

## Purification of Coronavirus Virions for Cryo-EM and Proteomic Analysis

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### Abstract

Purification of intact enveloped virus particles can be useful as a first step in understanding the structure and function of both viral and host proteins that are incorporated into the virion. Purified preparations of virions can be used to address these questions using techniques such as mass spectrometry proteomics. Recent studies on the proteome of coronavirus virions have shown that in addition to the structural proteins, accessory and non-structural virus proteins and a wide variety of host cell proteins associate with virus particles. To further study the presence of virion proteins, high-quality sample preparation is crucial to ensure reproducible analysis by the wide variety of methods available for proteomic analysis.

**Key words** Coronavirus, Cryo-EM, Proteomic, Virus purification, Density gradient centrifugation

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### 1 Introduction

The most important factor in Cryo-EM and proteomic studies of coronavirus virions is high-quality sample preparation. A useful guideline in planning to make a purified coronavirus sample is that the final preparation should be concentrated to at least  $10^{10}$  virions per milliliter if possible. For a simple proteomic analysis, less is sufficient, but as further digestions and purifications are often performed, higher amounts of infectious virus are recommended to recover enough sample for study, and to make it possible to recover a fairly complete virion proteome [1]. Likewise, the accuracy of cryo-EM results depends on both the concentration and intactness of the purified virions.

This requires a concentration step, since coronavirus growth seldom surpasses  $10^8$  PFU/ml, and can be much lower in some cases. It is also worth noting that cleaved viral spike proteins are sensitive to S1 shedding, though this susceptibility can vary considerably among viruses and even strains of the same viral species. The method of concentration and purification described here seeks to minimize virion disruption and spike loss, and has been used

successfully with several coronaviruses and other enveloped viruses derived from cells and in ovo culture. The method described here is a modification of our previously published method of concentrating samples for electron microscopy, and would still be useful for that purpose [2].

Serum used as part of cell culture medium can act as a carrier, potentially increasing virus yield, but can also lead to clumping and can add to the background of the sample. If purification in the presence of serum fails despite adequate starting virus titer, a serum-free preparation might be advantageous. For this reason an alternative serum-free purification protocol has been provided below. After concentration, but before further proteomics analysis, electron microscopy to look for virion density and spike coverage can be a useful quality control tool. The reliability of results from virion purification will depend on the percentage of cell-derived exosomal vesicles that are co-purified with the virions, although the contributions of exosome-derived proteins can be estimated by performing the same purification in parallel on uninfected cell culture supernatant. It is hoped that these techniques will facilitate further examination of coronavirus, torovirus, arterivirus, mesonivirus, and ronivirus virion proteomics.

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## 2 Materials

### 2.1 Virus Purification and Concentration

1. Vero cells.
2. Virus of interest.
3. DMEM: Dulbecco's Modified Eagle's Medium, 10 % fetal bovine serum, penicillin and streptomycin (100 U/ml each), 10 mM HEPES, adjusted to pH 6.7 using NaOH.
4. Polyethylene glycol-8000 or 10000, white flake type (PEG-8000 Ultra for Molecular Biology) (*see Note 1*).
5. NaCl, crystalline, high quality.
6. HEPES-saline: 0.9 % NaCl (w/v), 1 mM HEPES, pH adjusted to 6.7 using HCl, vacuum-sterilized through a 0.22  $\mu\text{m}$  pore size filter.
7. 3 $\times$  HEPES-saline: 2.7 % NaCl, 30 mM HEPES, pH adjusted to 6.7 using HCl.
8. 50 % (w/w) sucrose: 50 g sucrose, 50 ml HEPES-saline, vacuum-sterilized through a 0.22  $\mu\text{m}$  pore size membrane. Dilute with additional HEPES-saline to prepare 10, 20, and 30 % sucrose solutions.
9. If samples are to be inactivated by chemical fixation, prepare 25 % neutral buffered formalin: 10 ml of formalin (37–40 % formaldehyde), 5 ml of 3 $\times$  HEPES-saline.

