



# Proteomics as a tool for live attenuated influenza vaccine characterisation

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## ABSTRACT

Many viral vaccines, including the majority of influenza vaccines, are grown in embryonated chicken eggs and purified by sucrose gradient ultracentrifugation. For influenza vaccines this process is well established, but the viral strains recommended for use in vaccines are updated frequently. As viral strains can have different growth properties and responses to purification, these updates risk changes in the composition of the vaccine product. Changes of this sort are hard to assess, as influenza virions are complex structures containing variable ratios of both viral and host proteins. To address this, we used liquid chromatography and tandem mass spectrometry (LC-MS/MS), a flexible and sensitive method ideally suited to identifying and quantifying the proteins present in complex mixtures. By applying LC-MS/MS to the pilot scale manufacturing process of the live attenuated influenza vaccine (LAIV) FluMist® Quadrivalent vaccine (AstraZeneca), we were able to obtain a detailed description of how viral and host proteins are removed or retained at each stage of LAIV purification. LC-MS/MS allowed us to quantify the removal of individual host proteins at each stage of the purification process, confirming that LAIV purification efficiently depletes the majority of host proteins and identifying the small subset of host proteins which are associated with intact virions. LC-MS/MS also identified substantial differences in the retention of the immunosuppressive viral protein NS1 in purified virions. Finally, LC-MS/MS allowed us to detect subtle variations in the LAIV production process, both upstream of purification and during downstream purification stages. This demonstrates the potential utility of LC-MS/MS for optimising the purification of complex biological mixtures and shows that it is a promising approach for process optimisation in a wide variety of vaccine manufacturing platforms.

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**Abbreviations:** BSA, Bovine Serum Albumin; CHF, Clarified Harvest Fluid; CDPB, Centrifuge Diluent Phosphate Buffer; DCP, Dilute Centrifuge Pool; ECACC, European Collection of Authenticated Cell Cultures; EMEM, Eagle's Minimum Essential Medium; FFU, Fluorescent Focus Unit; FFA, Fluorescent Focus Assay; FVGM, FFA Viral Growth Medium; HA, Haemagglutinin; HCD, Higher-energy Collisional Dissociation; IAV, Influenza A virus; IBV, Influenza B virus; IIV, Inactivated Influenza Vaccine; LAIV, Live Attenuated Influenza Vaccine; LC-MS/MS, Liquid chromatography and tandem Mass Spectrometry; MDCK, Madin-Darby Canine Kidney; MDV, Master Donor Virus; MS, Mass Spectrometry; MVB, Monovalent Bulk; NA, Neuraminidase; PBS, Phosphate Buffered Saline; PBST, Phosphate Buffered Saline with 0.5% Tween; PHF, Pooled Harvest Fluid; QLAIV, Quadrivalent LAIV; RT, Room Temperature; TFF, Tangential Flow Filtration; TCEP, Tris (2-carboxyethyl) phosphine; WHO, World Health Organisation.

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## 1. Introduction

Influenza A viruses (IAV) and influenza B viruses (IBV) are highly transmissible human respiratory pathogens. In most years they kill 290,000–650,000 people globally, cause widespread morbidity and inflict major economic costs [1,2]. The primary method for controlling the disease is vaccination and the WHO recommends annual vaccinations for healthcare workers and those most at risk of severe complications from influenza [3], including pregnant women [4], young children [5], older adults [6] and individuals with certain underlying health conditions [7]. A number of governments also recommend the vaccination of school children and healthy adults to decrease the impact of seasonal epidemics [8].

This demand for influenza vaccines is largely met by propagating influenza viruses in embryonated chicken eggs [9]. Typically, harvested egg material is purified and different viral strains are blended to produce multivalent vaccines. These protect against the H3N2 and H1N1 subtypes of IAV, and either one (trivalent) or two (quadrivalent) lineages of IBV. Inactivated influenza vaccines (IIVs) and live attenuated influenza vaccines (LAIVs) both elicit a humoral immune response; LAIVs also elicit a mucosal immune response [10,11] which can lead to greater vaccine effectiveness in children [12–15]. For decades, egg-grown vaccines against influenza and many other viruses have been purified by sucrose gradient ultracentrifugation [16,17].

Influenza viruses evolve rapidly, and so the strains recommended for influenza vaccines are frequently updated. The strains can vary in their growth properties and responses to purification, which creates an ongoing manufacturing challenge. Some optimisation is possible during the 'upstream' part of manufacturing, in which virus is grown in the allantoic cavity of eggs for a variable period of secondary incubation and temperature. The 'downstream' manufacturing process, in which egg-grown virus is concentrated and purified, is strictly controlled by the process parameters and critical quality attributes described in the license, but insights into the behaviour of a particular strain in a manufacturing process can be facilitated by performing pilot-scale purifications and identifying issues, such as high pressures, filter blockages, or loss of material, that may affect full-scale purification.

Optimising the manufacturing process can be helped by understanding the composition of vaccine stocks at each stage of their purification. This is also an issue of quality control: the final product must be shown to contain acceptable levels of antigen (if an IIV) or infectious virus (if an LAIV) and acceptably low levels of process-related impurities such as host proteins (e.g. ovalbumin). Determining the proteins present in influenza vaccines is challenging because influenza virions (and virus-like particles) are complex biological structures. Their heterogeneous composition incorporates viral proteins and a great variety of host proteins, over a wide range of different abundances [18–20]. Rather than obtaining a complete description of such a complex mixture, commonly-used assays focus on a small set of specific proteins [9,20–22]. A single assay which could reliably detect and quantify all viral proteins and host-encoded proteins would clearly aid vaccine quality control [9,22]. It would also aid vaccine manufacturing process development if the assay could be performed not only on the highly-purified final product, but on process intermediates in which virions are heavily diluted in a complex mixture of other material.

Mass spectrometry (MS) is a highly sensitive and flexible approach for proteomics. MS approaches can be applied to influenza vaccines, but to date they have been applied only to purified final products and to the detection and quantification of the major viral surface antigens [23–25]. We previously used liquid chromatography and tandem mass spectrometry (LC-MS/MS) for the comprehensive proteomic characterisation of purified influenza virions [18]. As we were able to detect not only the major viral proteins but also very low-level host proteins, we reasoned that this method could be effective in more complex mixtures such as vaccine process intermediates [18,22]. We therefore decided to investigate whether LC-MS/MS was suitable for the proteomic profiling of a vaccine purification process. As a proof-of-concept, we considered several strains prepared for inclusion in the quadrivalent LAIV (QLAIV) produced by AstraZeneca – an intra-nasal spray marketed as FluMist® Quadrivalent in the United States and Canada and Flu-enz® Tetra in Europe. Here, we show that LC-MS/MS can be an effective, flexible and highly sensitive tool for monitoring protein composition during LAIV manufacturing. Because of this, we suggest that LC-MS/MS could be useful in many other viral vaccine manufacturing processes.

