Purification of influenza virions by haemadsorption and ultracentrifugation

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Method Article

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Abstract

It is often useful to concentrate and purify the pleomorphic, enveloped virions of influenza viruses prior to analysis. This protocol describes a standard method for purifying ‘spherical’ influenza virions, the most common morphology of laboratory-adapted strains, by rate-zonal ultracentrifugation through a discontinuous density gradient. The protocol, which takes around eight hours to complete, is designed for virions shed into the growth media of infected cells, and would also be suitable for concentrating virions from the allantoic fluid of embryonated chicken eggs. Two different density gradients are described: a conventional gradient of sucrose and a gradient of iodixanol \(OptiPrep™ density gradient medium). A high degree of virion purity can be achieved, but small quantities of contaminants will remain. These can be removed by an optional haemadsorption-elution \(HAd) step, which takes an additional one and half hours. This allows extremely efficient removal of the original contaminants, but introduces other, distinct contaminants at low levels.

Introduction

When working with influenza viruses, concentration of virions is often a useful step before analysis or further work. A single round of ultracentrifugation may be sufficient for some applications \(for example, layering 0.8 ml of sample onto an 0.5 ml cushion of 33% sucrose in PBS and centrifuging at 91,000 g for 45 min at 4 °C, then resuspending the pellet). However, greater purity can be achieved with a multi-step protocol, as described here. The basic protocol purifies material with specific hydrodynamic properties, and will remove the majority of contaminating material from a solution of influenza virions. We have found it to be suitable for most biochemical studies \(as in ref \(1)). However, the basic protocol will not remove low levels of virion-sized material, such as exosomes, that are present in the sample. Additional purification to remove these can be achieved by a haemadsorption/elution \(HAd) step, which first selects material with sialic acid binding properties \(as provided by the viral haemagglutinin, HA), and then selects material with sialic acid cleaving properties \(as provided by the viral neuraminidase, NA). However, it should be noted that this approach introduces alternative contaminants derived from chicken red blood cells. Details of the proteins present when virus is purified with and without HAd can be found in ref \(2). This protocol is designed for users already familiar with handling influenza viruses. Work with viruses should be performed under suitable conditions \(typically Biological Safety Level II or higher) and in compliance with all local biological safety regulations. Ultracentrifuges should only be used in accordance with the manufacturer’s instructions and buckets, with lids attached, should be balanced to within 10 µg. Thickwall tubes can be disinfected after use by a suitable approved route \(eg detergent soak) followed by several washes in 70% ethanol and then water, and can then be re-used unless they show signs of wear. Care should be taken to thoroughly clean the bottom of the tubes, for example by rubbing with paper towel before further cleaning. Thinwall tubes should not be re-used.

Reagents

Consumables required: • 0.45 µm syringe filters • 50 ml syringes • 50 ml plastic tubes • 15 ml plastic tubes – optional • Thickwall tubes for the SW28 rotor \(polyallomer centrifuge tubes, 25 × 89 mm; Beckman Coulter; 355642) • Thinwall tubes for the SW41 rotor \(polyallomer centrifuge tubes, 14 × 89 mm; Beckman Coulter; 331372) • 5 ml syringe and needle \(21 G or similar) • 1.5 ml tubes • Standard plasticware, pipettes, permanent marker pen Reagents required: • 10× NTC solution \(1 M NaCl, 0.2 M Tris-HCl pH 7.4, 50 mM CaCl2) • sucrose or OptiPrep™ density gradient medium \(Sigma; D1556) • sterile phosphate-buffered saline \(PBS) • ddH2O • adult chicken blood cells in Alsever's \(TCS Biosciences; FB010) - optional

