5 × 1 | SW27 | 8 | 8 | 8 | 6 |
| 3.5 × 9/16 | SW41 | 3 | 3 | 3 | 2 |
| 2 × 0.5 | SW50.1 | 1 | 1 | 1 | 1 |

* percentages as given by w/v.

**Figure 4.1.** Preparation of sucrose gradients (from Hames 1984).

2. Connect the vessel outlet tubing to the top of the centrifuge tube. The tubing may be held in place but as long as care is taken can often be left 'lodged' against the inner side of the top of the centrifuge tube.
3. Ensure stop-cock between chambers is closed and introduce required volume of less dense solution into the reservoir chamber. Add appropriate volume of the more dense solution to the mixing chamber.
4. Stir the solution in the mixing chamber using a helix stainless steel wire (or similar device) driven by an overhead motor.
5. Set a peristaltic pump to give a steady flow rate and open connecting stop-cock (watch out for trapped air). The solution flowing into the centrifuge tube becomes progressively more dilute, thus establishing a gradient.
6. The gradient is stored at the required temperature but should normally be used within an hour.

A gradient preformed in such a way is ready for use in rate-zonal centrifugation, hopefully leading to the formation of a visible band of pure virus. Such a band can often be observed by shining a beam of light up through the bottom of the clear centrifuge tube, this being carried out in a darkened room. For some purposes the band can be harvested by puncturing the tube with a hypodermic syringe and withdrawing the band, the sample being pelleted later by ultracentrifugation.

It is however often necessary to collect appropriately volumed fractions from sucrose gradients. For this purpose, again, commercial apparatus is available, but great success is achieved easily by the construction of a home-made apparatus. The authors have used the three designs discussed by Minor (1985) but prefer the apparatus referred to as 'c' in his article. This is briefly discussed below (see Fig. 4.2.)

The apparatus consists of a rubber bung, a bleed tube of steel tubing attached to a short piece of silicone rubber tubing (i), and a glass inlet tube attached to a repeating syringe adjustable to deliver



**Figure 4.2.** Apparatus for harvesting sucrose gradients (from Minor 1985).

0.5–2.0 ml of liquid paraffin (ii).

1. Clamp the centrifuge tube vertically.
2. Insert the rubber bung.
3. Pump liquid paraffin into the tube until it emerges from the bleed tube. Clamp the bleed tube.
4. Pierce the bottom of the centrifuge tube.
5. Collect fractions of appropriate volume by displacement with a known volume of liquid paraffin delivered by the repeating syringe.

Such a method is capable of giving good separation of 'bands', of e.g. empty and full capsids, etc.

Sucrose density gradient rate-zonal centrifugation has been used for the purification of a whole range of viruses, e.g. influenza virus (Barrett and Inglis 1985), adenovirus (Precious and Russell 1985), coronavirus (Collins and Alexander 1980), parainfluenzavirus (Ito et al 1987), rotavirus (McCrae 1985), rhabdovirus (Wunner 1985), togavirus (Gould and Clegg 1985), picornavirus (Minor 1985), and herpes viruses (Killington and Powell 1985).

Specific purification methods for a small capsid virus (e.g. picornavirus) and a large enveloped virus (e.g. a herpes virus) are detailed below.

## Picornavirus

(Minor 1985)

Preparations of virus for purification are usually derived following high m.o.i. of appropriate cell cultures (e.g. HeLa suspension cultures) which are freeze–thawed two or three times and then centrifuged to remove the debris. The supernatant is often treated with either ammonium sulphate or polythene glycol prior to purification. Picornaviruses are however often associated with membranes and thus detergent is usually added (e.g. 1% NP40) to free the virus particles. The purification process is as follows:

1. Prepare a solution to give final concentrations of 15 g of sucrose per 100 ml, 10 mM Tris-HCl pH 7.4, 50 mM NaCl. Prepare a similar solution at final concentrations of 45 g of sucrose per 100 ml, 10 mM Tris-HCl pH 7.4, 50 mM NaCl.
2. Prepare 30 ml linear gradients of sucrose from 15% to 45% in an ultracentrifuge tube of 35–40 ml capacity (e.g. Beckman SW28) using 15 ml of each of these solutions per gradient and a suitable gradient maker.
3. Prepare a solution of 10% NP–40 (BDH) in PBS. Add one-tenth of a volume to the sample, whose volume should not exceed 6 ml. The sample should clarify visibly as it is shaken with the detergent.
4. Layer the sample carefully onto the preformed gradient. Balance the tubes with liquid paraffin.
5. Centrifuge the tubes at 4°C at 80,000 $g$ for 4 h (for example at 25,000 rpm in a Beckman L8 ultracentrifuge, SW28 rotor).
6. Harvest the gradients as described above. Assay for virus. The infectious virus peaks should be about $\frac{2}{3}$ down the gradient.

Virus purified in such a way is suitable for most purposes. However some impurities may remain and if extreme purity is required it may be necessary to re-purify on caesium chloride gradients.

## Herpes simplex virus

(Killington and Powell 1985)

Herpes simplex virus provides a more difficult challenge than most viruses with regard to the preparation of pure virus particles. Techniques which work well with one virus strain in a particular cell line do not work for other strains of virus in the same cells or for the same strain

of virus in different cells. For this reason we give two methods here. Method 1 relies on the virus strain being released into the extracellular medium of e.g. Hep-2 infected cells. Method 2 appears to work for most strains.

# Method 1

(Powell and Watson 1975)

1. Infect confluent monolayers of Hep-2 cells in roller cultures at a m.o.i. of 20–25 (pfu cell$^{-1}$).
2. Wash the infected cells after virus adsorption, add fresh medium and incubate the cells at 32°C for 2–3 days.
3. At the end of the incubation, centrifuge the medium at low speed to remove cell debris.
4. Harvest the virus either by precipitation from the medium with polyethylene glycol (PEG, molecular weight 6000, 8% w/v in the presence of 0.5 M NaCl) or by centrifugation (12,000 rpm, 2 h in a GSA rotor, Sorvall RCS-5B centrifuge).
5. Resuspend the virus in a low molarity Tris buffer pH 7.8 containing 50 mM NaCl. It is preferable to allow re-suspension overnight if this is convenient.
6. Layer the suspension of virus over a 30 ml gradient of 5–45% sucrose in the same buffer and centrifuge for 1 h at 12,500 rpm (Sorvall AH627 rotor). At the end of this period a fluffy white band of purified virus should be clearly visible at the centre of the gradient. This visible band contains the single peak of infectious virus in the gradient.

At this point the virus is of adequate purity (about 50 µg of protein/$10^{10}$ particles virus) for many purposes including the preparation of virus DNA. The virus may be recovered by simple sedimentation. To produce high quality preparations of virus a wide variety of other techniques can be used, including a second sucrose gradient or caesium chloride gradients. Both these techniques yield virus with a protein/particle ratio better than 20 µg/$10^{10}$ particles.

# Method 2

(Spear and Roizman 1972; Heine et al 1972; Killington et al 1977)

1. Infect the cells exactly as described for method 1.
2. Incubate the cells at 37°C for 18–24 h and harvest by scraping from the glass and low-speed centrifugation.
3. Resuspend the cell pellet so obtained in Reticulocyte Standard Buffer (RSB) and allow the cells to swell for 10 min. The cytoplasm may then be obtained from the cells by Dounce homogenization.
4. Centrifuge the Dounce-homogenized cells at low speed to remove nuclei and cell debris.
5. Layer the supernatant (containing the majority of the infectious virus) on to a 5–40% Dextran gradient in Tris buffer and centrifuge at 12,500 rpm for 1 h (Sorvall AH 627 rotor); the virus is obtained by removing the visible band in the centre of the gradient.
6. Collect the virus from this band by sedimentation (20,000 rpm for 1 h, Sorvall AH 627 rotor).