## 2. Materials and methods

### 2.1. Cells, eggs and viruses

Madin-Darby Canine Kidney (MDCK) cells (ECACC) were passaged in cell culture medium (1 × Eagle's Minimum Essential Medium containing non-essential amino acids (EMEM; Biowest), supplemented with 1.1 g/L sodium pyruvate (Lonza), 2 mM L-glutamine (Life Technologies), 10% heat inactivated foetal bovine serum (FBS, VWR) and 50 µg/mL gentamycin (Life Technologies)). Specific Pathogen Free embryonated chicken eggs (Charles River Laboratories) were incubated in a rocking incubator at 37 °C and 70% humidity for 11 days prior to inoculation. The LAIV viruses listed in Table 1 were produced by reverse genetics to make '6:2 reassortants,' with six genome segments from a Master Donor Virus (MDV, for either IAV or IBV), and the two genome segments encoding HA and NA from a circulating strain [26].

### 2.2. Fluorescent focus assay (FFA)

Confluent MDCK cells in 96-well tissue culture plates were washed twice with FFA viral growth medium (FVGM; consisting of cell culture medium without FBS and with 0.5 µg/mL amphotericin B antimycotic (Life Technologies)) and duplicate wells infected with a 1:10 serial dilution of virus stock in FVGM. The cells were incubated at 33 °C and 5% CO<sub>2</sub> for 20 h, washed with cell culture medium and fixed with 80% acetone in water for 1 h at –20 °C. Cells were dried at 36 °C for 30 min and washed with Phosphate Buffered Saline (PBS) containing 0.5% Tween (PBST) before labelling for 1 h at 36 °C with sheep antibody against the relevant HA (obtained from NIBSC-UK or TGA) in PBS with 1% Bovine Serum Albumin (BSA). Cells were washed with PBST and incubated with Alexa Fluor 488 donkey anti-sheep IgG (Life Technologies, A-11015) in 1% BSA in PBS for 1 h at 36 °C, washed again with PBST and dried at room temperature. Titres were calculated from the mean number of HA-positive foci in two replicate wells, determined using a Nikon Eclipse Ti inverted fluorescent microscope.

### 2.3. Production of PHF from chicken eggs

For each virus approximately  $1.3 \times 10^2$  FFU of influenza virus in 0.1 mL was inoculated into the allantoic cavities of each of approximately 5500 viable 11 day-old eggs, which were then underwent secondary incubation at 33 °C for IAV strains and 31 °C for IBV strains. After secondary incubation (Table 1), allantoic fluid was harvested and pooled. Samples for analysis were stabilised with by mixing 100:1 with 6.9% (w/v) sucrose phosphate in water (200 mM sucrose, 3.8 mM potassium di-hydro phosphate and 7.2 mM di-potassium hydrogen phosphate, pH 7.2) and stored at –80 °C. To consider different secondary incubation times, 9 eggs per time point were inoculated and harvested under the same conditions as above, after 36, 60, 72 or 84 h of incubation at 33 °C.

**Table 1**  
Live Attenuated Influenza Vaccine (LAIV) Strains.

Short Name	Full Name	Genus (Subtype/Lineage)	Secondary incubation
A/BOL13	A/Bolivia/559/2013	A (H1N1)	48 h, 33 °C
A/CAL09	A/California/07/2009	A (H1N1)	72 h, 33 °C
A/TEX12	A/Texas/50/2012	A (H3N2)	48 h, 33 °C
B/BRIS08	B/Brisbane/60/2008	B (Victoria)	72 h, 31 °C
B/MASS12	B/Massachusetts/2/2012	B (Yamagata)	72 h, 31 °C

## 2.4. Concentration and purification of LAIV

The purification process for LAIV, used both commercially and in pilot scale process development laboratories, is outlined in Table 2. The work described here was carried out at pilot scale in a process development laboratory using harvest fluid from approximately 5500 eggs. Two methods were used: method 1 was used for A/BOL13 and A/CAL09, and method 2 for A/TEX12 and B/MASS12.

### 2.4.1. Generation of clarified harvest fluid (CHF)

A clarification filter chain was constructed using filters of decreasing size – 8.0 µm (PP2 filter, Sartorius), 1.2 µm (Milligard Opticap filter, Millipore) and 0.8 µm/0.45 µm (Sartopore 2 Midicap filter, Sartorius). This was pre-flushed with 15 L of purified water at approximately 200 mL/min. Approximately 40 L (method 1) or 25 L (method 2) of PHF were pumped through the clarification filter chain at a flow rate of 550 mL/min (method 1) or 275 mL/min (method 2). CHF was collected on an ice bath and mixed at 25–35 rpm for 5 min at 2–8 °C.

### 2.4.2. Generation of dilute centrifuge Pool (DCP)

A 500 kDa hollow fibre membrane cartridge (GE Healthcare) was pre-flushed with purified water at a flow rate of 2 L/min (method 1) or 1 L/min (method 2) for at least 5 min. CHF was passed through at 3 L/min (method 1) or 1.25 L/min (method 2) until a 4 – 5-fold reduction in volume was achieved by tangential flow filtration (TFF). The TFF retentate was purified by continuous flow sucrose gradient ultracentrifugation using a Hitachi CC40 continuous flow ultracentrifuge with an 800 mL (method 1; pilot-scale) or 3200 mL (method 2; equivalent to the production scale) core. The gradient was sampled between the refractive indices of 1.4183–1.3898 nD using an inline refractometer, generating approximately 140 mL (method 1) or 800 mL (method 2) of pooled centrifuge fractions. These were diluted with centrifuge diluent phosphate buffer (CDPB, 65 mM potassium phosphate dibasic and 35 mM potassium phosphate monobasic, pH 7.2) to reduce the sucrose content, creating approximately 1.0 L (method 1) or 2.0 L (method 2) of Diluted Centrifuge Pool (DCP).

### 2.4.3. Generation of monovalent bulk (MVB)

A sterilising-grade 0.45 µm/0.2 µm filter (Sartopore 2 capsule, Sartorius) was pre-flushed with approximately 150 mL of purified water at 45 mL/min then used to filter the DCP at a maximum flow rate of 50 mL/min (Method 1) or 60 mL/min (Method 2) to generate the MVB. To remove residual virus from the filter, 90 mL of CDPB containing 0.69% (w/v) sucrose phosphate (final concentration) was used to flush the filter at a flow rate of 51 mL/min and this was combined with the filtered MVB.