10. Centrifuges and rotors: A low-speed centrifuge rotor with a capacity  $\geq 1$  l (Sorvall GSA or GS-3, for example) and a high-speed centrifuge rotor with a total capacity  $\geq 100$  ml (SW32.1 Ti, Beckman-Coulter, for example).
11. Tracking dye: 10 $\times$  SYBR-gold dye (Life Technologies, sold as 10,000 $\times$ ), which will bind ssRNA in virions readily, and can be used to locate your virus pellet, and to distinguish the viral component of complex pellets that contain impurities with different sedimentation rates.

### **2.2 Serum-Free Virus Purification and Concentration**

1. Virus production serum-free medium (VP-SFM; Life Technologies) supplemented with penicillin and streptomycin (100 U/ml each).
2. All other reagents and equipment as in Subheading 2.1.

### **2.3 Quality Control EM**

1. Formvar/carbon coated 200-mesh or 300-mesh EM grids.
2. Negative stain: 2 % uranyl acetate in water, pH adjusted to 6.5 with 1 M NaOH, filtered freshly through a 0.22  $\mu$ m hole size membrane immediately before use, stored in a brown glass bottle away from light.
3. Parafilm M.
4. Fine forceps for EM grid manipulation.
5. Filter paper.

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## **3 Methods**

Since the quality of the virus preparation is the most important component of proteomics studies, two purification protocols are listed below. Either can yield high-quality coronavirus, but Subheading 3.1 is generally preferable because the serum proteins function as a “carrier” during the PEG precipitation step. Serum-free purification (Subheading 3.2) can be used with cell and virus combinations that tend to produce overly viscous purified virions, or as a means to reduce the complexity of “background” proteins before proteomics analysis. For best results, the purification process should be completed in 1 day, and the virus should be used immediately.

### **3.1 Virus Purification and Concentration**

This method is suitable for most coronaviruses that grow well in cultured cells, and has been used successfully with severe acute respiratory syndrome-coronavirus (SARS-CoV), feline coronavirus (FCoV) and mouse hepatitis virus (MHV), coronavirus virus-like particles, and torovirus in addition to several types of influenzavirus, arenavirus, and retrovirus-like particles [3, 4]. In the case of infectious bronchitis virus (IBV), this method has been used

successfully for the purification of virus from embryonated chicken eggs. For the purpose of this protocol, it is assumed that IBV is being prepared on Vero cells.

1. Culture Vero cells in DMEM to approximately 70–90 % confluency (*see Note 2*).
2. Inoculate with IBV at a multiplicity of infection of three or more.
3. Remove the inoculum after 1 h and replace with fresh medium.
4. Remove and discard the culture medium 24 h after inoculation. Replace with fresh DMEM (*see Note 3*).
5. Collect the cell culture supernatant 48 h after inoculation. Store a small sample for plaque assay titration (*see Note 4*).
6. Prepare three-step 10–20–30 % sucrose gradients in tubes appropriate to the high-speed centrifuge rotor. If using the Beckman SW-32 Ti rotor, use ~8 ml for each step, leaving ~10 ml for sample loading and balancing. Pipette the 10 % sucrose into the bottom of the tube. Refill the pipette with 1 ml more sucrose solution than you will need, tilt the tube as much as possible without spilling the sample and place the pipette tip just above the bottom of the tube. Dispense the 20 % sucrose very slowly using the gravity-only setting—the last 1 ml will be retained in the pipette. Carefully and slowly withdraw the pipette, and put the 30 % sucrose layer underneath the 20 and 10 % layers in the same way. There will be visible lines at the border between steps if this is done correctly (*see Note 5*).
7. Transfer the supernatant to the largest available screw-cap centrifuge bottles that will fit your rotor, noting the total volume. Pellet cellular debris at  $10,000\times g$ , 4 °C for 20 min. It is best to use a high-capacity rotor at this stage (Sorvall GSA, for example) to minimize preparation time.
8. During the centrifugation, prepare fresh screw-cap centrifuge bottles containing 10 g of dry PEG-8000 and 2.2 g of NaCl per 100 ml of culture medium to be added. Alternatively, prepare a large conical flask with sufficient PEG-8000, NaCl, and a heavyweight magnetic stir bar to bring the entire volume of virus-containing medium to a final concentration of 10 % PEG-8000, 2.2 % NaCl.
9. Chill HEPES-saline and neutral-buffered formalin on ice for later use (*see Note 6*).
10. After centrifugation (**step 7**), an off-white or yellow pellet of cell debris will be visible. Quickly decant the supernatant into the centrifuge bottles or conical flask prepared earlier with PEG and NaCl (*see Note 7*).