At this point the virus is of adequate purity for many purposes but can easily be improved by the methods mentioned above. Purified virus derived by either method may be resuspended in distilled water or a suitable buffer and aliquots taken for infectivity assay, total particle count and protein estimation. Virus can be frozen at −70°C, but on thawing this leads to partial disintegraton of the viral envelope.

# Caesium chloride gradients

Caesium chloride (CsCl) is one of the materials of choice used for the purification of viruses by a technique referred to as buoyant density-gradient sedimentation, isopycnic centrifugation or equilibrium density gradient centrifugation. This method is based on the premise that the layering of a virus suspension on to a pre-formed gradient followed by ultracentrifugation will result in the migration of virus particles until they reach an equilibrium according to their buoyant density. Alternatively, the gradient may be field formed by mixing the virus suspension with a known amount of CsCl in a suitable buffer prior to centrifugation when the gradient is produced. This technique contrasts with the procedure of rate-zonal centrifugation which separates particles on the basis of size. Other materials used in isopycnic centrifugation include organic molecules such as sugars (sucrose) or polysaccharides (Ficoll), iodinated aromatic compounds (Nycodenz) or colloidal silica (Percoll). Hence no one particular compound is of universal application. More far reaching applications of CsCl gradients include the purification of plasmid DNA and the separation of subviral components.

The widespread use of CsCl is attributed to the fact that solutions of up to 1.91 g cm$^{-3}$ can be prepared from it which is highly desirable for the purification of viruses whose buoyant densities range from 1.28–1.45 g cm$^{-3}$ (non-enveloped) to 1.18–1.25 g cm$^{-3}$ (enveloped). Suggestions for the concentrations of CsCl to be used for gradient formation range from 1.32 g cm$^{-3}$ (32% w/v) for a virus containing 5% RNA to 1.7 g cm$^{-3}$ (55.5%) w/v for a DNA virus.

In addition to its high level of solubility and density, CsCl is able to form solutions of low ionic strength and viscosity. Also, at a suitable pH its presence does not affect the biological activities of the virus. Optical grade CsCl does not absorb UV light allowing direct photometric analysis of gradient fractions. Further it does not interfere with the activity of scintillation fluids, thereby allowing analysis of radioactively labeled virus preparations taken from the gradient. CsCl can easily be removed from the virus fraction by dialysis, filtration or ultracentrifugation.

CsCl gradients may either be self formed or preformed and the latter type of gradient may either be discontinuous or continuous. For the discontinuous gradient, often referred to as a step gradient, different densities of CsCl are layered into a centrifuge tube, beginning either by underlaying the least dense with a more dense preparation or by layering the less dense preparation on the top of the more dense. The virus sample is loaded on to the top of the gradient and centrifugation begun immediately. The continuous gradient is prepared in the same manner but additionally it is held at 4°C overnight, allowing diffusion to occur. More often however, a gradient maker is used to form the continuous gradient thus saving time. For the self-forming gradient, the virus sample, the gradient solute and the buffer are mixed together in proportions that will generate a suitable density range on centrifugation.

Swing out, fixed angle and vertical rotors can all be used for the purpose of CsCl centrifugation. If limited time is available, the various fractions of the gradient are collected and their densities can be measured in an Abbé refractometer. Alternatively, known volumes of liquid may be weighed using a pycnometer. Some methods for virus purification using CsCl gradient centrifugation are given below.

## The purification of picornaviruses on a preformed CsCl gradient

(Minor 1985)

1. Prepare a 40% w/w solution of CsCl by dissolving 4 g of solid CsCl in 6 ml of 0.01 M Tris-HCl pH 7.4.

2. Prepare a 5% w/w solution of CsCl by dissolving 0.5 g of solid CsCl in 9.5 ml of 0.01 M Tris-HCl pH 7.4.
3. Pour a 10 ml 5–40% linear CsCl gradient in an ultracentrifuge tube of 12 ml capacity (e.g. Beckman SW41 tube) using 5 ml of each solution and a suitable gradient maker.
4. Layer the sample containing 1% NP-40 on to the gradient in a volume of 1 ml. Balance centrifuge tubes using liquid paraffin.
5. Centrifuge at 120,000 $g$ for at least 4 h and preferably overnight at 4°C in, for example, a Beckman L8 ultracentrifuge, SW41 head at 30,000 rpm.
6. Harvest the gradients.