In an additional step, not representative of LAIV purification, the PHF and CHF samples of A/TEX12 were concentrated by overlaying 3.6 mL of the sample virus onto an 0.5 mL 30% sucrose cushion in CDPB and centrifuging in a Beckman JA-25.50 rotor at 18 K rpm (27 K g) and 4 °C for 18 h. The pellet was resuspended with CDPB to achieve a final viral titre of at least 10<sup>9</sup> FFU/mL.

## 2.5. Mass spectrometry

Samples were lysed at room temperature in 8 M urea and frozen before processing. Processing was carried out as described previously [18,27], with the incorporation of Filter-Aided Sample Preparation [28], following a protocol described in detail in [29]. Briefly, lysed samples were clarified and proteins collected by centrifugation through a Vivacon 500<sup>®</sup> 10 kDa molecular-weight cut-off filter unit (Sartorius). Proteins remaining in the filter unit were reduced with 10 mM tris(2-carboxyethyl) phosphine (TCEP) for 30 min at RT and then alkylated with 50 mM (final) chloroacetamide. The sample was washed with 8 M urea in 100 mM ammonium bicarbonate, equilibrated by washing with 6 M urea in 25 mM ammonium bicarbonate, and digested with Lys-C for 1–3 h at 37 °C and then with trypsin overnight at 37 °C. Peptides were collected, pooled and lyophilised prior to analysis. As described previously [18], prior to analysis the majority of digested samples were mixed with peptides which had been prepared in the same fashion from protein mass standards (MS Qual/Quant QC Mix, Sigma Aldrich).

Samples were analysed by LC-MS/MS using an Ultimate 3000 RSLCnano HPLC system (Dionex) run in direct injection mode and coupled to a Q Exactive mass spectrometer (Thermo) in 'Top 10' data-dependent acquisition mode with fragmentation by higher-energy collisional dissociation (HCD). Charge state + 1 ions were rejected from selection and fragmentation and dynamic exclusion with 40 s was enabled.

Mass spectra were analysed using MaxQuant 1.5.8.3 analysis software [30], following a protocol described in [29]. Briefly, data files for each sample in a comparison were searched together as separate experiments, using standard settings and the following parameters: enzyme: trypsin/P; variable modifications: oxidation (M) and acetyl (Protein N-ter); and fixed modifications: carbamidomethyl (C). Label-free quantitation and the iBAQ algorithm [31] were enabled. Peptide spectra were matched to a database containing the predicted proteome of each virus in the comparison, the protein sequences of the protein mass standards (MS Qual/Quant QC Mix, Sigma Aldrich), a single repeat of the protein sequence of ubiquitin, and a custom database in which all instances of the ubiquitin sequence had been deleted from the *Galus gallus* UP000000539 proteome (retrieved from UniProt on 16/05/2017). Viral proteins were matched to the predicted consensus sequence, on the assumption that mutations and post-translational modifications of any individual residue would be low-frequency events [27]. Protein group data were further processed by deleting reverse (decoy) matches, potential host proteins, protein groups identified by site only and (except where stated) the protein standards.

Raw data, the databases used for the searches and tables containing the processed results can be downloaded from Enlighten

**Table 2**  
Downstream stages of LAIV production.

Stage	Abbreviation	Description
Pooled Harvest Fluid	PHF	Allantoic fluid is harvested from approximately 5500 infected chicken eggs after secondary incubation. Samples taken for analysis were stabilised with sucrose phosphate.
Clarified Harvest Fluid	CHF	Unstabilised PHF bulk material is pumped through a series of filters of decreasing pore size to exclude cellular material and tissue debris, giving CHF.
Diluted Centrifuge Pool	DCP	CHF is concentrated by tangential flow filtration with a high-molecular weight cut-off filter. The retentate is subjected to ultracentrifugation through a sucrose gradient. Fractions of the appropriate density are collected and diluted to reduce the sucrose concentration, giving the DCP.
Monovalent Bulk	MVB	The DCP is filtered to give a sterile stock of MVB.
Live Attenuated Influenza Vaccine	LAIV	The diluted combination of MVBs for an A/H1N1, A/H3N2, B/Victoria and B/Yamagata strain to give a final QLAIV.

at the University of Glasgow (<https://doi.org/10.5525/gla.research-data.707>).

## 2.6. Electron microscopy

For PHF and CHF samples, 30 mL of material was clarified at 1.6 K rpm at 4 °C for 10 min and then layered onto a 30% sucrose cushion, with the remainder of the centrifuge tube filled with PBS. For DCP and MVB samples 4 mL of material was added to a centrifuge tube which was then filled with PBS. Samples were centrifuged in a Beckman SW32Ti rotor at 30 K rpm (111 K g) at 4 °C for 3.5 h (PHF and CHF) or 3 h (DCP and MVB). After centrifugation, pellets were resuspended in 500 µL PBS, mixed 1:1 with 4% glutaraldehyde in PBS and stored at –80 °C before being adsorbed to carbon coated and plasma etched Pioloform® grids and stained with 2% aqueous uranyl acetate in PBS. Grids were examined using a Philips CM 100 Compustage (FEI) transmission electron microscope.

