Propagation and purification of influenza virus

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We have developed and tested a method for purifying live influenza virus from MDCK cell culture using established techniques that could be easily scaled up and adapted to industrial-scale production. We have used this method to purify three different seasonal vaccine strains of influenza virus. Application of this method resulted in the production of influenza virus with a purity comparable to international regulatory requirements.

Introduction

Traditionally, the propagation of influenza virus has been accomplished via infected fertilized hen eggs. The disadvantages of the traditional method includes (i) Time consumption; (ii) The need for large production facilities; (iii) Inherent risks from open handling and susceptibility to avian flu. In the future, the method of choice for the propagation of viruses for vaccine production would include large-scale mammalian cell culture. This is driven by advantages such as closed handling, ready scale-up, and fast deployment (1-3). However, the production of cell-based vaccines would pose both technical and regulatory challenges that may require the development and application of new techniques for downstream processing (4-7).

In this application note, we describe the development and verification of a method for the purification of live influenza virus derived from MDCK cells cultivated on Cytodex™ 3 microcarriers. The use of live attenuated virus for vaccination is a strategy that has been used for several commercially available vaccines against different disease targets. Figure 1 shows an overview of the influenza virus purification method. We tested the efficacy of this method by purifying four different strains of influenza virus (Table 1).

Fig 1. Schematic representation of the influenza virus purification procedure.

Table 1. Influenza strains

Basic development was performed using the following laboratory strain:

A/Puerto Rico/8/1934(H1N1)	(Denoted PR/8)

Verification was performed using the following vaccine strains:

A/Solomon Islands/3/2006(H1N1)	(Denoted A/Solomon Islands)
A/Wisconsin/67/2005(H3N2)	(Denoted A/Wisconsin)
B/Malaysia/2506/2004	(Denoted B/Malaysia)

Abbreviations

AIEX	Anion exchange chromatography
gDNA	Genomic DNA
FBS	Fetal bovine serum
HA	Hemagglutinin
LMH	Liters/square meter/hour
MDCK	Madine-Darby canine kidney
MWCO	Molecular weight cut-off
NFF	Normal flow filter
SRID	Single radial immunodiffusion
TCID50	Tissue culture infective dose
UF/DF	Ultrafiltration/diafiltration

Materials and methods

Biacore™ T100, Typhoon™, ImageQuant™ TL, ÄKTAexplorer™, QuixStand™, and ÄKTAcrossflow™ instruments were from GE Healthcare. Sensor chip CM5, agarose, Cy™3-labeling kit, Surfactant P20, ImageScanner™, illustra™ tissue and cells genomic Prep Midi Flow Kit, ULTA™ Prime GF 2, ULTA Prime GF 0.6 µm normal flow filters, Hollow fiber UPF-500-C, and Capto™ ViralQ medium (resin) were from GE Healthcare. The 15 L bioreactor for cell growth was from Applicon Inc. Cell factories were obtained from Thermo Fisher Scientific. Ultra MDCK cell medium was obtained from Lonza. Anti-influenza sera and virus antigens were obtained from the National Institute for Biological Standards and Control (NIBSC), except for the B/Brisbane/3/2007 serum and antigen, which were from Solvay Pharmaceuticals. Bradford solution was obtained from BioRad, and Zwittergent™ from Merck. NuPAGE™ SDS-PAGE system and primers were obtained from Invitrogen™. StepOnePlus™ instrument with StepOne software v2.0, TaqMan™ Universal PCR Master Mix, and FAM-MGB probes were from Applied Biosystems.

Virus production

Virus strains A/Solomon Islands, A/Wisconsin, and B/Malaysia (originally from WHO and produced in eggs) were adapted for propagation in MDCK cells that can grow in a low-serum medium (ECACC 84121903).

For pilot-scale production (i.e., up to 1.5 L), influenza virus was produced in cell factories, inoculated with 40 000 MDCK cells/cm^2 and grown for 2 d in Ultra MDCK medium supplemented with FBS at 37°C and 5% CO_2. After infection, the cells were incubated at 33°C and the virus was harvested on the third or fourth day after infection.