# The purification of Brome mosaic virus on a self forming CsCl gradient

(Hull, 1985)

1. Precipitate a suspension of virus extracted from infected leaves with polyethylene glycol 6000 (10% in 0.1 M NaCl and centrifuge at 10,000 $g$ for 10 min, resuspend in 1/10 the volume of 0.1 M sodium acetate pH 5.0 and centrifuge at 10,000 $g$ for 10 min to recover the supernatant.
2. Add CsCl to give a density of 1.36 g cm$^{-3}$ and centrifuge at 36,000 rpm for 18 h in a Beckman R40 rotor.
3. Recover the virus band from the gradient and dialyse against at least 100 volumes of 0.1 M sodium acetate pH 5.0 for 3–4 h to remove the CsCl.
4. Repeat steps 1 and 2 and resuspend the final product in 1 ml of sodium acetate pH 5.0 per 100 g of starting leaf material.

# The use of CsCl gradient centrifugation in the isolation and purification of viruses from tissue samples

Following the treatment of tissue samples to extract a virus of interest, CsCl gradient centrifugation can then be used for its purification. One such example of this was an examination of the structure of fish lymphocystis disease virus from skin tumours of pleuronectes following their purification on CsCl gradients (Samalecos 1986).

1. Tissue is homogenized (1–5 g) in TNE buffer and the suspension clarified by centrifugation at 3000 rpm for 20 min twice.
2. The cell free supernatant is centrifuged through a 30% (w/w) sucrose cushion in a Spinco SW 27 rotor at 25,000 rpm for 120 min at 4°C.
3. The virus pellets are resuspended in TNE buffer and layered on to 35 ml gradients of 25–60% (w/w) sucrose and recentrifuged for 20 h in a Spinco SW27 rotor at 25,000 rpm at 4°C.
4. The virus band is collected and centrifuged in a CsCl gradient (10–35% w/w) for 24 h in a Spinco SW41 rotor at 30,000 rpm at 10°C. The virus band in the middle of the gradient is harvested, dialyzed against TNE buffer and used for electron microscopy.

A second example of the use of this technique for the purification of a virus from biological material was in the isolation of rabbit picobirnaviruses from faecal material. These particles co-sediment with 32 nm virus particles which

have a buoyant density of 1.39 g cm$^{-3}$ (Gallimore et al 1993).

1. A 10% faecal suspension is clarified by centrifugation at 1500 rpm for 15 min.
2. The supernatant is extracted with an equal volume of trichlorotrifluoroethane.
3. The virus present in the aqueous phase is concentrated by centrifugation through a 45% sucrose cushion at 45,000 rpm for 2 h at 5°C in a Beckman SW55Ti rotor.
4. The pellet is resuspended in Tris/Ca buffer and loaded on to a 30% (v/v) CsCl gradient and centrifuged at 35,000 rpm for 17.5 h.
5. The gradient is harvested into 0.3 ml fractions and the buoyant density of each fraction is calculated from its refractive index.
   The sample is then ready for further examination by such techniques as polyacrylamide gel electrophoresis (PAGE).

Finally, CsCl centrifugation has been used for the separation on the basis of buoyant density of viruses isolated from faecal material whose description had previously been limited to that of 'small round viruses'. These were then further examined by electron microscopy and could be classified in more detail (Oliver and Phillips 1988).

1. 0.3 ml of virus supernatant was layered on to 4.5 ml of 45% aqueous CsCl and centrifuged at 100,000 g for 18 h in an MSE 65 Superspeed ultracentrifuge using a swing out rotor.
2. 2.5 µl of 0.3 ml fractions with densities between 1.2 and 1.5 g cm$^{-3}$ were placed on agar coated slides and allowed to dry. Samples were stained and examined by electron

microscopy. By carrying out this retrospective study, small round viruses found in faecal samples were classified into groups such as astrovirus, 'Norwalk-like' virus, parvovirus, enterovirus and hepatitis A.