## 3. Results

### 3.1. Mass spectrometry can be used to determine protein ratios during LAIV production

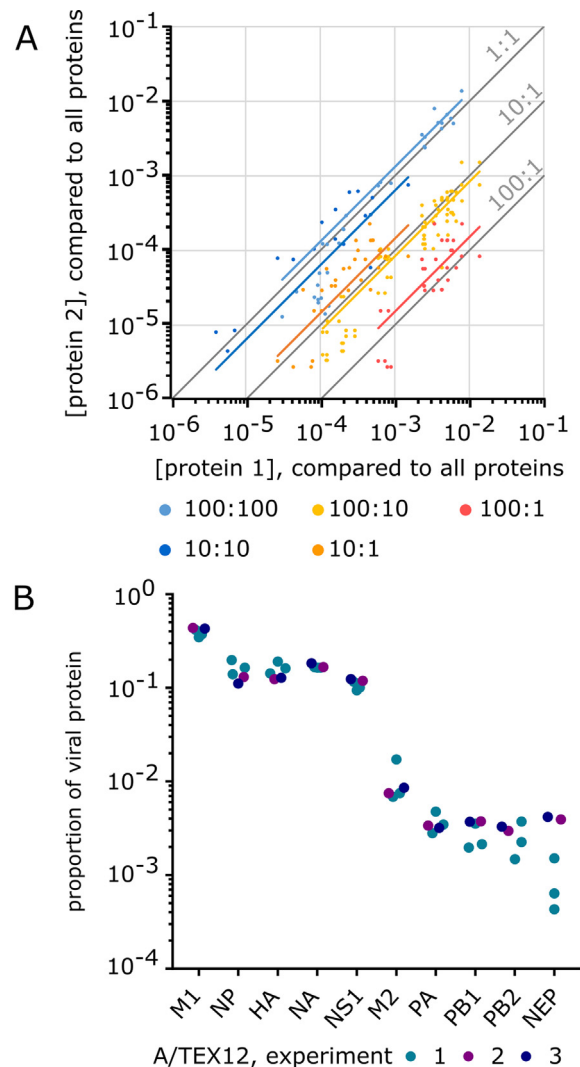
To determine the protein composition of LAIV production stages, we adapted an LC-MS/MS approach that we had previously used to identify and quantify proteins in purified influenza virions [18]. We first wished to determine whether LC-MS/MS could consistently determine protein ratios in LAIV in-process material, which was considerably more complex than the material previously considered. To do this we mixed a set of LAIV samples with protein standards. These were in a fixed ratio of abundance relative to each other (1:10:10:100:100) but varied considerably in their total abundance with a sample, allowing us to assess the reproducibility of protein quantitation over a wide dynamic range.

We could consistently and accurately measure 1:1 ratios of protein standards, provided they were within the top three orders of magnitude of total protein abundance (Fig. 1A). As expected, the amount of noise increased both with greater pairwise ratios and when the protein standards were increasingly diluted. Despite this, almost all measured ratios were still within ten-fold of their actual values even over a dynamic range of 100 for pairwise ratios and at a proportional abundance of less than  $10^{-5}$  in the sample. As in a previous study [18], we noted stochastic failures to detect very low abundance proteins, with the least abundant protein standards being worst affected (data not shown). We concluded that LC-MS/MS could consistently and accurately determine protein ratios in LAIV process intermediate samples, although caution was required when interpreting large pairwise ratios and failures to detect very low-abundance proteins.

### 3.2. Mass spectrometry can consistently quantify viral proteins in process intermediates

We next wished to determine whether the ratios of viral proteins could be reproducibly determined in process intermediates. To do this, we considered upstream LAIV material consisting of unpurified allantoic fluid from infected eggs (PHF). This is the manufacturing stage in which viral proteins are most dilute and contaminating host proteins most abundant.

In initial experiments, we analysed the PHF from eggs infected with a former influenza vaccine strain, A/TEX12 (Fig. 1B). We first quantified viral proteins in a sample concentrated through a sucrose cushion, making three replicate measurements of the same material. As we could reproducibly detect and measure the ratios



**Fig. 1.** Mass spectrometry can determine protein ratios in LAIV in-process material. (A) LC-MS/MS with label-free quantitation can consistently determine protein ratios over a wide dynamic range. 24 infected and 12 mock-infected samples of unpurified allantoic fluid, as well as 12 samples taken throughout the vaccine purification process, were mixed with purified protein standards and analysed by LC-MS/MS. In each sample the protein standards, which were always in a fixed 100:100:10:10:1 ratio, were quantified as a proportion of total protein, and all possible pairwise ratios between the standards within each sample were calculated (in each pair [protein 1]  $\geq$  [protein 2]). Grey lines show the expected pairwise ratios and coloured points and trendlines show the measured pairwise ratios. (B) LC-MS/MS with label-free quantitation can consistently measure the abundance of viral proteins in unpurified samples. The viral proteins in the allantoic fluid of eggs infected with A/TEX12 were quantified with respect to each other. Three independent experiments are shown, with triplicate technical repeats (repeat injections of sample into the instrument) for the first experiment. Samples in the first experiment were concentrated through a sucrose cushion prior to analysis.

of the viral proteins, we then compared this to two independent experimental repeats on which no concentration had been performed. We consistently measured the ratios of viral proteins over a wide dynamic range, albeit with greater noise at larger ratios (Fig. 1B).

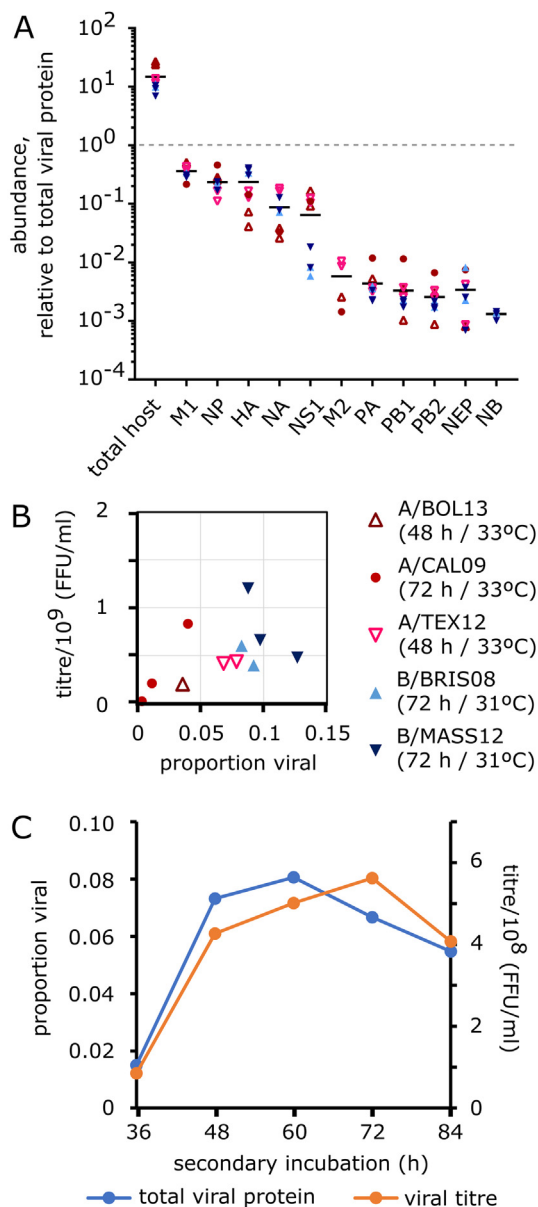
In further pilot experiments we encountered stochastic failures to detect the least abundant viral proteins (data not shown). We noted that a failure to detect the viral polymerase subunits PA, PB1 and PB2, which are among the least abundant viral proteins in the virion [18], correlated with a failure to detect other low-abundance virion proteins such as M2 and NEP. For this reason, we adopted the detection of at least two polymerase subunits as



an acceptance criterion for analysis. With this condition, we concluded that LC-MS/MS could reproducibly determine the abundance of viral proteins, even in unpurified allantoic fluid.