Equipment

• Rotating wheel – optional • Refrigerator • 37 °C water bath • Benchtop centrifuge with refrigeration \(e.g. a Rotanta 460 R, Hettich Lab Technology) • Mass balance • Ultracentrifuge \(Beckman Coulter) • Beckman Coulter SW28 swinging bucket rotor • Beckman Coulter SW41 swinging bucket rotor • Aspiration vessel suitable for infectious material \(e.g. an Integra Vacusafe; optional) • -80 °C freezer

Procedure

\(1) Prepare solutions in advance and store in the refrigerator – all should be used cold. Prepare: a. 1× NTC solution \(from 10× stock). b. Working dilutions for the density gradient. If using sucrose, make 50 ml aliquots of 30, 40, 50 and 60% solutions \(w/v) by adding the required mass of sucrose to 5 ml 10× NTC and topping up with ddH2O. Mix on a rotating wheel until dissolved, then pass through an 0.45 µm syringe filter into a clean 50 ml tube. Mix equal amounts of these solutions in additional tubes to produce 35, 45 and 55% solutions \(w/v). If using OptiPrep™, follow a similar procedure to prepare a 10 – 40% \(w/v) series of iodixanol solutions in NTC \(OptiPrep™ is 60% iodixanol, so 12 ml solutions at 10, 20, 30 and 40% should contain 1.2 ml 10× NTC and 2, 4, 6 and 8 ml OptiPrep™, respectively. Intermediate concentrations \(15, 25 and 35%) can be prepared by mixing, as above.) c. To prepare a step gradient, layer 1.5 ml of each of the seven solutions into a thinwall tube. Starting with the most concentrated solution, use a pipette to slowly add layers, 0.75 ml at a time, taking care not to mix layers \(the interfaces of different concentrations should remain visible). Gradients can be prepared in advance and stored at 4 °C \(some mixing by diffusion is acceptable); if prepared during the purification this should be done during the first ultracentrifugation step. If only one gradient is required a balance can be quickly prepared by mixing 5.25 ml of the most and least concentrated solutions, or using 10.5 ml of the middle concentration. d. If performing HAd, prepare chicken blood. To prevent lysis, blood should be centrifuged at no more than 1,250 g \(around 2,500 rpm if using 15 ml tubes in a benchtop centrifuge, depending on the rotor) at 10 °C; although it should be kept cold it should not be put directly on ice. On receipt cells should be rinsed twice in chilled PBS. Comparison of the cell pellet to liquid in another tube should be used to estimate the volume of cells, and the pellet resuspended in PBS to 20% packed cell volume \(pcv) for storage in the refrigerator. If haemolysis occurs during storage the cells should be rinsed again in PBS and resuspended in an appropriate volume. When about to begin purification, pre-warm an aliquot of PBS