# The purification of viruses by CsCl density centrifugation for use in immunological assays

CsCl density gradient centrifugation was used for the production of purified preparations of rotavirus antigens for use in T-cell proliferation assays (Bruce et al 1994). Virus was pelleted from 400 ml of clarified (5000 g for 30 min) cell culture lysates and resuspended in 3 ml of 20 mM-Tris-HCl pH 7.5 buffer containing 5 mM calcium chloride. The virus was purified further by differential centrifugation on a five-step caesium chloride gradient. Double and single-shelled rotavirus particles were harvested from the gradient, pooled and washed by ultracentrifugation. The virus purified thus was then used in T-cell proliferation assays.

# The purification of subviral components using CsCl gradient centrifugation

CsCl gradient centrifugation is effective in the production of purified subviral components from either biological fluids or from systems which over-express the protein. Three examples of this are given below.

1. HBsAg was purified from the plasma of high-titre chronic carriers of HBsAg

(Gavilanes et al 1990). Following treatment with octyl glucoside the suspension was layered onto a 12.8 ml CsCl linear gradient (density 1.15–1.32 g cm$^{-3}$). The CsCl had been previously filtered through GSWPO4750 Millipore filters before use.

2. Gradients were centrifuged for 4 h at 154,400 $g$ in a Beckman SW40 rotor.
3. Fractions of 0.4 ml were collected beginning from the bottom of the gradient and their absorbance at 280 nm measured. Antigen-positive fractions were pooled and dialyzed against 10 mM-Tris (pH 7.0)/50 mM NaCl.

Hepatitis B core antigen is composed of an envelope carrying the surface antigen and an internal capsid containing the circular, partially dsDNA genome and the viral polymerase. Empty cores have been produced in baculo-

virus and one cycle of CsCl gradient centrifugation was used for their purification. Three species were found in sedimentation velocity studies of a 3 mg ml$^{-1}$ solution. These had migrations of 71.3S, 62.5S and 11.0S respectively (Hilditch et al 1990).

Hepatitis delta virus antigen was expressed in a eukaryotic cell line (Macnaughton et al 1990) and the density of the antigen was found to be 1.19 g cm$^{-3}$ by equilibrium centrifugation in caesium chloride. The procedure was as follows:

1. A 200 µl sample of ammonium sulphate precipitated recHDAg was overlaid on a 10 ml gradient of preformed caesium chloride (1.1–1.5 g cm$^{-3}$ and centrifuged (120,000 $g$) for 25 h at 20°C and 0.5 ml fractions collected).
2. Fractions were tested for HDAg activity by RIA and its density was estimated from the refractive index.

# Positive density/negative viscosity gradients

## Rationale

Positive density/negative viscosity gradients were first proposed by Barzilai et al (1972) as a means of effectively separating viruses from cytoplasmic components with similar densities. His studies used CsCl to provide density and glycerol for viscosity, and the gradients were employed to purify foot-and-mouth disease virus, a picornavirus, from the host cell milieu in which it was grown. The method described here is patterned after Obijeski et al (1974), who found that potassium tartrate provided a gentler chemical environment than caesium or rubidium salts for enveloped viruses.