For large-scale production, the influenza virus was propagated in a stirred 15 L bioreactor using Cytodex 3 microcarriers. Upon expansion in cell factories as described above, the cells were inoculated on the microcarriers at a cell concentration of 0.5 × 10^6 cells/mL and cultured for 2 d with an agitation rate of 100 rpm. The cells were expanded up to a final cell density of 2 to 2.5 × 10^6 cells/mL prior to virus infection.

Harvest clarification

Two 2" ULTA Prime GF capsules with cutoff values of 2 and 0.6 µm were connected in series to a peristaltic pump and used to clarify the material after harvest.

For UF/DF of the clarifed harvests, a QuixStand system with a 650 cm^2 500 kDa MWCO 0.5 mm lumen i.d. hollow fiber unit and a peristaltic pump was used for diafiltration of clarified harvests.

The clarified harvest was concentrated 10 times and then discontinuously diafiltered 6-fold into 20 mM Tris, 0.5 M NaCl, pH 7.5, followed by additional concentration to the final volume. The permeate flow rate varied between 26 to 50 mL/min and the average flux LMH was approximately 45 L/m^2 × h. The final concentration varied from 10 to 20 times for the different harvests.

DNA-binding anion exchange chromatography

Screenings for optimal pH and NaCl conditions for the anion exchange chromatography step were performed in PreDictor™ 96-well plates and 1 mL HiTrap™ columns packed with Capto ViralQ media. Anion exchange chromatography runs for the verification step were carried out in an XK16 column packed with 20 mL (10 cm bed height) Capto ViralQ. ÄKTAexplorer 10 system with a Frac-910 and UNICORN™ 5.11 were used for all the chromatography experiments (8).

Sterile filtration

For sterile filtration, a stainless steel filter holder for 47 mm filter discs was connected to the P960 sample pump on an ÄKTAexplorer 10 instrument. Each filter was pre-wetted with approximately 30 mL of buffer before the application of 50 to 450 mL of sample. We used 0.2 µm ULTA disc Pure SG filters and the fractions were collected manually.

After sample application, both filter and housing were washed with an additional volume of 20 mL of buffer.

Analytical methods

TCID 50 assay

MDCK cells were grown in 96-well microplates and then infected with 10-fold serial dilutions of virus suspension followed by incubation for 5 d. Wells showing cytopathic effect were counted and the TCID50 titer was calculated according to the method of Reed and Münch (10).

SRID assay

Anti-influenza sera, unlabeled and Cy3-dye-labeled sera (< 0.5% of the total serum amount) were mixed with melted agarose (1%) and cast in a mould, which created holes in the gel (11). Samples and reference antigens treated with Zwittergent (1% for 30 min at 22°C) were allowed to diffuse into the gel over 15 to 18 h and the gel was subsequently dried and scanned at 450 nm with a Typhoon scanner. Evaluation was performed by comparing ring areas of samples with reference antigens using ImageQuant TL software.

Biacore biosensor SPR assay

Recombinant HA antigen was immobilized on sensor chip CM5 with standard amine coupling. Anti-influenza sera were mixed with samples or reference antigen and injected in 400 s, followed by regeneration of the surface (50 mM HCl, 0.05% P20 for 30 s). Standard curves were first run in the middle and end of the assays. An interpolated calibration performed by the software was used to ensure high precision in the calculated concentrations (9, 12).

SDS-PAGE

The samples were incubated in NuPAGE loading buffer for 10 min at 70°C and run on 4% to 12% NuPAGE gels for 10 min at 60 V and 70 min at 150 V. The gels were stained with GelCode™ Blue, scanned with Image Scanner, and evaluated with ImageQuant TL.

Bradford total protein assay

The assay was performed according to the manufacturer's instructions (13).

Quantitative PCR (qPCR)

The level of MDCK genomic DNA (gDNA) in the samples was quantitated on StepOnePlus using custom-designed primers and FAM-MGB probe. MDCK gDNA from uninfected cells was purified with illustra tissue and cells genomicPrep Midi Flow Kit and used as a reference.