11. Swirl the PEG-8000/NaCl/supernatant mixture gently until the PEG crystals are fully dissolved. This can be done by hand for individual bottles, or using a magnetic stirring plate which has been incubated in a 4 °C incubator or cold room.
12. Add a clean stir bar if one is not already present and incubate at 4 °C for a further 30 min with gentle stirring.
13. Transfer the solution to centrifuge bottles, if necessary. Pellet the PEG-precipitated protein, which will also contain the virus, by centrifugation for 30 min at approximately 10,000×*g* at 4 °C.
14. Decant and discard supernatants *immediately* to minimize the amount of virus that is lost by resuspending. A large opaque white pellet should be present in each of the flasks following centrifugation, and may run from the bottom to top of one side if you used a fixed-angle rotor.
15. Swirl each pellet by hand in 1–3 ml of cold HEPES-saline until dissolved. Avoid passing the sample through a pipette at this step, if possible. It is critical that the PEG pellets be completely resuspended before proceeding to the next step. The resuspended pellet will be viscous if this step is done correctly (*see Note 8*).
16. Optionally, add one-tenth the volume of each pellet of Tracking dye. This can be left for 10 min with the resuspended PEG pellets, and will penetrate virions to fluorescently label the RNA inside without any additional permeabilization. This will make it possible to locate the virus-containing fraction or section of the pellet in any subsequent step, simply by resting the tube in a bottomless tube rack or clear beaker and illuminating the sample briefly with a UV transilluminator. This step is useful for troubleshooting the purification procedure.
17. Tilt the tube containing your gradient as much as possible without spilling, to allow the resuspended pellet to run as slowly as possible down the side of the tube and onto the top of the 10 % gradient layer. It is important not to disturb the gradient layers at this stage. In this manner, overlay the resuspended PEG pellet carefully onto the sucrose gradients. Balance with remaining sample or additional HEPES-saline.
18. Pellet the virions through the sucrose cushions by centrifugation at 100,000×*g* for at least 90 min at 4 °C. Since the pellet will be compact, it is not important to brake the centrifuge slowly.
19. After centrifugation, decant and discard supernatants immediately. Invert the empty tubes on an absorbent surface for 5 min, and tap gently to wick away any remaining sucrose solution that may have gathered near the rim of the centrifuge tube.

20. Resuspend the virion pellet in as small a volume of HEPES-saline as possible (typically 100  $\mu$ l for a small pellet and 200  $\mu$ l for a large pellet). Do not use a pipette to resuspend the virus, as this may shear spikes and damage fragile viral envelopes (*see Note 9*).
21. When the pellet has been resuspended, the HEPES-saline will turn somewhat opaque and milky in color. Use a P-1000 pipette tip from which the pointed end has been cut off to gently transfer the virus suspension to a cryovial with minimal shearing. Discard any insoluble material that remains, as most of the authentic virus will resuspend quickly and easily in comparison. Set aside a small sample (typically 5–10 % of your sample for a diagnostic plaque assay).
22. At this stage, the virus should be monodisperse, and can be formalin inactivated if desired. Treatment with a 1 % final concentration of ice-cold neutral buffered formalin, 2-propiolactone treatment, or gamma-irradiation should all yield intact, inactivated EM-quality particles. Samples for cryo-EM may be stored at 4 °C for up to 24 h, but should not be frozen for storage. Purified virus for mass spectrometry should not be formalin fixed, but can instead be inactivated using the solvent that will be used for mass spectrometry, provided this has been validated for your virus (*see Note 10*).