The gradients are constructed such that the potassium tartrate yields increasing density from the top of the gradient to the bottom, while the glycerol yields decreasing viscosity from the top to the bottom; thus the designation 'positive density/negative viscosity'. The method is gentle and does not destroy labile viruses either by pelleting them or by a harsh chemical milieu. It has been found particularly useful for purifying enveloped viruses such as rhabdoviruses (Obijeski et al 1974), coronaviruses (Hierholzer 1976), parainfluenza and mumps viruses (Hierholzer et al 1993), and respiratory syncytial virus (Hierholzer et al 1994), and non-enveloped but nonstable viruses such as caliciviruses and astroviruses (Ashley and Caul 1982). In the 30% (v/v) glycerol-to-40% (v/v) potassium tartrate gradients used for enveloped viruses, the top of the gradient after centrifugation contains a band of flocculent material with a refractive index of ~1.356; followed by a faint, hazy band (~1.377); a sharp, compact virus band (~1.379); another faint, hazy band just below the virus (~1.380); and two very flocculent bands near the bottom of the tube (RI 1.381 and 1.385, respectively). The RI values correspond to a range of 15–32% sucrose. For non-enveloped viruses, the gradient is constructed with 30% glycerol and 60% tartrate to provide the greater density needed for these viruses (Ashley and Caul 1982).

## Protocol and interpretation

1. Construct gradients in cellulose nitrate centrifuge tubes holding at least 10 ml. The directions here will be for the Beckman SW41 tubes, which hold 12.5 ml to the top, and allow for 10 ml of gradient and 2.5 ml of previously-concentrated virus. The tubes should be situated vertically, as with all gradients.
2. To make three identical gradients, place 16 ml of 40% di-potassium tartrate in TES buffer, pH 7.2 (see page 176), in the right-hand cylinder to prevent an air trap, and then lead it into the three lines of tubing leading to the pump and centrifuge tubes. This provides a small cushion of 40% tartrate (highest density) which will prevent viruses with densities around 1.18 g cm$^{-3}$ from passing through.
3. Commence stirring in the right-hand cylinder, either with a stainless steel agitator rod or a magnetic mixing bar. Adjust the stirrer so that excessive air bubbles are not formed and forced into the tubing going to the pump and centrifuge tubes. Excessive bubbles can create air traps and also disrupt the linearity of the gradients.

4. Add 15 ml of 30% glycerol in the same TES buffer to the left-hand cylinder, open the stopcock at the bottom of the chamber joining the two cylinders, and allow the glycerol to begin feeding into the mixing chamber as the pump is slowly filling the tubes.

5. The linear gradients are best formed over a 40–50 minute period at ambient temperature.

6. Gently load the gradients with 2.5 ml of concentrated virus per tube.

7. Load the tubes onto the SW41 rotor and spin at 41,000 rpm (208,000 avg. $g$) for 18 h at 3°C. (Alternatively, for larger volumes, Beckman SW27.1 cellulose nitrate tubes can be filled with 15 ml of gradient and 3 ml of virus concentrate, and spun in an SW28 rotor at 27,000 rpm (130,000 avg. $g$) for 18 h at 3°C).

8. After centrifugation, study the tubes with a strong, narrow-beam light aimed upward from below the bottom of the tube in a darkened room. The virus band should stand out as a narrow, condensed band sandwiched between two hazy bands of cellular material of the same density.

9. Harvest the virus band, either by careful pipetting from the top of the gradient downwards, or by puncturing the bottom of the tube and collecting the bands dropwise.

10. Verify the virus band by refractive index, electron microscopy, some appropriate antigen or nucleic acid test, or preferably by all three.

# Filtration methods

Gel filtration, also referred to as size exclusion liquid chromatography (SEC), gel chromatography or gel permeation chromatography has proven to be one of a number of methods of choice for the purification of viruses and is extensively used in the purification of subviral components including antigens and virally encoded enzymes often expressed in either prokaryotic or eukaryotic systems. SEC is used in the separation of simple mixtures conveniently and rapidly when the components of the mixture have a sufficient difference in size. The technique uses a solid phase composed of a column packing of beads with pores of a defined average size. The beads have a specific size exclusion so that the separation of solute entering the column is dependent on its ability to enter the pores. The smaller components are able to enter the pores and are retained in the column and are eluted first. Solutes of intermediate size are less able to approach the walls of the pores and spend less time in the pores. These move through the column at speeds dependent on their relative size. Thus separation in SEC is strictly on the basis of molecular size. The retention of solutes by adsorption is undesirable and usually does not occur with the correct combination of substrate and mobile phase. A differential elution, proportional to the particle size (and consequently to the particle molecular weight) can be obtained.