Results

Cell culture and virus propagation

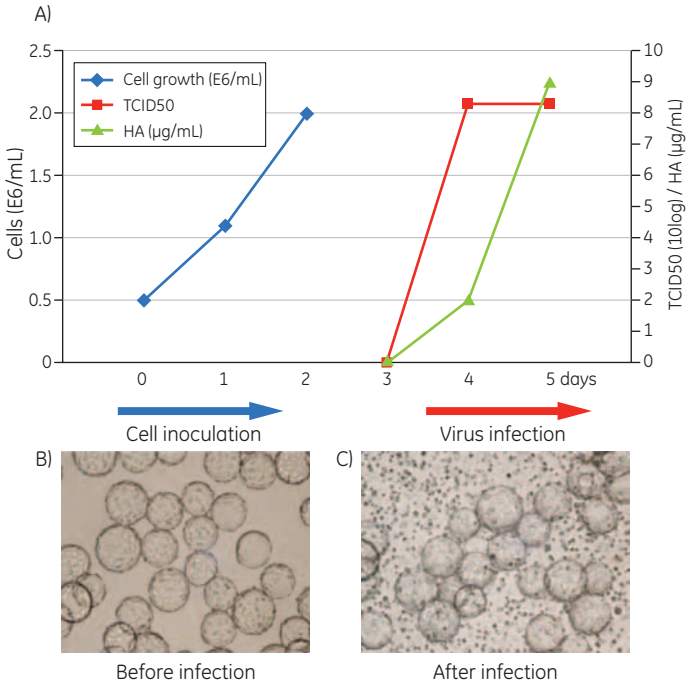


Fig 2. (A) Diagram showing cell density, TCID50, and HA level during the course of a virus production run in a stirred-tank reactor; (B) shows confluent cells on microcarriers in the expansion phase, while (C) shows cells after viral infection with typical lysis and release of cell debris to the medium.

Harvest and UF/DF conditioning

The combination of 2 µm and 0.6 µm ULTA Prime GF provided the maximum throughput in harvest measured as volume per filter area. In subsequent runs, we did not observe any significant increase in filter pressure over the two filter capsules, indicating that the filters were oversized for the volumes involved (5 to 10 L of harvest).

The 500 kD MWCO hollow fiber filter yielded good clearance of host cell proteins and gDNA fragments while providing full retention of influenza virus. On all the runs, HA in the permeate from the filters was below the detection limit, protein clearance was 80% to 92%, and gDNA was reduced by a factor of 2.8 to 5.8 (Table 2).

Table 2. Data from the harvest filtration steps

Strain	HA recovery (%)	Total protein recovery (%)	DNA recovery (%)
PR/8	63	ND	26
A/Solomon Islands (n = 5)	98 ± 26	12 ± 4	36
A/Wisconsin (n = 2)	82 ± 13	17 ± 3	17

DNA-binding anion exchange chromatography

Column: XK16 Capto ViralQ (10 cm bedheight)
Buffer A: 20 mM Tris, 0.5 M NaCl, pH 7.5
Buffer B: 20 mM Tris, 1.5 M NaCl, pH 7.5
Sample: 2 CV, concentrated Influenza A/Solomon Island, produced in MDCK cells
Flow: 60 cm/h

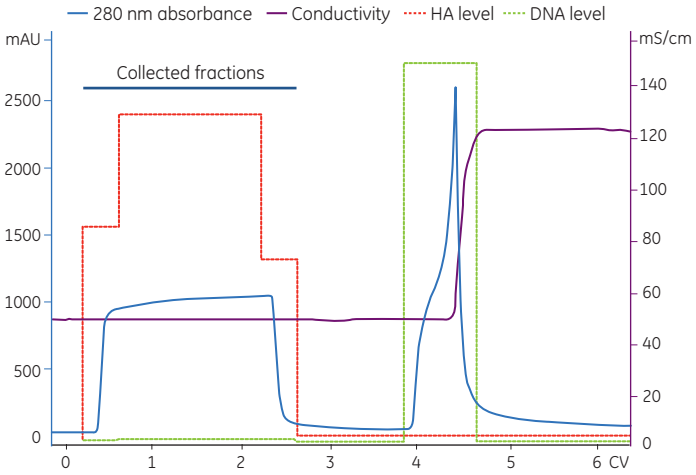


Fig 3. Representative chromatogram from the DNA-binding AIEX step showing levels of HA and DNA across different fractions.

