Purification and proteomics of influenza virions

Edward C. Hutchinson[1]* and Monika Stegmann[2]

*Edward.Hutchinson@glasgow.ac.uk

[1]MRC-University of Glasgow Centre for Virus Research

[2]University of Oxford Advanced Proteomics Facility

Running Head: Virion Purification and Proteomics

Abstract

This chapter describes a basic workflow for analysing the protein composition of influenza virions. In order to obtain suitable material, the chapter describes how to concentrate influenza virions from the growth media of infected cells and to purify them by ultracentrifugation through a density gradient. This approach is also suitable for purifying influenza virions from the allantoic fluid of embryonated chicken eggs. As a small quantity of microvesicles are co-purified with virions, optional steps are included to increase the stringency of purification by enriching material with viral receptor binding and cleaving activity. Material purified in this way can be used for a variety of downstream applications, including proteomics. As a detailed example of this, the chapter also describes a standard workflow for analysing the protein composition of concentrated virions by liquid chromatography and tandem mass spectrometry.

Key Words

Influenza, Virion, Ultracentrifugation, Mass spectrometry, LC-MS/MS, MaxQuant, Proteomics

1 Introduction

1.1 Virion concentration – a simple solution for simple assays

This chapter describes how to concentrate and purify influenza virions, and then describes a straightforward workflow for identifying and quantifying all of the proteins they contain in a "bottom-up" proteomics approach.

Detailed characterisation of influenza virions typically requires that they be concentrated. For some experiments purity is not an issue, and in such case a single round of ultracentrifugation can be very effective. As an example of how to do this (as shown in [1]):

1. Clarify virus-containing medium or allantoic fluid either by 0.4-µm filtration or by centrifugation at top speed in a microcentrifuge (ca. 13,000 rpm) for 5 min.

2. Layer 0.8 mL of the clarified sample onto an 0.5 mL cushion of 33% sucrose in PBS in an ultracentrifuge tube.

3. Centrifuge at 91,000×g for 45 min at 4 °C in an ultracentrifuge.

4. Aspirate the supernatant and resuspend the pellet in a suitable volume of buffer.

While this approach is quick and straightforward, it will result in the co-purification of considerable amounts of contaminating material. For applications which detect specific components of the virion (e.g. qRT-PCR of viral gene segments, or Western blotting for individual viral proteins) such contamination may not be a concern. In other applications, particularly "omics" approaches which are not limited to a single target, contamination can be a serious problem. This chapter therefore presents methods for both concentrating and purifying virions. This can be used to prepare material for mass spectrometry, as described below, as well as for a wide range of other contamination-sensitive techniques.

1.2 Virion purification

Influenza virions can be purified in a multi-step ultracentrifugation protocol using a density gradient. However, even this approach cannot completely remove contaminants, and without additional increases in stringency the purified virions will

be mixed with low but detectable levels of material whose protein composition is suggestive of ribosomes and microvesicles [2]. Microvesicles such as exosomes are shed by most host cells and due to their hydrodynamic radius and density they sediment at a similar position to influenza virions. To address this, this chapter presents two variants of the purification method which allow the stringency of purification to be adjusted (Fig. 1a, b).

Firstly, there is the option of purifying virions through a gradient of iodixanol (OptiPrep™ Density Gradient Medium, Sigma) rather than sucrose. This has been reported to separate virions slightly from co-purifying microvesicles [3]. Secondly, chicken red blood cells (RBCs), which bind to but do not take up virus, can be used to purify material with both viral receptor binding and cleaving activities. This haemadsorption (HAd) step is very efficient at removing contaminants from the initial sample, but does introduce additional contaminants from the RBCs themselves and so is not suitable for all purposes [2]. Material purified as described should be suitable for detailed proteomic analysis. Detailed descriptions of the stringency of purification with and without haemadsorption can be found in [2].

The multi-step approach described here was optimised for purifying virions of laboratory-adapted influenza strains, which typically have a spherical morphology, and is adapted from [4]. In principle it could be adapted to separate out filamentous forms of the virus, but while this has been reported, it is often challenging in practice [5, 6]. Readers interested in adapting the purification methods described here to other viruses may wish to consult an online series of ultracentrifugation protocols, including mini-reviews of methods for a wide variety of viruses, maintained at the time of writing by the density gradient medium manufacturer Axis-Shield (http://www.axis-shield-density-gradient-media.com/methodology.htm). Before

undertaking any work with viruses or ultracentrifugation, readers should familiarise themselves with the safety considerations for such work, as summarized in **Note 1**.

1.3 Virion proteomics

1.3.1 A simple proteomics workflow

A wide variety of approaches are available for proteomic analysis, but in recent years mass spectrometry has become particularly powerful (reviewed in [7]). This chapter outlines a simple three-part workflow for determining the proteome of virions by mass spectrometry.

First, sections 2.2 and 3.2 describe sample preparation, specifically how to prepare tryptic peptides from virions.

Next, sections 2.3 and 3.3 outline the process of liquid chromatography and tandem mass spectrometry (LC-MS/MS) using a Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific). Despite the growing popularity of mass spectrometry methods, it is still likely that most readers will send samples to a specialist facility for analysis. In addition, those readers already operating a mass spectrometer will not need to look to this chapter for guidance on its use. Instead, this chapter will outline a standard set of conditions suitable for influenza virion proteomics, so that readers who do perform their own mass spectrometry can confirm that the conditions they are using are appropriate, and readers who out-source this stage of analysis can gain a more detailed understanding of what is involved.

Finally, sections 2.4 and 3.4 present a standard data analysis workflow for protein identification and quantitation using the open-source platform MaxQuant. More sophisticated analyses of the data are also possible, for example to identify

sites of post-translational modification. For details of these, the reader is directed to a detailed recent protocol article on the various uses of MaxQuant [8].

The principles underlying LC-MS/MS, as well as the principles behind sample preparation for this method and the analysis of mass spectra, are briefly explained below.

1.3.2 Principles of LC-MS/MS

There are a wide variety of approaches for obtaining and analysing mass spectra. The approach set out here has the following key features:

- It is a bottom-up approach, which is to say that instead of analysing intact proteins (top-down proteomics) it analyses peptides generated by enzymatic digest. This approach is experimentally and computationally easier to implement [7].

- First, the peptides are separated by reversed-phase chromatography (liquid chromatography, LC). As they elute from the chromatography column, they are ionised and transferred into the vacuum of a mass spectrometer by electrospray ionisation (ESI) [9].

- In the instrument, peptide ions are captured and focused using magnetic fields. The method described here uses data-dependent acquisition (DDA). Under this method, ions that co-elute from the LC column are initially collected and their mass spectra are recorded (full scan, or MS[1] spectra). Before the composition of the eluting ions changes, the most abundant of these 'precursor' ions are sequentially selected from the LC column output using a quadrupole mass filter, fragmented using high-energy collisions, and the mass spectra of their 'fragment' ions also recorded (MS/MS, or MS[2] spectra) to

provide detailed information on the precursor ion's composition. The instrument cycles between mass analysis of precursor ions and fragment ions, with each MS^1 scan followed by a user-defined number of MS^2 scans (in the 'top 10' approach described here, ten MS^2 scans) [7, 9].

- The Q Exactive instrument described here detects mass spectra using an Orbitrap mass analyser. In an Orbitrap, ions are injected along a tangent into the cavity between two co-axial electrodes, the inner electrode being spindle-shaped. Ions orbit rapidly around the inner electrode at a frequency proportional to their mass to charge (m/z) ratio, inducing a current. A Fourier transform is performed on this output to determine the frequencies and amplitudes of the currents induced by each species of ion, which can then be used to calculate their m/z ratios and abundance, respectively [9, 10].

1.3.3 Principles of sample preparation by FASP

Bottom-up proteomics requires that proteins in the sample are digested into peptides. Trypsin is the most commonly used enzyme for generating peptides for mass spectrometry analysis. It has high proteolytic activity, cleaves specifically after lysine and arginine, unless followed by a proline, and the peptides generated have an optimal length and charge for analysis. However, trypsin digestion is rarely complete. For improved digestion of complex or difficult-to-digest samples, prior to trypsin digestion proteins can be digested with lysyl endopeptidase Lys-C, which cleaves after lysine regardless of the following residue. Lys-C proteolysis can occur in denaturing conditions of up to 6 M urea, allowing the digestion of proteolytically resistant proteins.

A number of protocols are available for digesting proteins with trypsin, including digests of proteins in solution and in small sections of polyacrylamide gels. In this chapter we describe a procedure for filter-aided sample preparation (FASP), which uses a 10 kDa molecular weight cut-off (MWCO) filter to retain undigested proteins during washing [11]. This offers a number of advantages including the ability to wash out residual detergents, to remove non-protein components such as nucleic acids, and to bypass precipitation steps which are associated with substantial protein losses. Avoiding contamination is critical at this stage, and the guidelines in **Note 2** should be carefully adhered to.

1.3.4 Principles of proteomic data analysis

A number of software platforms are available for identifying and quantifying proteins from mass spectra. Here, we provide a brief protocol for using MaxQuant, a widely-used open-source platform for the analysis of mass spectrometry-based proteomics. A detailed description of this powerful system has recently been published in a protocols paper, and the reader is referred to this for technical notes and further details of its capabilities [8].

Briefly, MaxQuant identifies proteins by matching mass spectra to a user-supplied list of proteins. An *in silico* digest is performed to predict the peptide sequences that could be generated from these proteins, and their predicted mass spectra are calculated (both for the precursor ions and the fragment ions). Peptide spectral matches are then made between experimentally observed mass spectra and these theoretical spectra, allowing proteins in the user's list to be identified. The false-discovery rate is controlled by a target-decoy database strategy. For this, a "decoy" database is generated by reversing the order of amino acids in the user-

supplied protein list and generating theoretical peptide mass spectra from that. These "decoy" spectra are also matched to the experimental data, and the stringency of acceptable matches is adjusted until only a small proportion of the protein groups identified (typically <1%) are from the decoy database.