The beads are composed of various materials. The ideal surface must be neutral and hydrophilic to minimize the possibility of adsorption. Highly cross-linked, mechanically stable, macroporous matrices from neutral, hydrophilic polymers such as dextrans or agarose have been used for such purposes. The commercially available Sepharose CL (Pharmacia) is a beaded agarose matrix which has been cross-linked. However, cross-linking agents introduce hydrophobic character into the matrix. Pore diameter and pore volume can also be reduced during cross-linking.

Sepharose gel filtration media give a broad range of fractionation (10,000–40,000,000 $M_r$) and have a high exclusion limit for the separation of biomolecules. The particle size is from 45–200 µm. The beaded agarose matrix shows low non-specific binding and the cross-linked forms have good chemical and physical stability. Sepharose is available with three different agarose contents: 2%, 4% and 6%. Increasing the agarose concentration decreases matrix porosity thus altering the fractionation range while increasing the rigidity.

Operating instructions and gel selection depend on the application and the desired resolution. The technique can be broadly separated into two categories, desalting or group separation and fractionation. For desalting, the molecule of interest is eluted in the void volume whilst smaller molecules are retained. Thus the exclusion size should be smaller than the molecule of interest. For fractionation, molecules of varying molecular weight are separated within the gel matrix and thus the molecules of interest should fall within the separation range of the gel. Gel filtration is a non-interactive technique which means that the conditions can be chosen to maintain the stability of the molecules being separated. This is a valuable means of separating unknown samples but, more pertinent here, can also be used for the purification of viruses such as poliovirus or rabies virus (van Wezel et al 1979). Filtration on Sephacryl S300 has also been used for the purification of influenza neuraminidase fragments.

The more efficient method of high performance SEC (HPSEC) is now frequently used where the system is closed and operated at high pressures. Thus separations take 10–20 min compared to hours. High pressure, rapid flow rates and sophisticated equipment are characteristic of this procedure. However, small more rigid porous particles will be used for column packing as opposed to the ones used for SEC which would collapse at high

pressure. For details on these systems see Snyder and Kirkland (1979) and Yau et al (1979).

The steps involved in gel filtration are:

a) bed preparation;
b) sample application;
c) sample flow-achieved by gravity feeding of the column;
d) detection and quantitation of the purified virus particle or protein.

Some applications of SEC are given below. McGrath et al (1978) used a Sepharose 4B chromatographic method for the purification of a retrovirus. This method gave increased purified virus yields, conserved the virus glycoprotein and the preparation had an increased recovery of biological infectivity in comparison with the previously used method of sucrose density gradient centrifugation.

1. Viruses are harvested from infected cells and cell debris pelleted by centrifugation at 10,000 g.
2. Clarified supernatant is concentrated 10–50 fold in an Amicon ultrafiltration apparatus using a PM10 membrane at an ultrafiltration rate of 1 ml/min. The concentrate is spun through a discontinuous sucrose gradient (25% to 40% w/w) in TEN buffer (20 mM Tris, 1 mM EDTA, 0.1 M NaCl, pH 7.5) for 2.5 h at 90,000 g and the virus band collected.
3. The supernatant concentrated by ultrafiltration corresponding to 5% of a pre-sterilized Sepharose C1 4B (Pharmacia) column bed volume is chromatographed at 4°C in TEN buffer. Virus appears in the void volume at a flow rate of 0.5 to 1.0 ml min$^{-1}$. The fractions containing the virus peak are monitored by optical density at 280 nm and by radioactivity.

Pinto et al (1991) used gel filtration techniques for the recovery and purification of a virus from the erythrocytes of sea bass.