### 3.3. Assessment of upstream LAIV material by LC-MS/MS

We next extended our analyses to a panel of influenza A and B virus LAIV strains (Table 1). The ratios of viral proteins in the PHF were broadly consistent across strains, allowing for increased noise and occasional losses of detection when considering the least abundant proteins (Fig. 2A). This consistency was expected, both because of the viruses' common genetic background and because



**Fig. 2.** Viral protein abundance in upstream LAIV material. Proteins in pooled harvest fluid (PHF), the starting material for live-attenuated influenza vaccine (LAIV) production, were assayed by LC-MS/MS with label-free quantitation and by focus-forming assay. Eggs were infected with different viruses and incubated under different conditions before harvest, as indicated. (A) Protein abundance in PHF, normalised by the total abundance of viral proteins in the sample (dashed line). (B) The ratio of viral protein to total protein in each sample, compared to the infectious titre. The same symbols are used to indicate each strain in both (A) and (B). (C) The ratio of viral protein to total protein, and the infectious titre, for PHF harvested from eggs infected with A/TEX12 and incubated at 33 °C for the indicated secondary incubation times prior to harvest.

of a propensity we have previously observed for egg-adapted viruses to assemble virions with a common 'core architecture' [18].

However, there were two striking differences between the PHF stocks. Firstly, we noted variations in the amount of viral NS1, with higher levels detected in IAV strains than in IBV strains (Fig. 2A). Secondly, the total proportion of viral protein in the samples varied, both between strains and between experiments (Fig. 2B). To examine more closely the ability of LC-MS/MS to detect differences in viral yield within PHF, we varied the yield of A/TEX12 by altering the secondary incubation time. The variation in total viral protein detected using LC-MS/MS correlated with infectivity, indicating that LC-MS/MS can detect variations in viral protein yield in PHF (Fig. 2C). Thus, LC-MS/MS can be used to assess the proteomic composition of upstream LAIV production material. Even in these complex samples, it can identify changes in both the relative abundance of individual proteins and in the overall viral yield.

### 3.4. Assessment of purified LAIV material by LC-MS/MS

We next analysed virions in purified (MVB) stocks (Fig. 3A; Table 3). As expected from purer material, we were able to determine viral protein ratios more consistently in samples of MVB than of PHF (Fig. 2A, 3A). Variations in relative host protein abundance were largely eliminated after virion purification (Fig. 3B) which, along with the correlation of viral protein with infectious titre in the PHF (Fig. 2B), indicated that the differences in the host protein abundance likely arose from differences in the total concentration of virions in the PHF rather than in differences in composition of the virions themselves. Although purification to MVB gave greater consistency in the concentration of viral protein it did not lead to a consistent infectious titre, indicating that the specific infectivity of virions after purification was variable (Fig. 3B).

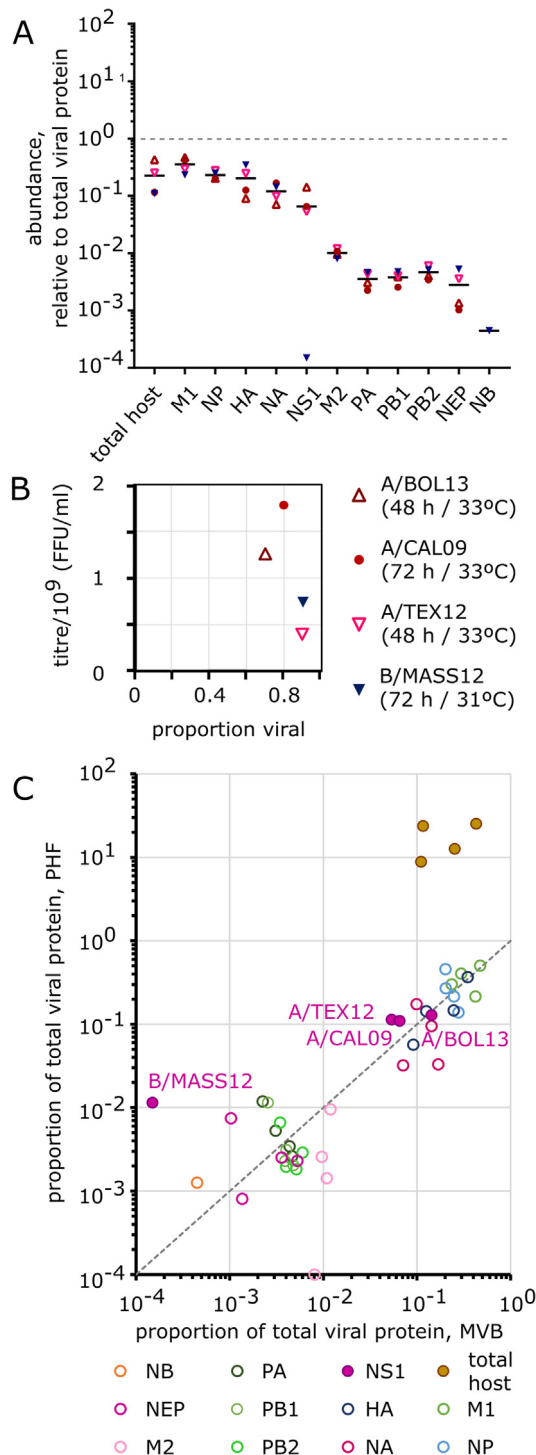
Most viral proteins maintained a consistent ratio during purification from PHF to MVB, while the amount of host protein present was dramatically reduced (Fig. 3C). For the IAV strains, purification did not change the relative abundance of NS1 (Fig. 3C), supporting the hypothesis that NS1 is a component of influenza virions [18]. However, this was not the case for the IBV strain B/MASS12. Here, NS1 was already less abundant in the PHF than it was for the IAV strains, and it was depleted nearly 80-fold by purification (Fig. 3C). This suggests that for B/MASS12 NS1 is not a component of the virion.

Together, these data show that LC-MS/MS can be used to assess the proteomic composition of purified LAIV stocks and can quantify viral and host proteins, including those of low abundance, in a single measurement.