2 Materials

2.1 Materials for virion concentration and purification

2.1.1 Equipment for virion concentration and purification

- Rotating wheel – optional.

- Gradient Master (BioComp Instruments) – optional.

- Refrigerator.

- 37 °C water bath.

- Benchtop centrifuge with refrigeration (e.g. a Rotanta 460 R, Hettich Lab Technology).

- Mass balance.

- Ultracentrifuge (e.g. Beckman Coulter).

- Beckman Coulter SW28 swinging bucket rotor or equivalent.

- Beckman Coulter SW41 swinging bucket rotor or equivalent.

- Aspiration vessel suitable for infectious material (e.g. an Integra Vacusafe) – optional.

- -80 °C freezer.

2.1.2 Consumables for virion concentration and purification

- 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 microfuge tubes.

- Standard plasticware, pipettes, permanent marker pen.

2.1.3 Reagents for virion concentration and purification

- Virus-containing growth medium or allantoic fluid (see section 3.1.2, steps 2-3).

- 10× NTC: 1 M NaCl, 0.2 M Tris-HCl pH 7.4, 50 mM $CaCl_2$.

- Sucrose, or OptiPrep™ density gradient medium (Sigma; D1556).

- Phosphate-buffered saline (PBS).

- H_2O: HPLC-grade water can be used, though double-distilled water can also be used provided detergent contamination is scrupulously avoided (*see* **Note 2**). Tap water should not be used.

- RBCs: adult chicken blood cells in Alsever's solution (TCS Biosciences; FB010) – optional.

2.2 Materials for filter-aided sample preparation (**FASP)**

2.2.1 Equipment for FASP

- Microcentrifuge.

- Vacuum centrifuge with cold trap for volatile organics (e.g. Thermo Scientific, SPD111V).

- Spectrophotometer (set to 595 nm) and cuvettes for Bradford assay.

2.2.2 Consumables for FASP

- 10,000 MWCO filter units (Vivacon 500, VN01H02).

2.2.3 Reagents for FASP

- For all buffers and solvents listed in this section use ultrapure water.

- Bradford assay kit, for example Bio-Rad Protein Assay (500-0001).

- ABC: Ammonium bicarbonate.

- TFA: Trifluoroacetic acid.

- TCEP: Tris(2-carboxyethyl)phosphine hydrochloride.

- IAA: Iodoacetamide. Note: IAA is light sensitive.

- ACN: Acetonitrile.

- Lysis Buffer: 8 M urea and cOmplete protease inhibitor cocktail (Sigma) in 100 mM ABC.

- Wash Buffer I: 8 M urea in 100 mM ABC.

- Wash Buffer II: 6 M urea in 25 mM ABC.

- Lys-C: Lysyl Endopeptidase (Wako).

- Sequence grade-modified trypsin (Promega).

2.3 Materials for LC-MS/MS

2.3.1 Equipment required for LC-MS/MS

- Trap column: 5 mm, PepMap RSLC, C18, 300 μm I.D. particle size 3 μm (Thermo Scientific).

- C18 reversed-phase capillary column: 50 cm, PepMap RSLC, EASY-spray column, C18, 75 μm I.D. particle size 3 μm (Thermo Scientific).

- Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific) with a Dionex UltiMate™ 3000 HPLC (Thermo Scientific).

2.3.2 Consumables for LC-MS/MS

- V-bottom 96-well plate.

2.3.3 Reagents for LC-MS/MS

- ACN: Acetonitrile.

- FA: Formic acid.

- DMSO: Dimethyl sulfoxide.

- Loading buffer: 5% FA, 5% DMSO.

- Mobile phase A: 0.1% FA, 5% DMSO.

- Mobile phase B: 100% ACN, 0.1% FA, 5% DMSO.

2.4 Materials for Data Analysis

2.4.1 Equipment for Data Analysis

- A personal computer: MaxQuant is typically run with parallel execution on multiple cores, with no upper limit to the number of cores used provided the disk performance scales up with it. Each core should have at least 2 GB RAM. In addition to sufficient disk space to store the raw data files, at least half as

much space should be available for temporary files generated during calculations.

2.4.2 Software for Data Analysis

- Operating System: Windows (64-bit) with .NET Framework 4.5 or higher, or Windows emulation software if using Mac computers. Linux is not currently supported.

- MaxQuant: MaxQuant can be downloaded from http://www.maxquant.org (free registration required). This chapter describes the use of version 1.5.8.3.

- MSFileReader, a standard library used to access raw files from Thermo instruments. At the time of writing, MaxQuant has been most thoroughly tested with MS File reader 3.0 SP2. Free registration is required at https://thermo.flexnetoperations.com/control/thmo/RegisterMemberToAccount after which it can be downloaded from https://thermo.flexnetoperations.com/control/thmo/login.

- Data analysis software: The data generated here can be analysed with standard software such as Microsoft Excel or R (https://www.rstudio.com/). However, particularly for more complex analyses the reader is directed to Perseus, a freely-available programme developed to analyse proteomics data from MaxQuant (http://www.perseus-framework.org).

3 Methods

3.1 Method for virion concentration and purification

3.1.1 Timings for virion concentration and purification

1. Solution preparation: when convenient.

2. Generation of virus: two to three days (*see* **Notes 3 and 4**).

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.

3.1.2 Protocol for virion concentration and purification

An overview of the standard protocol is given in Fig. 1a and of the haemadsorption
(HAd) protocol in Fig. 1b.

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.

      (i) If pouring the gradient manually, to use sucrose, make 50 mL
      aliquots of 30, 40, 50, and 60% solutions (w/v) by adding the required
      mass of sucrose to 5 mL of 10× NTC and topping up with ddH$_2$O. Mix
      on a rotating wheel until dissolved, then pass through a 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 of 10× NTC and 2, 4, 6, and 8 mL OptiPrep™, respectively). Intermediate concentrations (15, 25, and 35%) can be prepared by mixing, as above.

(ii) If mixing the gradients using a Gradient Master, only the highest and lowest densities listed above are required.

c. Pouring the density gradient (*see* **Note 5** for optimisation).

(i) To manually 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). **This is most easily done by tilting the tube to 45° and pipetting gently into the meniscus on the far (overhanging) wall of the tube** (the same position is shown being used to overlay media on to a cushion in Fig 2a, b). Gradients can be prepared in advance and stored at 4 °C (some mixing by diffusion is acceptable); if prepared during the purification this can be done during the first ultracentrifugation step.

(ii) Alternatively, a continuous gradient can be rapidly prepared using a Gradient Master (BioComp), if available. This tilts a stoppered tube containing two densities of solution until their interface runs diagonally from the meniscus to the bottom edge of the tube, then rotates around an axis parallel with the tube to mix the two solutions together. To do this, place the thinwall tube in the correct holder and use the rim of the

holder to mark the halfway point with a pen, fill with the upper (least dense) medium up to the mark, then use a syringe with a long cannula to gently underlay with the lower (most dense) medium until the interface reaches the mark. Then stopper the tube, place on the Gradient Master, and tilt to 80° before rotating for 2 min at 18 rpm (for 30 – 60% sucrose) or for 50 s at 20 rpm (for 10 – 40% iodixanol). (iii) 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 simply 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) **and at no colder than 10 °C**. Although the blood should be kept chilled (e.g. in a fridge) it should not be put directly on ice. On receipt, cells should be rinsed twice in chilled PBS. Comparison of the cell pellet to a measured volume of liquid in an equivalent 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 (visible by the PBS becoming pink) 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 (*see* **Note 4**). A maximum of 150 mL of virus-containing medium can be prepared without prior HAd. This can be obtained from six 175-cm^2 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, depending on the strain. Alternatively, cells can be seeded into roller bottles, which allow a larger surface area of cells to be maintained in a smaller volume of medium (e.g. 40 mL of medium in an 850-cm^2 bottle).

3. When the virus is ready to harvest, extensive cytopathic effect is likely be apparent by microscopy (though this is somewhat strain-dependent, and for some strains prior determination of the optimal growth conditions may be required; *see* **Note 3**). Transfer the media to six 50-mL tubes and centrifuge at 4 °C for 10 min at approximately 2,000× *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 10 min at 18,000×*g* (10,000 rpm) in an SW28 rotor (or equivalent) 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 5 – 9. Otherwise, proceed to step 10.

5. *Optional.* Transfer supernatants to six clean, chilled 50-mL tubes and add 325 µL of RBCs (20% pcv) to each tube. Mix by gentle inversion and refrigerate for 30 min, inverting regularly. Pellet cells with bound virus (1,250×*g* or around

2,500 rpm depending on the rotor, 5 min, 10 °C). Remove and discard supernatant, retaining 1 mL sample for analysis (optional).

6. *Optional (cont.).* Resuspend all six cell pellets 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 into a single 15-mL tube. Pellet cells (1,250×*g* or around 2,500 rpm depending on the rotor, 5 min, 10 °C). Remove and discard supernatant, retaining 1 mL of sample for analysis (optional).

7. *Optional (cont.).* Resuspend pellet in 10 mL of chilled PBS (some agglutination of cells may be apparent). Pellet cells (1,250×*g* or around 2,500 rpm depending on the rotor, 5 min, 10 °C). Remove and discard supernatant, retaining 1 mL 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), retain supernatant and take 100 µL 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) (*see* **Note 6**). Add 20 mL of PBS or water to the second tube to balance. Place the tubes in SW28 rotor buckets (or equivalent) 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 of chilled 30% sucrose or 10% iodixanol in each. As shown in Fig. 2a–c, 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) (*see* **Note 6**). Place the tubes in SW28 rotor buckets (or equivalent) and weigh with the lids on; adjust the volumes as necessary to balance across the rotor.