Flow-through anion exchange chromatography has been used to remove host cell DNA during influenza virus purification (8). The aim was to obtain maximum reduction of gDNA with optimal retention of virus in the flowthrough. Capto ViralQ medium was used for this step because it combines reliable scale-up capabilities with optimal DNA reduction.

The MDCK gDNA was reduced between 130 and 870 times. Less than 5% of HA was found in the peak that emerged after elution of the bound material from the column using 1.5 M NaCl, thus indicating that the loading concentration of 0.5 M NaCl was close to the optimum concentration required to prevent the virus from binding.

Table 3 shows that flow-through anion exchange chromatography is a robust and efficient method for gDNA removal in the purification of four different influenza strains: PR/8, A/Solomon Island, A/Wisconsin, and B/Malaysia.

Table 3. Results of the AIEX procedure

Strain	HA recovery (%)	DNA recovery (%)
PR/8 (n = 3)	83 ± 2	0.12 ± 0.01
A/Solomon Islands (n = 5)	80 ± 14	0.52 ± 0.47
A/Wisconsin (n = 2)	99 ± 1	0.09 ± 0.02
B/Malaysia (n = 1)	82	0.02

Sterile filtration

The final step in the purification process involved sterile filtration. The collected fractions from the Capto ViralQ procedure were pooled and sterile filtered. HA yields from the sterile filtration process were in the range of 83% to 94% (Table 4). The high yields we observed could be due to the relatively high ionic strength (0.5 M NaCl) of the buffer used because such a buffer would minimize interactions between the viral particles.

Table 4. Yields across the sterile filtration step using ULTA Pure SG 0.2 µm membrane

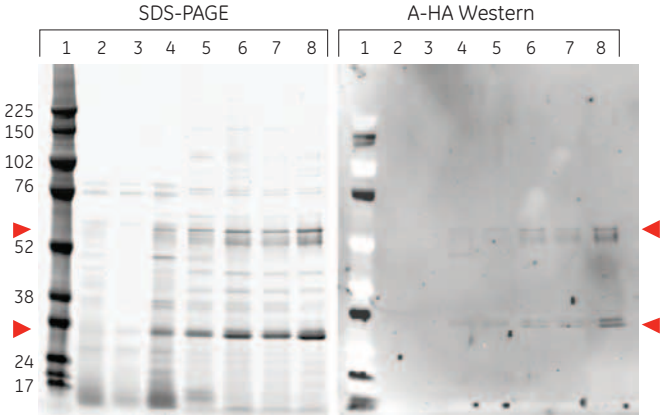
Strain	Harvest day (after infection)	HA yield (%) [a]Biacore [b]SRID
A/Solomon Islands	3	85a
A/Solomon Islands	4	94a
A/Wisconsin/	4	83b
B/Malaysia	3	84b

The combined performance over the different purification steps gave a total HA yield of 34%, to 84%, a consistent gDNA depletion over 99.9% and a total protein recovery of 2% to 14% (Table 5).

Table 5. Combined performance

Strain	HA yield (%)	DNA recovery (%)	Total protein recovery (%)
A/Solomon Islands (n = 5)	59 ± 25	0.07	6 ± 4
A/Wisconsin (n = 2)	56 ± 12	0.03	11 ± 3

The data in Figures 4 and 5 show that the purity of the final virus sample from this work was similar to that achieved through sucrose gradient centrifugation.



Lanes

1. Rainbow™ Marker
2. Harvest
3. ULTA Prime GF 2 µm + ULTA Prime GF 0.6 µm filtrate
4. UF/DF retentate UF 10-fold
5. UF/DF retentate UF 10-fold/DF 6-fold
6. Capto ViralQ flowthrough
7. ULTA disc SG 0.2 µm filtrate
8. Sucrose gradient: Virus band

The arrows denote the approximate molecular weight for HA1 and HA2, respectively.

Fig 4. SDS-PAGE gels comparing patterns from different intermediates in the purification procedure compared to the band from the 30%/40% interface of a sucrose gradient (lane 8).