### 3.5. Assessment of the downstream LAIV purification processes by LC-MS/MS

Having established that LC-MS/MS could be applied to both purified and unpurified material, we used it to examine the effects of the multi-stage LAIV downstream purification process (Table 2, see also Materials and Methods). We began by obtaining an overall impression of the stringency of LAIV purification. Using A/TEX12, we compared the effects of LAIV purification to one-step concentration through a cushion, an approach commonly used in experimental procedures that require concentrated influenza virions but do not require a particularly high degree of purity [29]. LC-MS/MS showed that while a simple concentration step increased the proportion of viral protein in the stock by 8-fold, LAIV purification removed a greater proportion of host proteins, increasing the proportion of viral protein by 12-fold (Fig. 4A). Importantly for an LAIV, this process left the virions intact (Fig. 4B).

We next considered each stage in the purification process (Table 2), using four LAIV strains (Fig. 5A). Filtration between



**Fig. 3.** Viral protein abundance in purified LAIV material. Proteins in monovalent bulk (MVB), the purified material resulting from LAIV production, were assayed by LC-MS/MS with label-free quantitation and by focus-forming assay. Prior to purification, eggs were infected with different viruses and incubated under different conditions before harvest, as indicated. (A) Protein abundance in MVB, normalised by the total abundance of viral proteins in the sample (dashed line). (B) The ratio of viral protein to total protein in each sample, compared to the infectious titre. The same symbols are used to indicate each strain in both (A) and (B). (C) A comparison of the abundance of proteins in unpurified (PHF) and purified (MVB) material, normalised to total viral protein and with each viral strain plotted as separate points. Proteins with the same ratio to total viral protein both before and after purification would lie on the dashed line. For the viral NS1 protein, annotations indicate the strain to which each point corresponds.

PHF and CHF did not change the concentration of host proteins but had a variable effect on viral titre. TFF and gradient ultracentrifugation between CHF and DCP caused most of the reduction in host protein concentration and substantially reduced viral titre. Finally, sterile filtration between DCP and MVB caused a slight but consistent reduction in host material without substantial reductions in viral titre, suggesting that even after gradient ultracentrifugation some filterable host material remained. Overall, the purification process greatly reduced the concentration of host proteins in all cases.

Most viral proteins maintained a consistent ratio at each stage of the purification process (Fig. 5B), with the exception of the NS1 protein of B/MASS12 (Fig. 5C). For B/MASS12, the largest decrease in NS1 concentration was during the initial filtration step (PHF to CHF), even though this left the bulk concentration of host proteins largely unaffected (Fig. 5A, C). This suggests that the NS1 of B/MASS12 was associated mainly with large particulate material, such as cellular debris.

As described in the Materials and Methods, our data were collected using four different virus strains and two differently scaled-down LAIV purification methods. We were able to detect differences in the efficiency of host protein depletion under these different conditions (Fig. 5A). Our experimental design was not intended to systematically compare the effects of these conditions, but the fact that differences were detectable highlights the utility of LC-MS/MS for comparing methods during downstream process optimisation. Overall, these data indicate that LC-MS/MS is suitable for assessing the efficiency of LAIV downstream purification stages.

### 3.6. LAIV purification efficiently depletes most egg proteins

Finally, we used our data to produce a detailed description of how LAIV purification depletes specific host proteins. We compared the abundance of host proteins in PHF and MVB of the LAIV strains A/BOL13, A/CAL09, A/TEX12 and B/MASS12, excluding A/TEX12 samples that had undergone additional concentration through a sucrose cushion. To express protein abundance in terms of maximum copy number per virion we assumed that each virion incorporated no more than eight copies of the viral polymerase [18,32].

LAIV purification efficiently removed the majority of host proteins (Fig. 6A). This included ovalbumin (mean depletion 640-fold), ovomucoid (1360-fold) and ovomucoid (730-fold), as well as haemoglobin beta (465-fold) and apolipoprotein A1 (1880-fold), plausible host proteins from the blood and yolk content of the egg, respectively. Purification depleted 82% of host proteins by more than 10-fold, and most of the remainder were present only at very low abundance (Fig. 6A). When considering purified (MVB) samples, only a small number of host proteins were present at a greater abundance than the viral polymerase (Fig. 6B). Among these ubiquitin, the membrane proteins uroplakin-1b and -3a [33] and mucin-16 have all previously been identified as components of the influenza virion [18]. Actin, tubulins, lysozyme C, ovalbumin and BPI fold-containing family B member 2, though present, were all depleted by tens to hundreds of fold during the purification process. In this way, LC-MS/MS provided a protein-level description of the depletion of host proteins during LAIV purification and of the proteomic composition of material prepared in a comparable way to the final LAIV product.

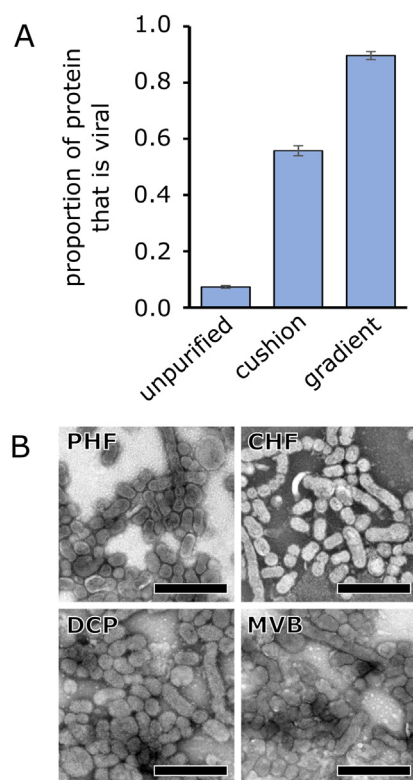
## 4. Discussion

LAIVs such as the FluMist® vaccine require the purification of a range of influenza strains using a single manufacturing process.

**Table 3**  
Maximum viral proteins per virion.

Protein	A/BOL13	A/CAL09	A/TEX12	B/MASS12	Mean $\pm$ s.d.
M1	1064	529	1320	342	814 $\pm$ 456
NP	455	471	568	386	470 $\pm$ 75
HA	178	384	386	522	367 $\pm$ 142
NA	160	146	508	179	249 $\pm$ 174
M2	22	21	32	13	22 $\pm$ 8
NEP	3	6	3	8	5 $\pm$ 3
NS1	322	90	194	0	152 $\pm$ 139
NB	0	0	0	1	N/A
PB2	9	10	10	9	9 $\pm$ 1
PB1	9	7	7	8	8 $\pm$ 1
PA	6	7	7	8	7 $\pm$ 0

NB: Data from MVB stocks, scaled to assume no more than eight polymerase subunits per virion.