1. Red blood cells from 40 ml of blood are collected by centrifugation at 2000 g for 20 min and resuspended in 40 ml of TNE (50 mM Tris-HCl, 150 mM NaCl and 1 mM EDTA, pH 7.4) buffer with 0.1% SDS.
2. Samples are freeze–thawed and sonicated three times and clarified by two centrifugation steps at 3000 g and 6000 g for 10 min.
3. The supernatant is concentrated to a final volume of 8–10 ml by ultrafiltration through CX30 immersible units. The complete removal of haemoglobin is accomplished by Sephadex G-100 gel filtration in a 20 × 6.5 cm column, with TNE/0.1% SDS as the eluent.
4. The volume of the first peak of UV light absorbing material to emerge corresponds to the first 10 ml of the void volume. The eluted volume is reconcentrated to 1 ml by ultrafiltration through immersible filters. The presence of virus particles is determined by staining with 2% phosphotungstic acid and electron microscopy. Protein concentration determined by the Lowry's method ranges from 25–250 μg ml$^{-1}$.

Whilst being useful for the purification of whole virus particles, gel filtration can be manipulated so that subviral components can be obtained in a pure form. One such example is work carried out on the oligomeric form of the gp160 glycoprotein of simian immunodeficiency virus. It is possible to purify oligomeric forms of SIV gp160 to greater than 90% purity using a simple gel filtration method (Rhodes et al 1994). Concentrated samples of CHO supernatants from cells expressing gp160 are filtered through Sepharose 6B (Sigma). 0.5 ml volumes from the column are collected and analysed for the presence of gp120/160 by ELISA. These results demonstrated that the major form of sgp160 existed as a 660 K

species (tetramer). Gel filtration was then used to purify this further. To do this, protein from one-litre batches of supernatant was precipitated with 85% ammonium sulphate, resuspended in 30 ml of water and dialyzed for 24 h against 3 changes of PBS. Six-millilitre aliquots (0.5 mg gp160) were separated by gel-filtration chromatography using a Sephacryl S-400HR column (90 × 2.5 cm). Three-millilitre fractions were collected and each screened for gp 160 by ELISA. Fractions containing gp160 were pooled and concentrated by ultrafiltration centrifugation through a Centricon filter with a 10 K size exclusion. Protein was stored at −20°C in 50 mM Tris-HCl pH 7.6.

For further applications of gel filtration techniques for the purification of viral proteins see HSV-1 UL8 protein (Parry et al 1993), Epstein-Barr virus Nuclear Protein 2A (Tsui and Schubach 1994), HTLV1 recombinant protease (Daenke et al 1994), HIV-1 reverse transcriptase (Sharma et al 1994), HIV-2 Nef Protein (Du Bois et al 1993).

# Criteria of purity

When designing a purification protocol it is essential to apply stringent tests to ensure that the virus particles produced are pure. Once the protocol has been tried and tested the purity of each batch of virus should be assessed by at least one criterion.

Protein/particle ratios are good indications of purity. Estimations of particle weight and percent protein allows the calculation of a target protein/particle ratio, e.g. for herpes simplex approximately 13.5 $\mu g/10^{10}$ particles. Whilst such figures are not totally accurate they do give a valid target for which to aim. The observation of particles in the electron microscope, whilst not a good criterion of purity, does allow the detection of 'unwanted structures'.

It would be expected that constituents of the medium would form a major part of the contaminants of purified virus preparations. This can be monitored by gel diffusion tests, where antisera raised against e.g. calf serum, or uninfected cells can be reacted with virus preparation.

Another method designed to detect free antigen contaminants in the purified virus is non-SDS electrophoresis, where pure virus should yield no detectable stained bands moving into the gel.

Comparison of radioactively labeled polypeptide profiles with stained profiles on SDS polyacrylamide gels can also be a useful monitor of purity.

Finally, another method of assaying virus purity is to use prelabeled cells. Cells are labeled with e.g. $^{14}$C-amino acids for a considerable period prior to infection and then chased with unlabeled amino acids. Following infection, infected cells are labeled with $^{3}$H-amino acids. The degree of incorporated label is monitored throughout the purification procedure. This method, again, is a rough guide to the degree of purity.

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