11. Pellet virions by ultracentrifugation at 4 °C for 90 min at 112,000×*g* (25,000 rpm). As shown in Fig. 2d, aspirate the supernatants, taking care not to disturb the pellet (which may not be visible, *see* **Note 7**). When nearing the bottom of the tube, run the pipette around the meniscus and tilt the tube to separate the supernatant from the pellet. As shown in Fig. 2e, add 50 µL of 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 (*see* **Note 7**). Pool the suspension in a clean 1.5 mL microfuge tube and then, as shown in Fig. 2f, layer it gently onto the top of a density gradient (see step 1c, above) taking care not to mix the layers. Place the gradient tube and a balance tube into SW41 rotor buckets (or equivalent) and weigh with the lids on; adjust the volumes as necessary to balance across the rotor.

12. Pellet virions by ultracentrifugation 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 (Fig. 2g). Illuminating the gradient from above and holding the tube against a dark background may make the band easier to see. **Use a pen to mark the position of the virions and of the meniscus, then mark a point below the virions where the tube will be punctured and a point below the meniscus to indicate how much fluid**

should be withdrawn (around 1 cm of the gradient height, or 1 – 2 mL).

Prepare an autoclavable sharps disposal bin, secure the tube above a waste pot and, using a 5-mL syringe and needle, carefully pierce the side of the tube a few millimeters below the virions, with the bevelled opening of the needle facing upwards. While moving the needle tip gently forwards and backwards, withdraw fluid from the gradient, including the band of virions, using the pen mark below the meniscus as a guide to the amount taken (Fig. 2h). Withdraw the needle and quickly transfer the pierced tube into the waste pot. As shown in Fig. 2i, eject the contents of the syringe into a new thinwall tube containing 9 mL of chilled 1× NTC, and rinse out the syringe once in this solution. Transfer the needle and syringe immediately to the sharps bin. Place the tube and a balance tube in SW41 rotor buckets (or equivalent) and weigh with the lids on; adjust the volumes as necessary to balance across the rotor.

13. Pellet virions by ultracentrifugation 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 of 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.

3.1.3 Anticipated results of virion concentration and purification

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 total recovery of material is likely to be better than this. Electron microscopy shows that virions generally remain intact during purification (Fig. 1c) and SDS-PAGE and silver staining of a small proportion

(around 1 – 5%) of the recovered material should show good recovery of viral proteins (Fig. 1d) [1, 11]. For guidance on troubleshooting, *see* **Note 8**.

Purification without HAd is sufficient to remove the majority of contaminating material, as can be seen by SDS-PAGE and silver staining [2, 12], and this is likely to be sufficient for the majority of applications. However, low levels of contaminants are still present when material is purified in this way [2]. These contaminants can be excluded by HAd, though this method introduces additional contaminants derived from chicken red blood cells [2]. Readers interested in the presence of specific protein contaminants after purification with and without HAd are referred to the supplementary tables of [2].

3.2 Method for FASP

1. Lyse purified virions by mixing 1:1 with Lysis Buffer and incubating for 30 min at room temperature, vortexing occasionally. As well as rendering the samples suitable for analysis, this will render the virus non-infectious. **Do not heat above room temperature**; this accelerates carbamylation of proteins and is unnecessary for inactivating influenza viruses. If a substantial insoluble pellet remains, detergent may be added (e.g. SDS), but this will increase the number of washing steps required for FASP and should be kept to a minimum. **Never use polyethylene glycol (PEG) for inactivating the virus** (*see* **Note 2**).

2. Clear lysate (15,000×*g*, 15 min) and collect the protein-containing supernatant.

3. Take an aliquot and estimate the protein concentration by Bradford assay. If dilution is necessary, dilute the aliquot, not the total lysate. This assumes the

lysate is free of detergent, as recommended. If not, a BCA assay should be used.

4. Assemble the filter units (Fig. 3a, b) and wash them with 200 µL of 0.1% TFA in 50% ACN (14,500× *g*, 15 min). There should be no more than a thin film of liquid remaining on the filter after centrifugation. Increase the centrifugation time if necessary.

5. Discard flow-through from the collection tube.

6. Equilibrate the filter units by spinning through 200 µL of Wash Buffer I.

7. Load the lysate onto the filter by spinning it through (Fig. 3c). Note that:
   a. Proteins with a MW greater than 10 kDa will be retained on the filter, but smaller proteins will be lost.
   b. A maximum of 400 µg of total protein can be loaded per filter, but if no further downstream peptide enrichment or fractionation is carried out (neither is required for the standard virion analysis) aim to digest 10-20 µg lysate.
   c. Multiple loading steps may be required if the volume exceeds 400 µL.
   d. High amounts of proteins can increase the centrifugation time.

8. To reduce the sample with 10 mM TCEP, add 4 µL of 0.5 M TCEP into 200 µL of Wash Buffer I, and add to the filter and incubate for 30 min at room temperature (Fig. 3d).

9. To alkylate the protein with 50 mM IAA, further add 20 µL of 0.5 M IAA and incubate for another 30 min at room temperature (Fig. 3d). Keep the samples in the dark for this step as IAA is light sensitive (*see* **Note 9**).

10. Spin to remove the reduction and alkylation buffer, and discard this.

11. Detergent-containing samples need to be washed extensively with 200 µL Wash Buffer I until the flow-through no longer forms bubbles when shaken.

12. Wash the filter with 200 µL of Wash Buffer II to reduce the urea concentration to 6 M.

13. Repeat step 12.

14. Change to a fresh collection tube for peptide collection.

15. To digest the lysate with Lys-C, add 100 µL of Wash Buffer II containing Lys-C at a final Lys-C:protein ratio of 1:40 (w/w), and incubate for 1–3 h at 37 °C.

16. To digest the lysate with trypsin, add 300 µL of 25 mM ABC to dilute the urea to 1.5 M, including trypsin at a final trypsin:protein ratio of 1:40 (w/w). Digest overnight at 37 °C.

17. Harvest peptides by spinning them through the filter (Fig. 3e). Retain the peptide-containing flow-through and pool with the flow-through from subsequent steps 18 and 19.

18. Wash remaining peptides off the filter with 200 µL of 0.1% FA and retain.

19. Repeat step 18 with 200 µL of 0.1% FA in 50% ACN. Pool flow-through from steps 17, 18, and 19.

20. Dry down peptides in a vacuum centrifuge until the solution has evaporated.

3.3 Method for LC-MS/MS

1. Reconstitute the peptides in loading buffer and add the sample to a V-bottom 96-well plate.

2. Place the plate in the Dionex UltiMate™ 3000 autosampler (Fig. 3f).

3. Inject sample. Peptides should be desalted on-line by a trap column, separated on an analytical C18 reversed-phase capillary column (flow rate:

200 nL/min, gradient: 7–30% (v/v) mobile phase B over 60 min, column

temperature: 45 °C) and analyzed by the Q Exactive Hybrid Quadrupole-

Orbitrap mass spectrometer (Fig. 3g).

4. Acquire data in data-dependent acquisition (DDA) mode, automatically

switching from MS to collision induced dissociation MS/MS on the top 10 most

abundant ions with a precursor scan range of 350 – 1650 m/z.

5. Reject 1+ charge states.

6. Acquire full scan MS spectra at a resolution of 70,000 and MS/MS scans at

17,000 at a target value of 3×10^6 and 1×10^5 ions respectively.

7. Enable dynamic exclusion with an exclusion duration of 40 s.

3.4 Method for data analysis

3.4.1 Preparation for data analysis

1. To analyse the virion proteome supply the viral proteome, the host proteome

and, if HAd was used, the chicken proteome. The host and chicken (*Gallus*

gallus) proteomes can be downloaded from UniProt

(http://www.uniprot.org/proteomes/). Proteome IDs of organisms commonly

used to grow influenza virus in culture are:

Host Organism	Proteome ID (UniProt)
Human (*Homo sapiens*)	UP000005640
Chicken (*Gallus gallus*; from chicken RBCs and infected eggs)	UP000000539
Dog (*Canis lupus familiaris*; from MDCK cells)	UP000002254
Cow (*Bos taurus*; from MDBK cells)	UP000009136

Download proteomes by navigating to the relevant page on UniProt and selecting "Download all protein entries / FASTA (canonical)."

2. To generate a viral proteome write a FASTA file. This can be done simply on any text editor. Readers unfamiliar with the format are referred to **Note 10**. Users will often already know the sequence of their virus. If not, it can often be found through searching online repositories such as the NCBI Influenza Virus Resource (https://www.ncbi.nlm.nih.gov/genome/viruses/variation/flu/) or the Global Initiative on Sharing All Influenza Data (GISAID; platform.gisaid.org/), though be aware that viral strains typically acquire mutations during passage, and any peptides which do no match precisely to the supplied sequence will not be identified. To date, the only viral proteins to have been identified in influenza virions are PB2, PB1, PA, HA, NP, NA, M1, M2, NS1 and NEP [2]. However, the virus encodes other proteins, both experimentally confirmed and predicted, and the user may wish to search for these as well. As an example of this, and as an example of the FASTA format, the proteome of the commonly used laboratory strain influenza A/Puerto Rico/8/1934 (H1N1) virus is given in **Note 11**.

3. Next, configure the database so that MaxQuant can read it. To do so, open MaxQuant and go to the Configuration/Sequence databases tab. Click "Add" to add a new database configuration (many standard databases are already configured). This will create a new line at the bottom of the database table, and create editable settings on the right. Use "Fasta file name" to select your fasta file (do not change its name, as it is required for MaxQuant to recognize it). If you wish you may specify the source and taxonomy of the file. Clicking

arrows will fill in the taxonomy name from the taxonomy number, and *vice versa*. Importantly, you must insert the correct identifier parse rule to allow MaxQuant to extract information from the headers (sequence names) in your fasta file. These are written using regular expressions, and can either be copied from one of the existing entries in the sequence configuration table, entered using the "Select" buttons next to each rule, or copied from this table:

Identifier parse rule	Meaning
>(.*)	Everything after the ">". Includes the full header supplied for any fasta sequence, creating a rather cluttered output table but including gene names, *etc.*, as well as accessions.
>([^])	Everything after the ">" up to the first space (suitable for the custom virus database in **Note 11**, as is the rule above).
>IPI:([^|\.]*)	IPI accession.
>(gi|[0-9]*)	NCBI accession.
>([^\t]*)	Everything after the ">" up to the first tab.
>.*|(.*)|	Uniprot ID (suitable for the UniProt databases suggested above).