### **3.2 Serum-Free Virus Purification and Concentration**

This alternative method is suitable for purification of viruses that grow to lower titers. Serum-free culture and preparation can also be used to remedy solutions that fail for cryo-EM or proteolysis due to high viscosity, non-viral protein contamination, or large amounts of insoluble material. Percentage recovery will typically be lower than that with Subheading 3.1.

1. Perform **steps 2–15** of Subheading 3.1 as above, substituting VP-SFM for DMEM starting at the time of inoculation (*see Note 11*).
2. The PEG-protein pellets should be white, and may be quite small and susceptible to resuspending quickly upon standing for even a few minutes. Decant and discard the supernatants immediately. Resuspend by swirling gently in 10 ml HEPES-saline (*see Note 12*).
3. Perform **steps 16–22** of Subheading 3.1 as above. The final translucent pellet may be small and quite difficult to see, but the presence of the virus can be confirmed using the Tracking dye and a transilluminator after removing the supernatant, if the dye was added at **step 16** of Subheading 3.1.

### **3.3 Quality Control for EM Analysis**

The number of infectious virus particles in the final preparation should be directly assessed by plaque assay or similar means as a retrospective measure of quality. The quickest way to assess sample

quality is by EM of a negatively stained sample. Ideal samples for proteomics or cryo-electron microscopy should contain a high density of virions with intact spikes, and relatively little non-viral material. This protocol describes how to prepare a negatively stained coronavirus specimen for transmission-EM.

1. Lay down a piece of paper towel on a work surface that is designated for work with slightly radioactive materials. Label the paper towel with the names of your samples.
2. Lay a piece of Parafilm M large enough to cover your labels, backing side facing up, on top of the paper towel and separate the backing from the Parafilm M at one corner. Trace lines through the backing paper on a piece of Parafilm M using the back of a pair of EM forceps to create channels for droplets of stain or wash buffer. The places where lines intersect will naturally hold droplets, and the labels will help keep the samples organized.
3. Peel off the rest of the backing paper, leaving the Parafilm stuck to the paper towel. On the Parafilm, place one ~20  $\mu$ l droplet of 2 % uranyl acetate and up to three droplets of HEPES-saline wash buffer for each sample. Highly viscous samples will give a cleaner appearance after one or more wash steps, but washing is not necessary if the sample is quite pure (*see Note 13*).
4. Place 2–5  $\mu$ l of the viral sample onto each grid, and leave each sample for 5 min to allow the sample to adsorb onto the grid.
5. If you are washing your sample, balance each grid on top of each droplet of HEPES-saline for 30 s. Do not reuse buffer or stain droplets, to avoid sample contamination. In between droplets, dry the grids. Hold the grid perpendicular to a clean piece of filter paper and touch the edge of the grid to the paper. Most of the wash buffer should be carried off.
6. After the washing steps, float each grid on a uranyl acetate stain droplet for 1 min. Longer staining will lead to more particles that appear to fill up with stain, if this is desired.
7. Touch the edge of the grid to the filter paper several times to remove excess stain (*see Note 14*).
8. Samples should be visualized immediately, and can be best stored for longer periods in a grid box that is kept under a vacuum.
9. Ideal preparations for both proteomics and cryo-electron microscopy will contain monodisperse virions as opposed to clumps of virions, few smooth-walled exosomal vesicles, little or no stringy released ribonucleoprotein, and will have virions covering roughly one-tenth of the grid surface area.

### **3.4 Quality Control for Proteomic Analysis**

1. If possible, western blot analysis should be performed against the structural proteins of the virus to confirm the presence of viral proteins in the final preparation.
2. If infectivity of the virus is important, the number of infectious virus particles in the final preparation should be directly assessed by plaque assay or similar means as a retrospective measure of quality.
3. Coomassie and silver stained SDS-PAGE can also be performed to show the complexity of the sample. As recent proteomic studies have shown, the presence of host cell proteins associated with coronavirus virions is to be expected, but a prominent band between 40 and 50 kDa is indicative of N protein.