to 37 °C. \(2) Prepare virus to be concentrated. A maximum of 150 ml of virus-containing medium can be prepared without prior HAd. This can be obtained from six 175 cm2 tissue culture flasks which have been seeded with a suitable cell line \(e.g. Madin-Darby Bovine Kidney or Madin-Darby Canine Kidney cells), infected when sub-confluent with an influenza virus at a low multiplicity of infection, and maintained at 37 °C in a humidified incubator for approximately 48 h. \(3) When the virus is ready to harvest, extensive cytopathic effect will be apparent by microscopy. Transfer the media to six 50 ml tubes and centrifuge at 4 °C for 30 min at approximately 2000 g \(around 4,000 rpm, depending on the rotor) to pellet cellular debris. \(4) Transfer the supernatant to six \(thickwall) tubes and place in the buckets of an SW28 rotor. Weigh the buckets \(with the lids on) and transfer media between them as necessary to balance. Centrifuge at 4 °C for 30 min at 18,000 g \(10,000 rpm) in an SW28 rotor to further pellet cellular debris. Retain 1 ml sample for analysis \(optional). If extremely high purity is required \(and chicken red blood cell contaminants are not problematic) perform HAd as described in steps \(3) – \(9). Otherwise, proceed to step \(10). \(5) Optional. Transfer supernatants to six clean, chilled 50 ml tubes and add 325 µl of chicken red blood cells \(20% pcv) to each tube. Mix by gentle inversion and refrigerate for 30 min, inverting regularly. Pellet cells \(1,250 g or around 2,500 rpm depending on the rotor, 5 min, 10 °C). Remove supernatant, retaining 1ml sample for analysis \(optional). \(6) Optional \(cont.). Resuspend all pellet in a single aliquot of 10 ml chilled PBS – to do so, sequentially rinse each tube with the same 5 ml aliquot of PBS, repeat with a second 5 ml aliquot, and pool both aliquots a single 15 ml tube. Pellet cells \(1,250 g or around 2,500 rpm depending on the rotor, 5 min, 10 °C). Remove supernatant, retaining 1ml sample for analysis \(optional). \(7) Optional \(cont.). Resuspend pellet in 10 ml chilled PBS \(some agglutination of cells is likely to be apparent). Pellet cells \(1,250 g or around 2,500 rpm depending on the rotor, 5 min, 10 °C). Remove supernatant, retaining 1ml sample for analysis \(optional). \(8) Optional \(cont.). Resuspend pellet in 10 ml 37 °C PBS, transfer to a clean, room-temperature 50 ml tube and increase the volume to 20 ml with 37 °C PBS. Incubate at 37 °C for 15 min, mixing regularly by gentle inversion. Pellet cells \(1,250 g or around 2,500 rpm depending on the rotor, 5 min, 10 °C), and take 100 µl of the supernatant for analysis \(optional). \(9) Optional \(cont.). Prepare two thickwall tubes with 5 ml chilled 30 % sucrose or 10 % iodixanol. Gently layer the supernatant onto the 5 ml cushion, using gravity flow and taking care not to mix the layers \(the interface should remain visible). Add 20 ml PBS or water to the second tube to balance. Place the tubes in SW28 rotor buckets and weigh with the lids on; adjust the volume of the balance tube as necessary. Proceed to step \(11). \(10) If HAd was not performed, prepare 6 thickwall tubes with 5 ml chilled 30 % sucrose or 10 % iodixanol in each. Gently layer the supernatants onto the 5 ml cushions, using gravity flow and taking care not to mix the layers \(the interface should remain visible). Place the tubes in SW28 rotor buckets and weigh with the lids on; adjust the volumes as necessary to balance across the rotor. \(11) Pellet virions by ultracentrifugation in an SW28 rotor at 4 °C for 90 min at 112,000 g \(25,000 rpm). Aspirate the supernatants, taking care not to disturb the pellet \(which may not be visible). When nearing the bottom of the tube, run the aspiration pipette around the meniscus and tilt away from the pellet to remove the supernatant. Add 50 µl chilled 1× NTC to each tube \(or around 500 µl to the single tube if HAd was used) and resuspend by pipetting up and down. Pool the suspension and layer gently onto the top of a density gradient \(see 1.c, above) taking care not to mix the layers. Place the gradient tube and a balance tube into SW41 rotor buckets and

weigh with the lids on; adjust the volumes as necessary to balance across the rotor. \(12) Pellet virions by ultracentrifugation in an SW41 rotor at 4 °C for 150 min at 209,000 g \(35,000 rpm). Afterwards, the virions should be visible as a 'milky' band of material at around 40% sucrose or 30% iodixanol. Hold the tube against a dark background and use a pen to mark the position of the meniscus and of the virions. Secure the tube and, using a 5 ml syringe and needle, carefully pierce the side of the tube a few millimetres below the virions. While moving the needle tip gently forwards and backwards, withdraw around 1 cm height of the gradient \(1 – 2 ml), including the band of virions. Eject the contents of the syringe into a new thinwall tube containing 9 ml chilled 1× NTC, and rinse out the syringe once in this solution. Place the tube and a balance tube in SW41 rotor buckets weigh with the lids on; adjust the volumes as necessary to balance across the rotor. \(13) Pellet virions by ultracentrifugation in an SW41 rotor at 4 °C for 60 min at 154,000 g \(30,000 rpm). Aspirate the supernatant, taking care not to disturb the pellet. Add around 80 µl chilled 1×NTC and resuspend by pipetting; this should give around 150 µl of a milky solution of purified virions. Reserve around 10% for analysis, and store the rest in a 1.5 ml tube at -80 °C.