To check the entry is correct, click "Test rules" and use the button to download entries from your fasta file to check that, at a minimum, the Header, Sequence and Identifier can be correctly read.

4. To save your changes, first click "Modify Table" (to transfer changes from the editing box on the right to the table on the left) then click "Save changes". Finally, close MaxQuant for the changes to take effect.

3.4.2 Protocol for Data Analysis

1. Open MaxQuant. Most default settings will be suitable, but the following points are either experiment-dependent or important for understanding the analysis.

2. Set the number of threads (bottom left) to the number of files to be searched or the highest number supported by your computer, whichever is lower.

3. Click the Raw Files / Load button and load raw files for each virus to analyse. If loading more than one raw file, set an experimental design using the buttons in the Raw Files tab. For analysing separate sets of virions it may be simplest to set each raw file as a separate experiment ("No fractions").

4. In the Group Specific Parameters / General tab the Type should be 'Standard' and the Multiplicity '1' (assuming no labelling strategy, such as SILAC or tandem mass tagging, has been used). 'Variable Modifications' (post-translational modifications (PTMs) which are only occasionally found) should include Acetyl (Protein N-term) and Oxidation (M). Other PTMs can be added, including biologically relevant ones such as phospho (STY) or artifactual ones such as deamidation (NQ) by selecting the modification on the left-hand menu and using the arrow button to add it to the list on the right. Note that increasing the number of PTMs simultaneously searched for will significantly

increase processing time. Digestion mode should be Specific, with the enzyme Trypsin/P (*i.e.* tryptic (KR) cleavage sites, including sites followed by proline that would be cleaved by Lys-C) and a maximum number of missed cleavages of 2.

5. In the Group Specific Parameters / Label-Free Quantitation tab, label-free quantitation should be set to 'LFQ.' This will allow relative protein abundance to be estimated based on the intensity with which peptide ions were detected in the instrument, incorporating several layers of normalisation [13].

6. In the Global Parameters / General tab, upload the FASTA files for your host and viral proteomes (these can be uploaded as multiple separate files). MaxQuant will also include a list of common contaminants in the search provided the relevant box is ticked. The Fixed Modifications list (PTMs applying to all relevant amino acids) should contain only 'Carbamidomethyl (C).'

7. In the Global Parameters / Label-Free Quantitation tab, select iBAQ. This enables intensity-Based Absolute Quantitation, an algorithm which adjusts peptide intensities based on the number of theoretically observable peptides from each protein [14]. LFQ provides better comparison between samples due to its normalisation steps, while iBAQ is a better indicator for absolute protein abundance [15].

8. Press 'Start' (bottom left). Depending on the computer and number of samples the programme may take some hours to run (days for larger experiments or slower machines).

9. MaxQuant will typically write a large number of output files to the directory the raw files are in. For identifying and quantifying proteins, the most relevant files

are tab-delimited tables written as text files in the subdirectory …/combined/txt. In particular, the file proteinGroups.txt contains information about the proteins identified. These files can be opened in MaxQuant's Perseus package, or in a general purpose spreadsheet program such as Microsoft Excel.

10. The proteinGroups file presents rows of 'protein groups,' or proteins that could be reconstructed from a common set of peptides. Of the many output columns in the proteinGroups file, the following are most likely to be of interest during an initial examination of the data:

   a. Protein IDs. Lists the proteins in the protein group, ranked by the number of peptides matched to each and separated by semicolons. Proteins with at least half as many peptides matched as the leading protein are also listed in the Majority Protein IDs column.

   b. Some protein groups should be disregarded, either because they are found in a list of common contaminants or because they are matches to the reversed decoy database. The columns 'Potential contaminant' and 'Reverse' mark these proteins, whose names in the Protein IDs column will also start with CON and REV, respectively. Proteins marked in the column 'Only identified by site' have only been identified by a modification site, and the user may choose to disregard these as well.

   c. Fasta Headers returns the original descriptions of the proteins.

   d. Peptide counts (all) gives the number of peptides matched to each member of the protein group.

   e. The Molecular Weight can be useful for some calculations.

f. The LFQ Intensity returns the LFQ score and iBAQ returns the iBAQ score.

3.4.3 Anticipated results of data analysis

As an example of the results that may be obtained, a sample of influenza A/Puerto Rico/8/1934 (H1N1) virions was grown on MDCK (canine) cells and purified without haemadsorption. This has been described in a previous publication [2] and the raw data file C130708_055.raw may be downloaded from the Mass spectrometry Interactive Virtual Environment (MassIVE; Center for Computational Mass Spectrometry at University of California, San Diego) at http://massive.ucsd.edu/ProteoSAFe/datasets.jsp using the MassIVE ID MSV000078740. A copy of MaxQuant was downloaded and configured to analyze the data, as described above, using the UniProt dog proteome described above (downloaded on the 25th April 2017) and the viral proteome described in **Note 11**.

The proteinGroups.txt file contained 653 proteins groups. Of these, 11 were only identified by site, 7 were from the decoy database and 12 were potential contaminants, with some overlap between these categories. Removing these left a list of 10 viral proteins and 615 host proteins (most of very low abundance). The abundance of these proteins is summarised in Fig. 4, using the relative iBAQ (riBAQ) score – a normalised abundance score obtained by dividing each protein's iBAQ score by the total iBAQ scores of all proteins under consideration [16].

4 Notes

1. Safety Considerations: This protocol is written for influenza viruses which can be handled at Advisory Committee on Dangerous Pathogens Containment

Level 2 / Biological Safety Level 2 (specifically, for low-pathogenicity strains of influenza virus), and assumes that users are familiar with the safe handling of such viruses. Changes to the protocol would be required if a highly-pathogenic strain of influenza virus was to be used. In all cases, work should be carried out in compliance with all applicable local and national biological safety regulations.

Ultracentrifuges should only be used in accordance with the manufacturer's instructions and full buckets, with lids attached, should be balanced across the rotor to within 0.1 g using a mass balance. **This is often easiest if a lightweight pot or rack is placed on the balance and used to hold the bucket.**

Thickwall centrifuge tubes can be re-used unless they show signs of wear (check particularly around the joint between the straight and curved sections). After use they should be disinfected by an approved route (e.g. soaking in 1% Virkon™) followed by several washes in water, then in 70% ethanol, and finally by wiping out thoroughly with paper towel, paying particular attention to the bottom of the tube. Thinwall centrifuge tubes should not be re-used.

2. Avoiding Contaminants: Mass spectrometry is an extremely sensitive approach which can detect even minute levels of contaminants. Two in particular regularly cause problems.

   a. Keratin. Human skin, hair and the resulting dust make keratin a ubiquitous contaminant – it is, as one researcher put it, always snowing in the lab. It is almost impossible to exclude keratin, but it can be minimised by keeping plasticware covered when not in use, working wherever possible in a

laminar flow hood or microbiological safety cabinet, wearing and frequently

changing gloves, and avoiding the use of communal chemical stocks.

b. Polyethylene glycol (PEG). This widely-used compound is strongly ionised

in the mass spectrometer and causes a repeating 44 Da signal that can

swamp informative mass spectra. PEGylated detergents include

commercial soaps, Tween, SDS, Triton X-100 and NP-40. Even minute

quantities of PEG can compromise an experiment, and while some PEG

can be removed by washing during sample preparation for mass

spectrometry it should be avoided wherever possible. Disposable

plasticware should be used where possible. When making buffers, do not

re-use glassware that could previously have contained PEG and ensure

any traces of soap have been washed off the glass with hot water followed

by a rinse with an organic solvent such as acetonitrile. Finally, do not store

buffers containing organic solvents in plastic containers, as this can leach

PEG from the plastic.

3. Virus Growth: Growth of virus can be assessed before beginning by

microscopic examination of cells. For laboratory-adapted strains if no

cytopathic effect (i.e. cell rounding or detachment) is visible it is unlikely that

there will be enough virus to purify, though if working with an unfamiliar strain

it is worth optimising growth conditions before proceeding to the purification.

4. Quantities of Virus: For a well-adapted laboratory strain such as PR8 or WSN,

we typically find that around 10^9 PFU ($10^{10} - 10^{11}$ particles) of unpurified stock

will give a clear band on a gradient as in section 3.1.2, steps 12 and 13.

However the band may be faint and a dark background may be necessary in

step 12. 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 by pipetting from the meniscus (step 13 is then not required).

5. Gradient Mixing: If gradient mixing needs to be checked a test gradient can be prepared in which one or more of the densities contain a dye such as Coomassie Brilliant Blue.

6. As when pouring a step gradient, this can be easier to do if pipetting into the meniscus on the far (overhanging) side of a tilted tube (see Fig 2 a,b).

7. Visibility of Pellets: Pellets may not be visible before the final concentration step (section 3.1.2, step 11) but can still be resuspended by pipetting. The final concentrated stock of virus should appear at least faintly milky. Methods for resuspending the pellet can vary. The author (EH) pipettes rapidly up and down twenty times, moving the tip around the area of the pellet while gently touching the bottom of the tube and taking care not to introduce bubbles.

8. Troubleshooting: 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 and protein yield; Fig. 1c). If chicken blood is available 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**:

a. 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).

b. In the first column add 100 µL of sample or PBS and perform twofold serial dilutions of this by transferring 50 µL into the second column, mixing, changing pipette tip and repeating across the dish. Up to eight samples can be assayed per dish; material from the final step in the purification is highly concentrated and so should only be used if heavily diluted beforehand, for example by 100-fold.

c. Add 50 µL chicken red blood cells (at 1% pcv) to each well, mix and leave to stand, preferably at 4 °C.

d. 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 directly compared to other HA assays. Note also that the assay is rather insensitive to low concentrations of virus. Typical end-points (for a laboratory-adapted virus) 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).