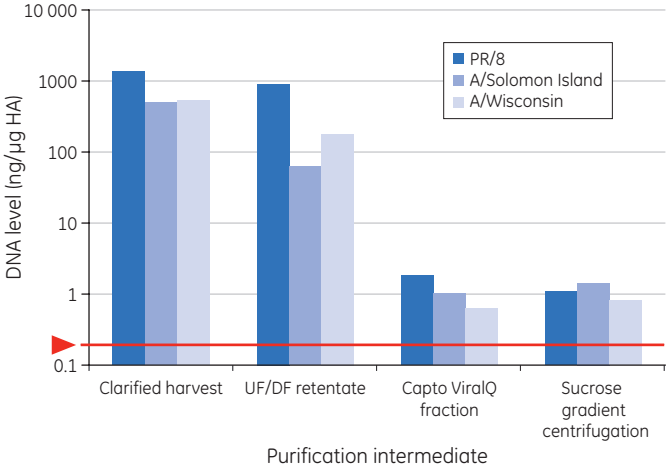


Fig 5. Total DNA reduction was between 2.2 and 3.1 $\log_{10}$. The bars to the right show data from the virus band of a sucrose gradient centrifugation of the corresponding harvests, and they are included for comparison. The red line corresponds to the WHO acceptance level for parenteral vaccines of 10 ng gDNA/dose, assuming a vaccine dose of 45 µg HA. If we assume a nasal route of administration with a dose of 10^7 infectious virus particles in 0.2 mL, the amount of DNA produced would be below the 10 ng/dose acceptance level.

TCID50 levels show that the purification procedure described in this work is suitable for the production of a live influenza virus (Fig 6).

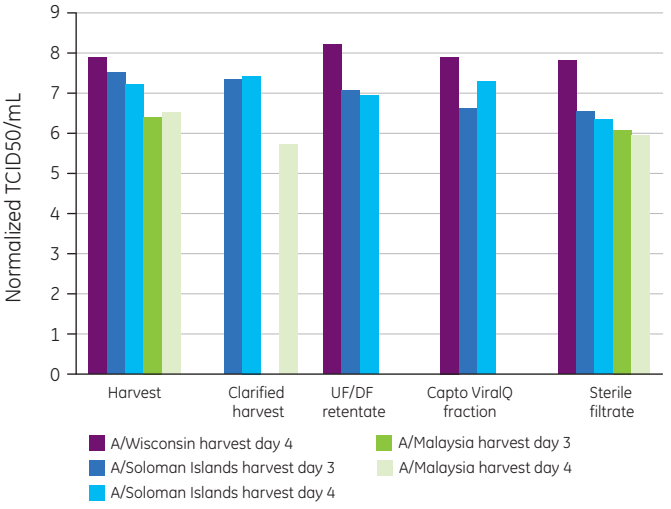


Fig 6. TCID50 viability through the downstream procedure normalized by volume. The drop in viability is lower than expected. It should also be noted that the inherent variability in the TCID50 method is roughly one order of magnitude.

Discussion

In this work, we have established and verified a scalable method for the preparation of influenza virus from MDCK cell culture. The method was used to purify three different strains of influenza virus under identical experimental conditions.

The use of two coupled NFF filters resulted in the extensive removal of any remaining microcarrier beads as well as intact cells and debris that may have affected downstream processes. The ultrafiltration part of the UF/DF process reduced the presence of small molecules, media components as well as host cell proteins and short DNA strands. In the diafiltration process the viral particles were transferred into the appropriate buffer for further processing. The ultrafiltration process adjusted the volume and concentration to suitable levels.

Capto ViralQ was used to reduce the presence of gDNA in the sample via anion exchange chromatography. Finally, the virus fraction was filtered through a 0.2 µm sterile filter.

The method described in this work may be scaled up and used to purify multiple influenza virus strains for use in the vaccine industry. Please note that the purification steps described are not fully developed for use as a platform process in vaccine production. Traditionally, ultracentrifugation is used for density separation of viruses from cell debris in vaccine purification, and when this technique is not applied, cell debris particles that are similar in size to the viral particles may still be found in the product. Further optimization of the purification process is therefore necessary prior to clinical use. However, Figures 4 and 5 show that the DNA levels and PAGE fingerprint of the purified material were comparable to material obtained through gradient centrifugation.