**Fig. 4.** The effects of purification on influenza virions. (A) The abundance of viral proteins in the allantoic fluid of eggs infected with A/TEX12 determined by LC-MS/MS, as in Fig. 1B, comparing allantoic fluid when unpurified, concentrated through a 30% sucrose cushion by ultracentrifugation, and purified using sucrose density gradient ultracentrifugation as for vaccine production. For unpurified and gradient-purified samples the mean and range of two independent experiments are shown; for the cushion the mean and SEM of three replicate measurements. (B) Negative-stain transmission electron microscopy (TEM) showing virions of A/TEX12 at each of the stages of vaccine purification: pooled harvest fluid (PHF), clarified harvest fluid (CHF), dilute centrifuge pool (DCP) and monovalent bulk (MVB). Scale bars are 500 nm. PHF and MVB correspond to the 'unpurified' and 'gradient' steps in (A), respectively.

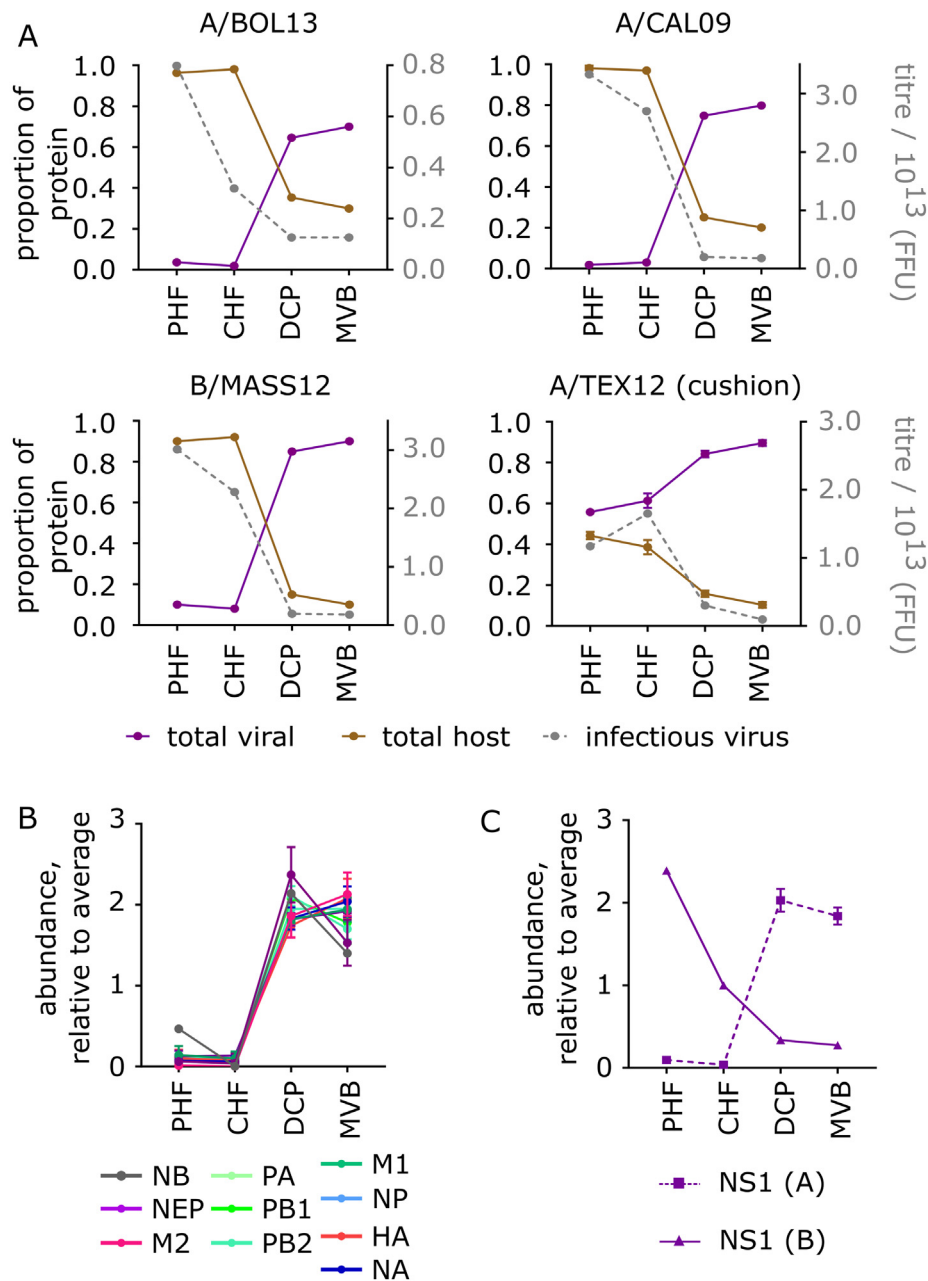
Frequent updates to strain recommendations mean that maintaining high levels of purity along with an acceptable viral yield can be a major challenge. This makes a simple method to produce detailed measurements of compositional changes during LAIV purification highly desirable. Firstly, such a method would offer the possibility of optimising the current manufacturing process, and secondly it could facilitate the development of a more efficient subtype- or strain-specific downstream purification process in the future. The current sucrose-gradient ultracentrifugation process is extremely costly, time-consuming and requires a high level of expertise to

execute. There may be significant benefits in developing an alternative downstream purification process using diafiltration or chromatography [34], but any new processes would need to be thoroughly characterised to allow process development and to demonstrate that an optimised process produces a product suitable for market. Here, we show that LC-MS/MS is a suitable tool for the sensitive and detailed characterisation of LAIV-in process material in a single measurement. We therefore suggest that it could be of use in the optimisation and development of vaccine purification processes.

LC-MS/MS was sensitive enough to be applied to upstream (pre-purification) material, where it could detect differences in both total yield and in the initial purity of virions of the sort that routinely arise when preparing vaccines from eggs (Fig. 2). Batch-to-batch variation of eggs is an acknowledged issue in influenza vaccine production, with anecdotal evidence suggesting that differences in farm, flock lineage and flock age can all affect viral yields [35]. LC-MS/MS offers an attractive process development tool with the potential to help understand and therefore reduce this variability, as well as helping to optimise the secondary incubation time and temperature for new strains.

LC-MS/MS was also used to profile downstream purification stages. We demonstrated proof-of-concept for the use of LC-MS/MS in downstream process optimisation by comparing the purification of four different viral strains under two different methods, and by identifying an unanticipated increase in virion purity during final sterile filtration. It should be noted that strain-specific process changes do not always have identical effects at pilot scale and commercial scale. Scale-driven differences in critical quality attributes, such as product titre and the clearance of impurities, can require remedial process changes. As the composition and complexity of pilot scale material resembles that of material produced at commercial scale, we would expect that LC-MS/MS would also be suitable for the assessment of commercial scale material.