9. Spurious Ub sites: If the data are likely to be searched for sites of ubiquitination (not described in detail here, but a straightforward modification to the MaxQuant search is described in [8]), the iodoacetamide (IAA) used in the alkylation step should be substituted for a different alkylating agents such as chloroacetamide. This is because in addition to alkylating cysteines as

intended, iodoacetamide can alkylate lysine residues. This can create a 2-acetamidoacetamide covalent adduct with the same atomic composition as the diglycyl tag which remains after tryptic cleavage of covalently-bound ubiquitin. Reducing with IAA can therefore lead to the identification of spurious ubiquitination sites [17].

10. The FASTA file format: This is simply a text file in the format below. The file extension should be unimportant, but if required the file extension of a plain text file can be changed to '.fasta'.

>NAME_1

sequence 1

>NAME_2

sequence 2

>NAME 3

sequence 3

11. The proteome of Influenza A/Puerto Rico/8/1934 virus in FASTA format, based on the reverse genetics virus with GenBank Accession numbers EF467817 - EF467824 [18]:

>PB2

MERIKELRNLMSQSRTREILTKTTVDHMAIIKKYTSGRQEKNPALRMKWMMA

MKYPITADKRITEMIPERNEQGQTLWSKMNDAGSDRVMVSPLAVTWWNRN

GPITNTVHYPKIYKTYFERVERLKHGTFGPVHFRNQVKIRRRVDINPGHADLS

AKEAQDVIMEVVFPNEVGARILTSESQLTITKEKKEELQDCKISPLMVAYMLE

RELVRKTRFLPVAGGTSSVYIEVLHLTQGTCWEQMYTPGGEVRNDDVDQS

LIIAARNIVRRAAVSADPLASLLEMCHSTQIGGIRMVDILRQNPTEEQAVDICK

AAMGLRISSSFSFGGFTFKRTSGSSVKREEEVLTGNLQTLKIRVHEGYEEFT

MVGRRATAILRKATRRLIQLIVSGRDEQSIAEAIIVAMVFSQEDCMIKAVRGDL

NFVNRANQRLNPMHQLLRHFQKDAKVLFQNWGVEPIDNVMGMIGILPDMTP

SIEMSMRGVRISKMGVDEYSSTERVVVSIDRFLRIRDQRGNVLLSPEEVSET

QGTEKLTITYSSSMMWEINGPESVLVNTYQWIIRNWETVKIQWSQNPTMLY

NKMEFEPFQSLVPKAIRGQYSGFVRTLFQQMRDVLGTFDTAQIIKLLPFAAA

PPKQSRMQFSSFTVNVRGSGMRILVRGNSPVFNYNKATKRLTVLGKDAGTL

TEDPDEGTAGVESAVLRGFLILGKENKRYGPALSINELSNLAKGEKANVLIGQ

GDVVLVMKRKRDSSILTDSQTATKRIRMAIN

>PB2-S1

MERIKELRNLMSQSRTREILTKTTVDHMAIIKKYTSGRQEKNPALRMKWMMA

MKYPITADKRITEMIPERNEQGQTLWSKMNDAGSDRVMVSPLAVTWWNRN

GPITNTVHYPKIYKTYFERVERLKHGTFGPVHFRNQVKIRRRVDINPGHADLS

AKEAQDVIMEVVFPNEVGARILTSESQLTITKEKKEELQDCKISPLMVAYMLE

RELVRKTRFLPVAGGTSSVYIEVLHLTQGTCWEQMYTPGGEVRNDDVDQS

LIIAARNIVRRAAVSADPLASLLEMCHSTQIGGIRMVDILRQNPTEEQAVDICK

AAMGLRISSSFSFGGFTFKRTSGSSVKREEEVLTGNLQTLKIRVHEGYEEFT

MVGRRATAILRKATRRLIQLIVSGRDEQSIAEAIIVAMVFSQEDCMIKAVRGDL

NFVNRANQRLNPMHQLLRHFQKDAKVLFQNWGVEPIDNVMGMIGILPDMTP

SIEMSMRGVRISKMGVDEYSSTERVVPLHQSKVECSSPHLL

>PB1

MDVNPTLLFLKVPAQNAISTTFPYTGDPPYSHGTGTGYTMDTVNRTHQYSE

KGRWTTNTETGAPQLNPIDGPLPEDNEPSGYAQTDCVLEAMAFLEESHPGI

FENSCIETMEVVQQTRVDKLTQGRQTYDWTLNRNQPAATALANTIEVFRSN

GLTANESGRLIDFLKDVMESMNKEEMGITTHFQRKRRVRDNMTKKMITQRT

MGKKKQRLNKRSYLIRALTLNTMTKDAERGKLKRRAIATPGMQIRGFVYFVE

TLARSICEKLEQSGLPVGGNEKKAKLANVVRKMMTNSQDTELSFTITGDNTK

WNENQNPRMFLAMITYMTRNQPEWFRNVLSIAPIMFSNKMARLGKGYMFE

SKSMKLRTQIPAEMLASIDLKYFNDSTRKKIEKIRSLLIEGTASLSPGMMMGM

FNMLSTVLGVSILNLGQKRYTKTTYWWDGLQSSDDFALIVNAPNHEGIQAG

VDRFYRTCKLLGINMSKKKSYINRTGTFEFTSFFYRYGFVANFSMELPSFGV

SGINESADMSIGVTVIKNNMINNDLGPATAQMALQLFIKDYRYTYRCHRGDT

QIQTRRSFEIKKLWEQTRSKAGLLVSDGGPNLYNIRNLHIPEVCLKWELMDE

DYQGRLCNPLNPFVSHKEIESMNNAVMMPAHGPAKNMEYDAVATTHSWIP

KRNRSILNTSQRGVLEDEQMYQRCCNLFEKFFPSSSYRRPVGISSMVEAMV

SRARIDARIDFESGRIKKEEFTEIMKICSTIEELRRQK

>PB1-N40

MDTVNRTHQYSEKGRWTTNTETGAPQLNPIDGPLPEDNEPSGYAQTDCVL

EAMAFLEESHPGIFENSCIETMEVVQQTRVDKLTQGRQTYDWTLNRNQPAA

TALANTIEVFRSNGLTANESGRLIDFLKDVMESMNKEEMGITTHFQRKRRVR

DNMTKKMITQRTMGKKKQRLNKRSYLIRALTLNTMTKDAERGKLKRRAIATP

GMQIRGFVYFVETLARSICEKLEQSGLPVGGNEKKAKLANVVRKMMTNSQD

TELSFTITGDNTKWNENQNPRMFLAMITYMTRNQPEWFRNVLSIAPIMFSNK

MARLGKGYMFESKSMKLRTQIPAEMLASIDLKYFNDSTRKKIEKIRSLLIEGT

ASLSPGMMMGMFNMLSTVLGVSILNLGQKRYTKTTYWWDGLQSSDDFALI

VNAPNHEGIQAGVDRFYRTCKLLGINMSKKKSYINRTGTFEFTSFFYRYGFV

ANFSMELPSFGVSGINESADMSIGVTVIKNNMINNDLGPATAQMALQLFIKDY

RYTYRCHRGDTQIQTRRSFEIKKLWEQTRSKAGLLVSDGGPNLYNIRNLHIP

EVCLKWELMDEDYQGRLCNPLNPFVSHKEIESMNNAVMMPAHGPAKNMEY

DAVATTHSWIPKRNRSILNTSQRGVLEDEQMYQRCCNLFEKFFPSSSYRRP

VGISSMVEAMVSRARIDARIDFESGRIKKEEFTEIMKICSTIEELRRQK

>PB1-F2

MGQEQDTPWILSTGHISTQKREDGQQTPKLEHRNSTRLMGHCQKTMNQVV

MPKQIVYWRRWLSLRNPILVFLKTRVLKRWRLFSKHE

>PA

MEDFVRQCFNPMIVELAEKTMKEYGEDLKIETNKFAAICTHLEVCFMYSDFH

FINEQGESIIVELGDPNALLKHRFEIIEGRDRTMAWTVVNSICNTTGAEKPKFL