Timing

\(1) Solution preparation. When convenient. \(2) Generation of virus. Two days. \(3) Harvesting and clarification. 40 min. \(4) Transfer, balance and clarification. 50 min. \(5) – \(9) HAd \(optional). 90 min. \(10) Layering onto cushions. 15 min. \(11) Ultracentrifugation. 120 min. \(12) Ultracentrifugation. 180 min. \(13) Ultracentrifugation. 80 min. Approximate time for purifications \(steps 3 – 13): 8 hours without HAd, 9 and a half hours with HAd.

Troubleshooting

• Growth of virus can be assessed before beginning by microscopic examination of cells \(step 3). If no cytopathic effect is visible it is unlikely that there will be enough virus to purify. • With the volumes described, concentrated virus should be visible in steps 12 and 13 \(though a dark background may be necessary in step 12, and the band may be faint). Additional bands may appear in step 12 depending on the mixing of the gradient steps and on the morphology of the virus; for most applications the major band can be harvested, but if this is ambiguous the entire gradient can be harvested in aliquots from the top \(step 13 is then not required). • Samples taken throughout the purification can help to identify problems. A number of assays can be used to detect virions, including plaque assay \(to detect fully infectious virions – typically recovery of infectious material is around 10 – 30%) and SDS-PAGE followed by silver-staining \(to assess purity). If chicken blood is being used a quick assay for the presence of virions \(whether infectious or not) can be performed while other steps are being carried out using a modified haemagglutination \(HA) assay. In a 96-well round-bottomed dish add 50 µl PBS to 11 of the 12 columns, leaving the first column empty \(a multi-channel pipette is helpful throughout this assay). In the first column add 100 µl of sample or PBS \(up to eight samples per dish; material from the final step in the purification should only be used if heavily diluted beforehand), and perform twofold serial dilutions of

this by transferring 50 µl into the second column, mixing, changing pipette tip and repeating across the dish. Then add 50 µl chicken red blood cells \(at 1% pcv) to each well, mix and leave to stand, preferably at 4 °C. For each sample the last dilution in which the blood cells agglutinate and cannot settle to the bottom of the well contains one haemagglutinating unit \(HAU). Note that, as this is a modification of the standard assay, the HAU values here are useful only for relative measures and should not be compared to other experiments. Note also that the assay is rather insensitive to low concentrations of virus. Typical end-points are 1/128 for the input material \(in 120 ml) and 1/512 after HAd \(in 20 ml – i.e. around 2/3 recovery of material).

Anticipated Results

The method described should result in a thousand-fold decrease in volume with, typically, a 10 – 30% recovery of infectious material \(plaque-forming units). As some virus will be inactivated during purification the physical recovery of material is likely to be better than this. Electron microscopy shows that virions generally remain intact during purification \(see Fig 1b of ref \(1) and Fig 1b of ref \(2)). Purification without HAd is sufficient to remove the majority of contaminating material, as can be seen by SDS-PAGE and silver staining in Fig 1a of ref \(1) and Fig 1a of ref \(2), and this is likely to be sufficient for the majority of applications. However, as shown in Fig 3b of ref \(2) low levels of contaminants are still present when material is purified in this way. These contaminants can be excluded by HAd, as shown in Fig 3c of ref \(2), though this method introduces additional contaminants derived from chicken red blood cells. Readers interested in the presence of specific protein contaminants after purification with and without HAd are referred to the supplementary data of ref \(2).

References

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