### **3.5 Proteomic Analysis**

1. Protease digestion of purified virions can be used to remove proteins outside the coronavirus virion. This should be optimized for each virus and each protease individually. Proteinase K is common, and following digestion, repurification by pelleting through a 30 % sucrose cushion can remove the unwanted proteinase K. It should be noted that after digestion, removal of extra-virion proteins would lighten the density of the virion and therefore a longer centrifugation is required to repurify virus particles.
2. A wide variety of methods are available to study the coronavirus proteome. Gel based methods are still common and virions purified by these methods are compatible with gel based analysis. Direct LC-MS analysis of coronavirus virions is also possible. Success has been achieved by lysing the virions using Rapigest™ (Waters) followed by alkylation, reduction, and tryptic digestion before MS.

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## **4 Notes**

1. Larger molecular weight polyethylene glycol preparations tend to be too heterogeneous to give the best results.
2. Assuming an average IBV cell culture titer of  $\sim 10^6$ – $10^7$  PFU/ml, and a desired total of  $\sim 10^{10}$  infectious particles, preparations should be made to produce between 1 and 10 l of cell culture medium. One-well plates (available from Nalgene) can be a cost- and space-effective alternative to standard tissue culture flasks for large-scale virus preparation. Due to the simplicity of the proteome of the allantoic fluid of embryonated chicken eggs and the high titer of IBV that can be recovered, lower volumes of starting material can be expected.
3. Early time points containing little virus can be discarded. Both sample purity and virus recovery are dependent on concentration.



4. Virus can be collected at 2 h intervals beginning 48 h after inoculation to increase the final yield. However, for best results, each virus sample should be processed immediately. In general, IBV inoculated into embryonated chickens eggs can be harvested from 16 h after inoculation, but can only be harvested at a single time point.
5. Virus can also be banded at the interface of a 30–50 % two-step sucrose gradient. The use of lower-concentration sucrose cushions can be used to study wild-type virions and virus particles containing defective mini-genomes that are of lower buoyant density [5].
6. For viruses with cleaved spike proteins, such as most strains of IBV, the amount of NaCl added should be reduced to 1 g/100 ml to minimize damage to the spikes.
7. Decanting quickly reduces the likelihood that some components of the pellet will resuspend. Ideally pellets should be separated from supernatants within 1 min of the end of centrifugation.
8. In general, rapid resuspension, cold temperature, and the minimization of mechanical stress will all improve the quality of the preparation. Soluble proteins contained in the pellet may alter the color to a yellowish hue.
9. The translucent virion pellet may be difficult to see and will probably not be visible before the supernatant is removed. The presence of the pellet can be confirmed by fluorescence under UV light after the supernatant is decanted, if the tracking dye was used. If a pellet is present, the viscosity of the added HEPES-saline will increase noticeably upon resuspension.
10. Inactivation techniques should be validated beforehand. Both formaldehyde and 2-propiolactone can lose effectiveness over time. Amines in some buffers such as Tris-HCl will react with formaldehyde, which is why HEPES buffer is recommended for use throughout the purification process.
11. The growth of SARS-CoV, FCoV and MHV is not affected by short-term treatment with VP-SFM, as outlined here. However, cells do grow more slowly in VP-SFM as compared to DMEM, and thus VP-SFM is not recommended for the initial cell culture step.
12. If left in contact with the supernatant, serum-free PEG pellets will resuspended much more quickly than serum-containing pellets, and the sample may be lost. It is therefore very important to decant the supernatants quickly with serum-free pellets.
13. Alternatively, the sample can be applied as a droplet on which the grid is floated for 5–10 min. Grids that continually sink in the saline or stain droplets likely have suffered extensive damage to the support surface and should not be used.

14. The effects of the electron beam on large amounts of residual stain can cause a “blowout” of the carbon-formvar support surface. It is therefore important to remove all excess uranyl acetate before visualization. If there is excess stain present, you will notice because the grid will stick to the forceps. If this happens, give the grid one more blot by gently touching the face of the grid containing the sample directly onto the filter paper, dry the forceps with another piece of filter paper, and try again.

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