PDLYDYKENRFIEIGVTRREVHIYYLEKANKIKSEKTHIHIFSFTGEEMATKAD

YTLDEESRARIKTRLFTIRQEMASRGLWDSFRQSERGEETIEERFEITGTMR

KLADQSLPPNFSSLENFRAYVDGFEPNGYIEGKLSQMSKEVNARIEPFLKTT

PRPLRLPNGPPCSQRSKFLLMDALKLSIEDPSHEGEGIPLYDAIKCMRTFFG

WKEPNVVKPHEKGINPNYLLSWKQVLAELQDIENEEKIPKTKNMKKTSQLK

WALGENMAPEKVDFDDCKDVGDLKQYDSDEPELRSLASWIQNEFNKACEL

TDSSWIELDEIGEDVAPIEHIASMRRNYFTSEVSHCRATEYIMKGVYINTALLN

ASCAAMDDFQLIPMISKCRTKEGRRKTNLYGFIIKGRSHLRNDTDVVNFVSM

EFSLTDPRLEPHKWEKYCVLEIGDMLIRSAIGQVSRPMFLYVRTNGTSKIKM

KWGMEMRRCLLQSLQQIESMIEAESSVKEKDMTKEFFENKSETWPIGESPK

GVEESSIGKVCRTLLAKSVFNSLYASPQLEGFSAESRKLLLIVQALRDNLEPG

TFDLGGLYEAIEECLINDPWVLLNASWFNSFLTHALS

>PA-N155

MATKADYTLDEESRARIKTRLFTIRQEMASRGLWDSFRQSERGEETIEERFE

ITGTMRKLADQSLPPNFSSLENFRAYVDGFEPNGYIEGKLSQMSKEVNARIE

PFLKTTPRPLRLPNGPPCSQRSKFLLMDALKLSIEDPSHEGEGIPLYDAIKCM

RTFFGWKEPNVVKPHEKGINPNYLLSWKQVLAELQDIENEEKIPKTKNMKKT

SQLKWALGENMAPEKVDFDDCKDVGDLKQYDSDEPELRSLASWIQNEFNK

ACELTDSSWIELDEIGEDVAPIEHIASMRRNYFTSEVSHCRATEYIMKGVYIN

TALLNASCAAMDDFQLIPMISKCRTKEGRRKTNLYGFIIKGRSHLRNDTDVVN

FVSMEFSLTDPRLEPHKWEKYCVLEIGDMLIRSAIGQVSRPMFLYVRTNGTS

KIKMKWGMEMRRCLLQSLQQIESMIEAESSVKEKDMTKEFFENKSETWPIG

ESPKGVEESSIGKVCRTLLAKSVFNSLYASPQLEGFSAESRKLLLIVQALRDN

LEPGTFDLGGLYEAIEECLINDPWVLLNASWFNSFLTHALS

>PA-N182

MASRGLWDSFRQSERGEETIEERFEITGTMRKLADQSLPPNFSSLENFRAY

VDGFEPNGYIEGKLSQMSKEVNARIEPFLKTTPRPLRLPNGPPCSQRSKFLL

MDALKLSIEDPSHEGEGIPLYDAIKCMRTFFGWKEPNVVKPHEKGINPNYLL

SWKQVLAELQDIENEEKIPKTKNMKKTSQLKWALGENMAPEKVDFDDCKDV

GDLKQYDSDEPELRSLASWIQNEFNKACELTDSSWIELDEIGEDVAPIEHIAS

MRRNYFTSEVSHCRATEYIMKGVYINTALLNASCAAMDDFQLIPMISKCRTK

EGRRKTNLYGFIIKGRSHLRNDTDVVNFVSMEFSLTDPRLEPHKWEKYCVLE

IGDMLIRSAIGQVSRPMFLYVRTNGTSKIKMKWGMEMRRCLLQSLQQIESMI

EAESSVKEKDMTKEFFENKSETWPIGESPKGVEESSIGKVCRTLLAKSVFNS

LYASPQLEGFSAESRKLLLIVQALRDNLEPGTFDLGGLYEAIEECLINDPWVL

LNASWFNSFLTHALS

>PA-X

MEDFVRQCFNPMIVELAEKTMKEYGEDLKIETNKFAAICTHLEVCFMYSDFH

FINEQGESIIVELGDPNALLKHRFEIIEGRDRTMAWTVVNSICNTTGAEKPKFL

PDLYDYKENRFIEIGVTRREVHIYYLEKANKIKSEKTHIHIFSFTGEEMATKAD

YTLDEESRARIKTRLFTIRQEMASRGLWDSFVSPREEKRQLKKGLKSQEQC

ASLPTKVSRRTSPALKILEPMWMDSNRTATLRASCLKCPKK

>HA

MKANLLVLLCALAAADADTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDS

HNGKLCRLKGIAPLQLGKCNIAGWLLGNPECDPLLPVRSWSYIVETPNSENG

ICYPGDFIDYEELREQLSSVSSFERFEIFPKESSWPNHNTNGVTAACSHEGK

SSFYRNLLWLTEKEGSYPKLKNSYVNKKGKEVLVLWGIHHPPNSKEQQNLY

QNENAYVSVVTSNYNRRFTPEIAERPKVRDQAGRMNYYWTLLKPGDTIIFEA

NGNLIAPMYAFALSRGFGSGIITSNASMHECNTKCQTPLGAINSSLPYQNIHP

VTIGECPKYVRSAKLRMVTGLRNNPSIQSRGLFGAIAGFIEGGWTGMIDGWY

GYHHQNEQGSGYAADQKSTQNAINGITNKVNTVIEKMNIQFTAVGKEFNKLE

KRMENLNKKVDDGFLDIWTYNAELLVLLENERTLDFHDSNVKNLYEKVKSQL

KNNAKEIGNGCFEFYHKCDNECMESVRNGTYDYPKYSEESKLNREKVDGV

KLESMGIYQILAIYSTVASSLVLLVSLGAISFWMCSNGSLQCRICI

>NP

MASQGTKRSYEQMETDGERQNATEIRASVGKMIGGIGRFYIQMCTELKLSD

YEGRLIQNSLTIERMVLSAFDERRNKYLEEHPSAGKDPKKTGGPIYRRVNGK

WMRELILYDKEEIRRIWRQANNGDDATAGLTHMMIWHSNLNDATYQRTRAL

VRTGMDPRMCSLMQGSTLPRRSGAAGAAVKGVGTMVMELVRMIKRGINDR

NFWRGENGRKTRIAYERMCNILKGKFQTAAQKAMMDQVRESRNPGNAEFE

DLTFLARSALILRGSVAHKSCLPACVYGPAVASGYDFEREGYSLVGIDPFRLL

QNSQVYSLIRPNENPAHKSQLVWMACHSAAFEDLRVLSFIKGTKVLPRGKL

STRGVQIASNENMETMESSTLELRSRYWAIRTRSGGNTNQQRASAGQISIQ

PTFSVQRNLPFDRTTIMAAFNGNTEGRTSDMRTEIIRMMESARPEDVSFQG

RGVFELSDEKAASPIVPSFDMSNEGSYFFGDNAEEYDN

>NA

MNPNQKIITIGSICLVVGLISLILQIGNIISIWISHSIQTGSQNHTGICNQNIITYKN

STWVKDTTSVILTGNSSLCPIRGWAIYSKDNSIRIGSKGDVFVIREPFISCSHL

ECRTFFLTQGALLNDKHSSGTVKDRSPYRALMSCPVGEAPSPYNSRFESVA
WSASACHDGMGWLTIGISGPDNGAVAVLKYNGIITETIKSWRKKILRTQESE
CACVNGSCFTIMTDGPSDGLASYKIFKIEKGKVTKSIELNAPNSHYEECSCYP
DTGKVMCVCRDNWHGSNRPWVSFDQNLDYQIGYICSGVFGDNPRPEDGT
GSCGPVYVDGANGVKGFSYRYGNGVWIGRTKSHSSRHGFEMIWDPNGWT
ETDSKFSVRQDVVAMTDWSGYSGSFVQHPELTGLDCMRPCFWVELIRGRP
KEKTIWTSASSISFCGVNSDTVDWSWPDGAELPFSIDK
>M1
MSLLTEVETYVLSIIPSGPLKAEIAQRLEDVFAGKNTDLEVLMEWLKTRPILSP
LTKGILGFVFTLTVPSERGLQRRRFVQNALNGNGDPNNMDKAVKLYRKLKR
EITFHGAKEISLSYSAGALASCMGLIYNRMGAVTTEVAFGLVCATCEQIADSQ
HRSHRQMVTTTNPLIRHENRMVLASTTAKAMEQMAGSSEQAAEAMEVASQ
ARQMVQAMRTIGTHPSSSAGLKNDLLENLQAYQKRMGVQMQRFK
>M2
MSLLTEVETPIRNEWGCRCNGSSDPLTIAANIIGILHLTLWILDRLFFKCIYRRF
KYGLKGGPSTEGVPKSMREEYRKEQQSAVDADDGHFVSIELE
>M3
MGVQMQRFK
>M4
MSLLTEVETYVLSIIPSGPLKAEIAQRLEDVFAGKNTDLEAYQKRMGVQMQR
FK
>M42
MSLQGRTPILRPIRNEWGCRCNGSSDPLTIAANIIGILHLTLWILDRLFFKCIYR
RFKYGLKGGPSTEGVPKSMREEYRKEQQSAVDADDGHFVSIELE
>NS1