Another major regulatory challenge for a vaccine process based on a live virus is the risk of adventitious viruses. This potential risk can be mitigated by performing extended characterization of cell banks and virus seed stocks, testing of raw materials and the final product. However, these solutions may not be adequate during a pandemic outbreak. Inclusion of virus inactivation steps could speed up the regulatory approval process. For many viral vaccines, inactivation is neither recommended nor possible, therefore a well-developed adventitious virus handling procedure may be required. However, this is outside the scope of the work described in this application note.

Conclusions

- The reduction of host cell-derived impurities suggests that the procedure presented in this work can be used as a suitable starting point for vaccine production.
- The procedure is highly reproducible and provides similar data with different strains of influenza virus.
- The DNA-binding AIEX step using Capto ViralQ media efficiently removed gDNA from the virus sample.
- Normalized TCID50 values were in the same range over the entire process for different influenza virus strains, indicating good retention of infectious particles.

References

1. Youil R. *et al.,* Comparative study of influenza virus replication in Vero and MDCK cell lines, *J. Virol. Methods* **120,** 23-31 (2004).
2. Pau M.G. *et al.,*The Human Cell Line Per.C6 Provides a New Manufacturing System for the Production of Influenza Vaccines, *Viral Vectors and Vaccines* ***2000,*** (2000).
3. Kistner O. *et al.,* Development of a mammalian cell (Vero) derived candidate influenza virus vaccine, *Vaccine* ***16,*** 960-8 (1998).
4. Kalbfuss B. *et al.,* Harvesting and concentration of human influenza A virus produced in serum-free mammalian cell culture for the production of vaccines, *Biotechnology and Bioengineering* ***97,*** 73-85 (2007).
5. Nayak D.P. *et al.,* Downstream processing of MDCK cell-derived equine influenza virus, *Journal of Chromatography B* ***823,*** 75-81 (2005).
6. Kalbfuss B. *et al.,* Purification of cell culture-derived human influenza A virus by size-exclusion and anion-exchange chromatography, *Biotechnology and Bioengineering* ***96****, 932-44 (2007).*
7. Bardiya, N and Bae, J.H. *Influenza vaccines: recent advances in production technologies, Appl. Microbiol. Biotechnol.* ***67,*** 299-305 (2005).
8. Application note: Use of Capto ViralQ for the development of a chromatographic step to remove DNA from a culture of influenza virus, GE Healthcare, 28-9769-69, Edition AA (2010).
9. Application note: Biacore biosensor assays for quantitation of influenza virus and HCP, GE Healthcare, 28-9771-57, Edition AA (2010).
10. Flint S.J. *et al.,* Principles of Virology, ASM Press, Washington D.C. pp. 31-36 (2004).
11. Wood J. M. *et al.,* An improved single-radial-immunodiffusion technique for the assay of influenza haemagglutinin antigen: application for potency determinations of inactivated whole virus and subunit vaccines. *J. Biol. Stand.* ***5,*** 237-247 (1977).
12. Estmer Nilsson C. *et al.,* A novel assay for influenza virus quantification using surface plasmon resonance, *Vaccine* ***28,*** 759-766 (2010).
13. Bradford, M. M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal. Biochem.* ***72,*** 248-254 (1976).

Ordering information

Product	Quantity	Code number
Capto ViralQ	25 mL	28-9032-30
Capto ViralQ	200 mL	28-9032-31
Capto ViralQ	1 L	28-9032-32
HiTrap Capto ViralQ	5 × 5 mL	28-9078-09
Xampler UFP-500-C-4A HF Cartridge	1	56-4102-01
ULTA disc SG 47 mm 0.2 µm	1	28-4002-11
ULTA SG 0.2 µm 2"	3	28-4002-70
ULTA Prime GF 2 µm 2"	1	28-9083-47
ULTA Prime GF 0.6 µm 2"	1	28-9083-29

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