The production of highly purified LAIV requires the removal of host proteins such as ovalbumin, which as well as being an allergen can reduce viral titre [36]. Considerable work has already been done to optimise LAIV process development, and it is important to note that current commercial purification methods reduce the concentration of ovalbumin to a point where LAIV can be safely administered to people with egg allergies [37–39]. Indeed, a recent study found that commercially available LAIV had the lowest levels of ovalbumin of any influenza vaccine available on the market [40]. The proteomic profiles produced by LC-MS/MS extend our ability to assess the purity of the final product. By characterising MVB stocks, comparable to those used in formulating a commercial LAIV product, we have provided what is, to our knowledge, the most detailed published proteomic assessment of a vaccine prepared by sucrose-gradient ultracentrifugation. By their very nature, the virions of an enveloped virus such as influenza contain some host



**Fig. 5.** Viral protein abundance during LAIV purification. Influenza vaccine strains were purified from allantoic fluid as for LAIV production; the strain A/TEX12 underwent additional concentration through a 30% sucrose cushion prior to analysis. (A) The effects of purification on viral and host protein abundance (determined by label-free LC-MS/MS) and infectious titre for four different LAIV strains. For A/TEX12 the mean and SEM of 3 (PHF) or 2 replicate measurements are shown. (B) The abundance of viral proteins at each stage of purification, compared to their average abundance across all stages, showing the means and standard deviations from A/BOL13, A/CAL09 and B/MASS12. (C) The abundance of NS1, compared to its average across all stages, showing the mean and range of A/BOL13 and A/CAL09 as NS1 (A) and B/MASS12 as NS1 (B).

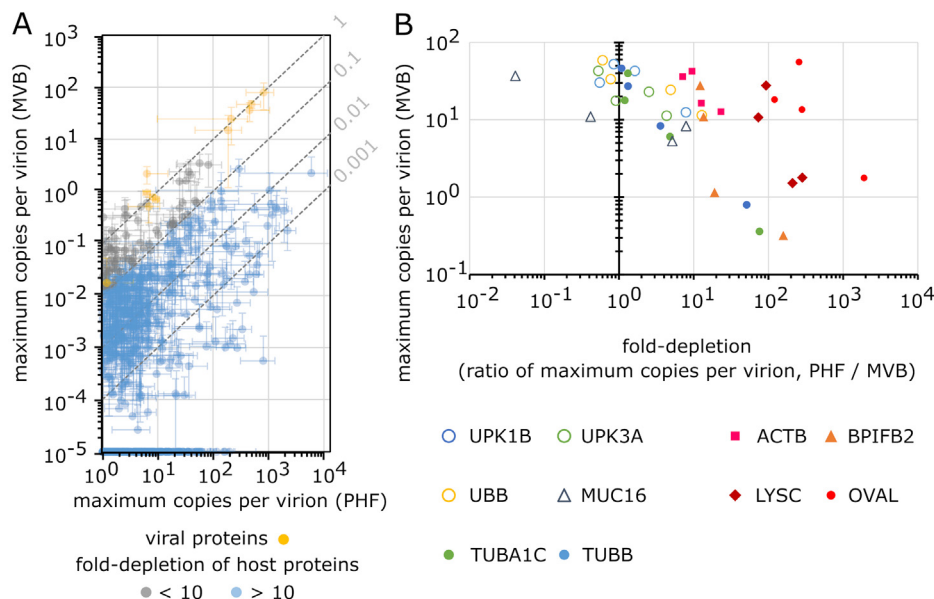
proteins (Fig. 6) [18,19]. Although these host proteins do not pose a risk in a vaccine [37–39], it is clearly desirable that we know what they are, and a comprehensive proteomic description by LC-MS/MS allows for this.

Our data confirm the expectation that LAIV strains, possessing the same MDV ‘backbone,’ produce virions with the same broadly conserved ‘core architecture’ of proteins. However, we noted that the incorporation of the viral protein NS1 into virions varied markedly (Figs. 2, 3). We previously detected NS1 in purified IAV and IBV virions at levels close to that of the viral polymerase [18]. In contrast, in this study, NS1 associated with IAV virions at higher levels, closer to that of the viral NA (Fig. 3), while for B/MASS12 NS1 appeared to be separable from virions by filtration

(Figs. 3, 5C). Together, these data suggest that, unlike the broadly consistent incorporation of the other viral proteins, influenza virions incorporate variable amounts of NS1. NS1 is a multi-functional and potentially immunosuppressive protein [41] but the functions of extracellular NS1 are unknown. However, we note that LAIV strains, including those used in this study, are extensively tested in release assays. We therefore know that the reduced association of NS1 with B/MASS12 does not significantly affect the strain’s capacity to generate an immune response and provide protection from circulating strains [14].

In summary, LC-MS/MS provides a suitable platform for optimising viral vaccine purification processes. Applied at the pilot scale, as here, it can be used when optimising current methods





**Fig. 6.** Host protein abundance during LAIV purification. Comparison of protein abundance before (PHF) and after (MVB) purification, normalised by assuming a maximum 8 polymerase subunits per virion. (A) Average PHF and MVB protein abundance in the strains A/BOL13, A/CAL09, A/TEX12 and B/MASS12 (excluding A/TEX12 PHF samples that had been purified through a cushion), showing means and standard deviations. Dashed lines show the degree of depletion after purification. (B) Host proteins whose mean abundance across strains, after purification, is greater than that of the viral polymerase. Abundance in the MVB and the degree of depletion during purification is shown for each of the viral strains. UPK1B: uroplakin-1b; UPK3A: uroplakin-3a; UBB: ubiquitin; Muc16: mucin-16; TUBA1C: tubulin alpha chain; TUBB: tubulin beta chain; ACTB: actin; BPIFB2: BPI fold-containing family B member 2; LYSC: lysozyme C; OVAL: ovalbumin.

for the purification of LAIV and of many other viral vaccines. It can also be used for assessing the scalability of these methods to commercial processes, which is currently a significant challenge in biologics [42–44]. LC-MS/MS therefore provides a powerful and flexible tool for vaccine optimisation and development.

### Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Edward Hutchinson and the University of Oxford Advanced Proteomics facility received consultancy fees from AstraZeneca Liverpool for the initial work that led to this project. Amy Hawkworth and Mahesh Jayachander are employees of AstraZeneca at Liverpool, UK. AstraZeneca are the manufacturers of FluEnz®/FluMist® intranasal influenza live virus vaccine.

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All authors attest they meet the ICMJE criteria for authorship.

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