MDPNTVSSFQVDCFLWHVRKRVADQELGDAPFLDRLRRDQKSLRGRGSTL

GLDIKTATRAGKQIVERILKEESDEALKMTMASVPASRYLTDMTLEEMSRDW

SMLIPKQKVAGPLCIRMDQAIMDKNIILKANFSVIFDRLETLILLRAFTEEGAIV

GEISPLPSLPGHTAEDVKNAVGVLIGGLEWNDNTVRVSETLQRFAWRSSNE

NGRPPLTPKQKREMAGTIRSEV

>NEP

MDPNTVSSFQDILLRMSKMQLESSSEDLNGMITQFESLKLYRDSLGEAVMR

MGDLHSLQNRNEKWREQLGQKFEEIRWLIEEVRHKLKITENSFEQITFMQAL

HLLLEVEQEIRTFSFQLI

>NS3

MDPNTVSSFQVDCFLWHVRKRVADQELGDAPFLDRLRRDQKSLRGRGSTL

GLDIKTATRAGKQIVERILKEESDEALKMTMASVPASRYLTDMTLEEMSRDW

SMLIPKQKVAGPLCIRMDQAIMDHTAEDVKNAVGVLIGGLEWNDNTVRVSET

LQRFAWRSSNENGRPPLTPKQKREMAGTIRSEV

>NEG8

MLFAQNYSLLSSVCVSLLQSTILFLQTSDLIVPAISRFCFGVSGGLPFSLLLLQ

ANLCRVSETRTVLSFHSSPPMRTPTAFLTSSAVCPGREGNGEISPTIAPSSV

KALSNIRVSSRSKITLKFAFSMMFLSMIAWSILIQRGPATFCLGMSMDQSLDI

SSRVMSVR

Figures

**Figure 1: Methods of Influenza Virion Purification**
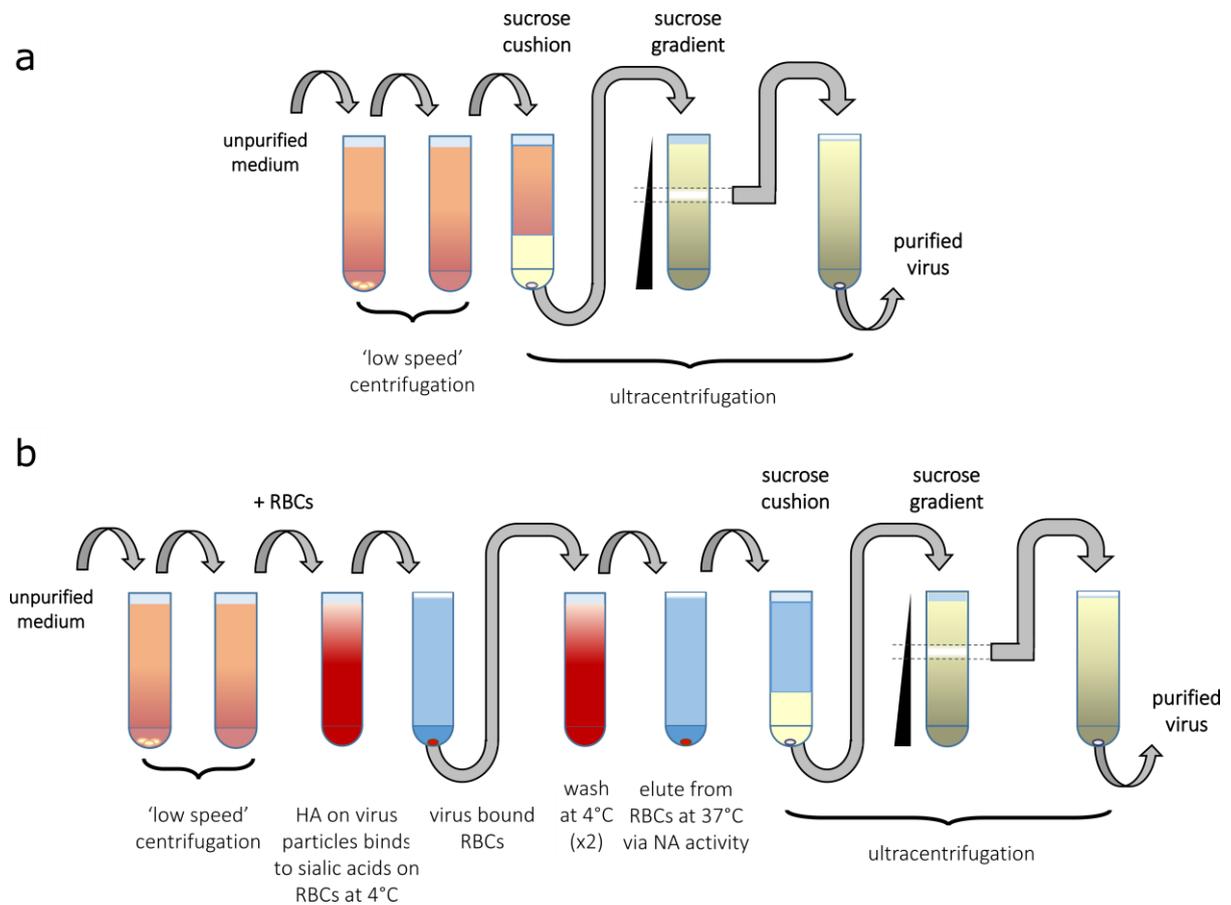
Schematics outlining the stages in purifying influenza virions (a) with and (b) without haemadsorption (HAd). (c) A silver-stained SDS-PAGE gel showing proteins present in a sample of virions purified using HAd, with molecular weight markers shown in kDa. (d) Negative-stain transmission electron micrograph showing intact virions purified without HAd. Scale bar 200 nm; adapted from [12].

**Figure 2: Stages in Influenza Virion Purification**
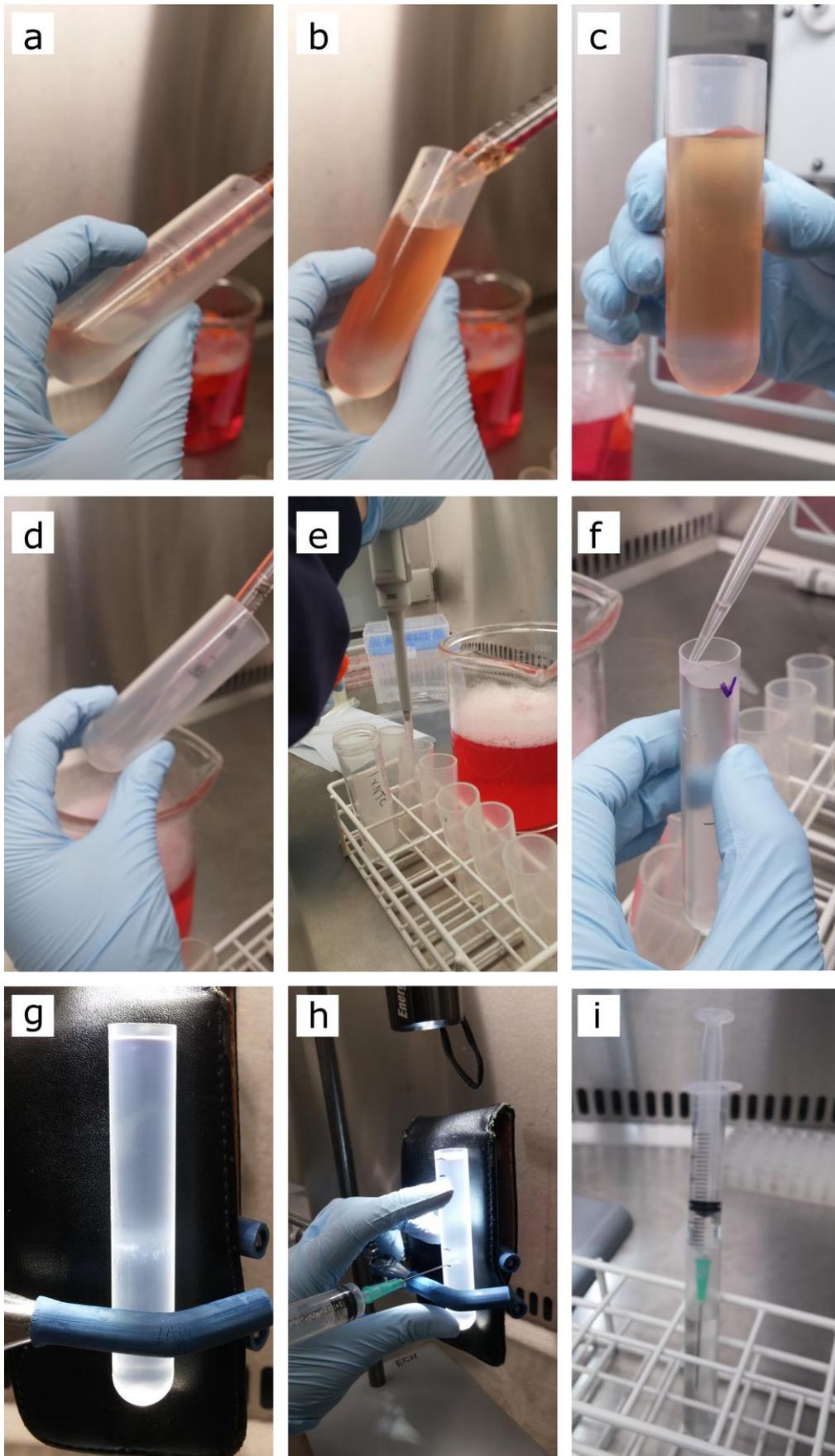
Photographs showing stages in the purification procedure outlined in Fig. 1a, purifying viruses grown in tissue culture using an OptiPrep density gradient. (a)-(c) Layering clarified virus growth medium onto a cushion, showing the overhanging angle used to minimise mixing. (d) Removal of the cushion after centrifugation, angling to avoid disrupting the pellet. (e) Addition of buffer to the pellets from six tubes for resuspension. Note the pot of Virkon solution for the immediate disposal of virus-contaminated waste. (f) Layering the pooled, resuspended pellets onto the top of a density gradient. (g) Density gradient after centrifugation. Virions are visible as a band just below the mid-point of the tube. (h) Harvesting virions by side puncture (fingers were kept well clear during the puncturing). Note the guide-marks made on the tube with pen (showing the initial levels of the meniscus and of the band of virions, the point just below the virions where the tube will be punctured, and an indication below the meniscus of how much fluid should be withdrawn), the torch clamped above and the use of a black background (in this case, a broken smartphone case). (i) Ejection of harvested virions into a fresh tube containing buffer.

Figure 3: Sample Preparation for Mass Spectrometry
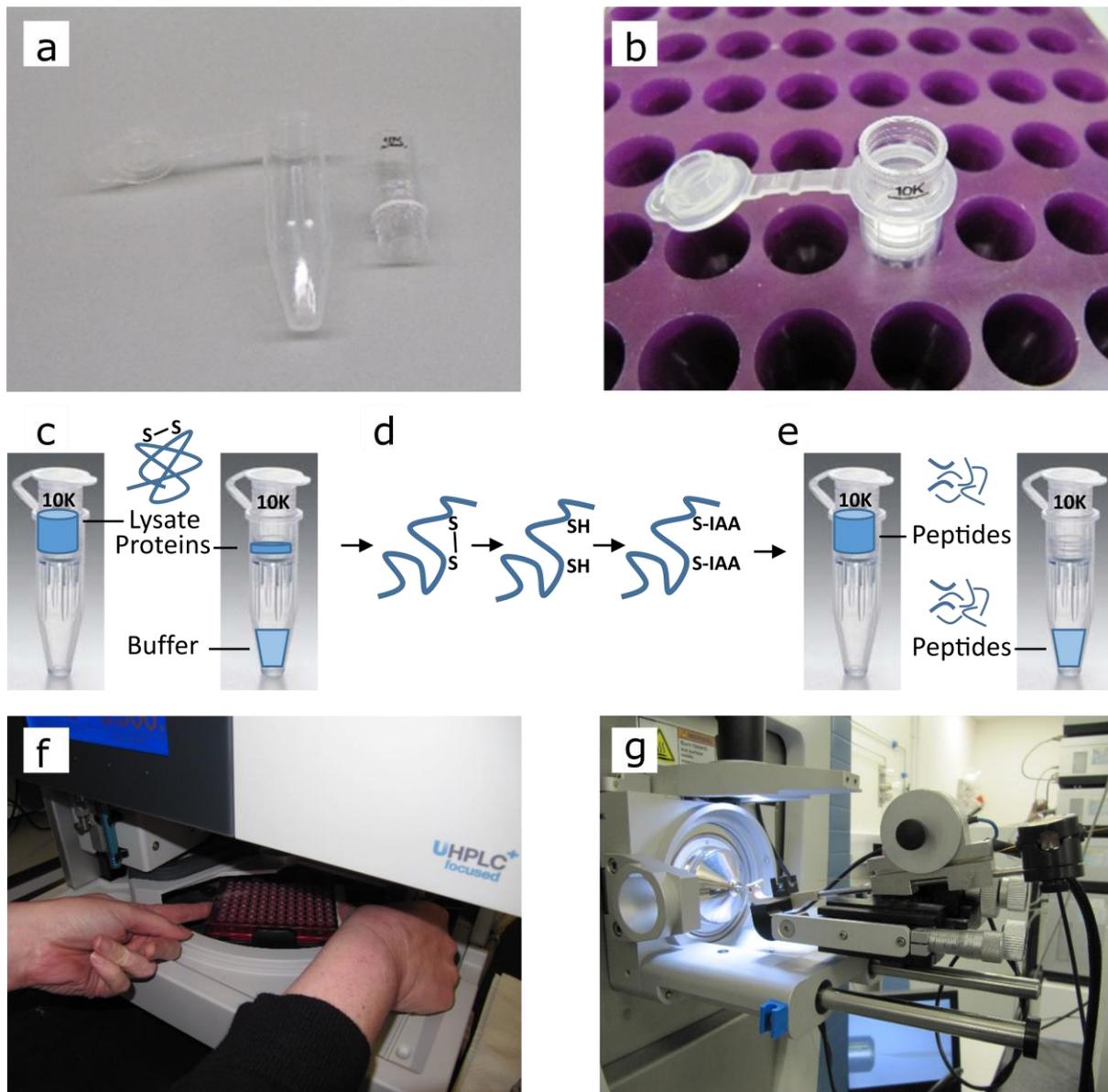
A collection tube and 10 kDa MWCO filter, shown (a) separately and (b) assembled in a rack. (c) Illustration of how a filter can be used to concentrate and wash proteins. (d) Effect on a protein of reduction (with tris(2-carboxyethyl)phosphine hydrochloride, TCEP, affecting disulphide bonds) and alkylation (with iodoacetamide, IAA, to leave *S*-acetamide (AA) alkylations). (e) Illustration of how a filter can be used to collect peptides after digest. (f) Placing samples, loaded in a v-bottom 96-well plate, into a Dionex UltiMate™ 3000 autosampler for HPLC. (g) Injection of sample into a Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer.

Figure 4: Summary of an influenza virion proteome.
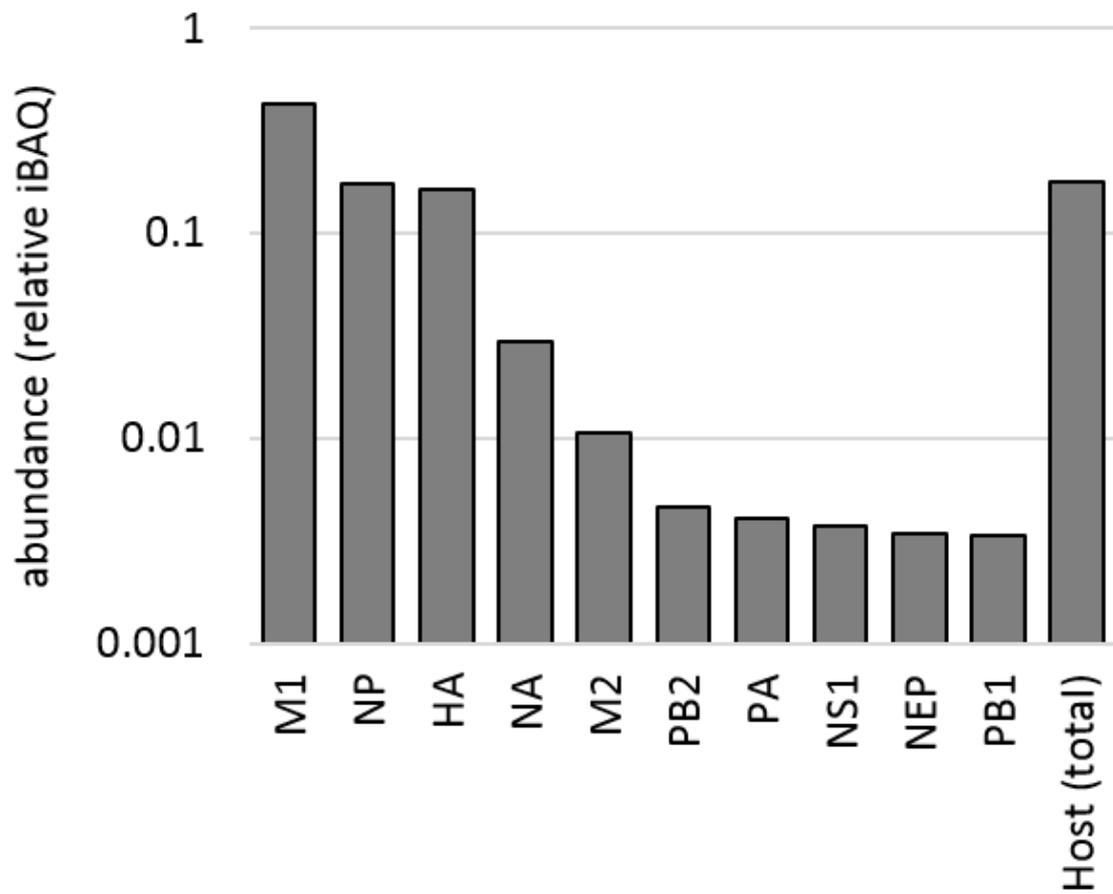
Viral and host proteins identified and quantified in a sample of MDCK-grown

influenza A/Puerto Rico/8/1934 virus, using the methods described in this chapter.

Acknowledgments

References

1.   Hutchinson EC, Curran MD, Read EK, Gog JR, Digard P. 2008. Mutational analysis of cis-acting RNA signals in segment 7 of influenza A virus. J Virol 82:11869-11879.

2.   Hutchinson EC, Charles PD, Hester SS, Thomas B, Trudgian D, Martinez-Alonso M, Fodor E. 2014. Conserved and host-specific features of influenza virion architecture. Nat Commun 5:4816.

3.   Shaw ML, Stone KL, Colangelo CM, Gulcicek EE, Palese P. 2008. Cellular proteins in influenza virus particles. PLoS pathogens 4:e1000085.

4.   Hutchinson E, Fodor E. 2014. Purification of influenza virions by haemadsorption and ultracentrifugation. Protocol Exchange doi:10.1038/protex.2014.027.

5.   Roberts PC, Lamb RA, Compans RW. 1998. The M1 and M2 proteins of influenza A virus are important determinants in filamentous particle formation. Virology 240:127-137.

6.   Sieczkarski SB, Whittaker GR. 2005. Characterization of the host cell entry of filamentous influenza virus. Archives of Virology 150:1783-1796.

7.   Aebersold R, Mann M. 2016. Mass-spectrometric exploration of proteome structure and function. Nature 537:347-355.

8.   Tyanova S, Temu T, Cox J. 2016. The MaxQuant computational platform for mass spectrometry-based shotgun proteomics. Nat Protoc 11:2301-2319.

9.   Michalski A, Damoc E, Hauschild JP, Lange O, Wieghaus A, Makarov A, Nagaraj N, Cox J, Mann M, Horning S. 2011. Mass spectrometry-based proteomics using Q Exactive, a high-performance benchtop quadrupole Orbitrap mass spectrometer. Mol Cell Proteomics 10:M111 011015.

10.   Zubarev RA, Makarov A. 2013. Orbitrap mass spectrometry. Anal Chem 85:5288-5296.

11.   Wisniewski JR, Zougman A, Nagaraj N, Mann M. 2009. Universal sample preparation method for proteome analysis. Nat Methods 6:359-362.

12.   Hutchinson EC, Denham EM, Thomas B, Trudgian DC, Hester SS, Ridlova G, York A, Turrell L, Fodor E. 2012. Mapping the phosphoproteome of influenza A and B viruses by mass spectrometry. PLoS Pathog 8:e1002993.

13.   Cox J, Hein MY, Luber CA, Paron I, Nagaraj N, Mann M. 2014. Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. Mol Cell Proteomics 13:2513-2526.

14.   Schwanhausser B, Busse D, Li N, Dittmar G, Schuchhardt J, Wolf J, Chen W, Selbach M. 2011. Global quantification of mammalian gene expression control. Nature 473:337-342.

15.   Hettinga KA, Reina FM, Boeren S, Zhang L, Koppelman GH, Postma DS, Vervoort JJ, Wijga AH. 2015. Difference in the breast milk proteome between allergic and non-allergic mothers. PLoS One 10:e0122234.

16.   Shin JB, Krey JF, Hassan A, Metlagel Z, Tauscher AN, Pagana JM, Sherman NE, Jeffery ED, Spinelli KJ, Zhao H, Wilmarth PA, Choi D, David LL, Auer M, Barr-Gillespie PG. 2013. Molecular architecture of the chick vestibular hair bundle. Nat Neurosci 16:365-374.

17.   Nielsen ML, Vermeulen M, Bonaldi T, Cox J, Moroder L, Mann M. 2008. Iodoacetamide-induced artifact mimics ubiquitination in mass spectrometry. Nat Methods 5:459-460.

18.   de Wit E, Spronken MI, Bestebroer TM, Rimmelzwaan GF, Osterhaus AD, Fouchier RA. 2004. Efficient generation and growth of influenza virus A/PR/8/34 from eight cDNA fragments. Virus Res 103:155-161.