NRC·CNRC

NRC Publications Archive
Archives des publications du CNRC

Accelerated mass production of influenza virus seed stocks in HEK-293 suspension cell cultures by reverse genetics

Milián, Ernest; Julien, Thomas; Biaggio, Rafael; Venereo-Sanchez, Alin; Montes, Johnny; Manceur, Aziza P.; Ansorge, Sven; Petiot, Emma; Rosa-Calatrava, Manuel; Kamen, Amine

This publication could be one of several versions: author's original, accepted manuscript or the publisher's version. / La version de cette publication peut être l'une des suivantes : la version prépublication de l'auteur, la version acceptée du manuscrit ou la version de l'éditeur.
For the publisher's version, please access the DOI link below./ Pour consulter la version de l'éditeur, utilisez le lien DOI ci-dessous.

Publisher's version / Version de l'éditeur:
https://doi.org/10.1016/j.vaccine.2017.04.065
Vaccine, 35, 26, pp. 3423-3430, 2017-05-08

NRC Publications Record / Notice d'Archives des publications de CNRC:
https://nrc-publications.canada.ca/eng/view/object/?id=cfbf971a-e649-47f9-9357-a6501636ecd3
https://publications-cnrc.canada.ca/fra/voir/objet/?id=cfbf971a-e649-47f9-9357-a6501636ecd3

Access and use of this website and the material on it are subject to the Terms and Conditions set forth at https://nrc-publications.canada.ca/eng/copyright
READ THESE TERMS AND CONDITIONS CAREFULLY BEFORE USING THIS WEBSITE.

L'accès à ce site Web et l'utilisation de son contenu sont assujettis aux conditions présentées dans le site https://publications-cnrc.canada.ca/fra/droits
LISEZ CES CONDITIONS ATTENTIVEMENT AVANT D'UTILISER CE SITE WEB.

Questions? Contact the NRC Publications Archive team at PublicationsArchive-ArchivesPublications@nrc-cnrc.gc.ca. If you wish to email the authors directly, please see the first page of the publication for their contact information.

Vous avez des questions? Nous pouvons vous aider. Pour communiquer directement avec un auteur, consultez la première page de la revue dans laquelle son article a été publié afin de trouver ses coordonnées. Si vous n'arrivez pas à les repérer, communiquez avec nous à PublicationsArchive-ArchivesPublications@nrc-cnrc.gc.ca.

National Research Council Canada | Conseil national de recherches Canada

Canada

Vaccine 35 (2017) 3423–3430

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Accelerated mass production of influenza virus seed stocks in HEK-293 suspension cell cultures by reverse genetics

Ernest Milián [a,b], Thomas Julien [c], Rafael Biaggio [a], Alina Venereo-Sanchez [b], Johnny Montes [b], Aziza P. Manceur [b], Sven Ansorge [b], Emma Petiot [c], Manuel Rosa-Calatrava [c], Amine Kamen [a,b,*]

[a] Department of Bioengineering, McGill University, Montréal, Québec, Canada
[b] Vaccine Program, Human Health Therapeutics, National Research Council, Montréal, Québec, Canada
[c] Virologie et Pathologie Humaine - VirPath Team, Centre International de Recherche en Infectiologie (CIRI), INSERM U1111, CNRS UMR5308, ENS Lyon, Université Claude Bernard Lyon 1, Université de Lyon, Lyon, France

ARTICLE INFO

Article history:
Received 29 January 2017
Received in revised form 14 April 2017
Accepted 23 April 2017
Available online 8 May 2017

Keywords:
Influenza virus
Vaccines
Pandemic
Candidate Vaccine Virus (CVV)
Suspension cell culture
Reverse genetics
Transient transfection
Large-scale

ABSTRACT

Despite major advances in developing capacities and alternative technologies to egg-based production of influenza vaccines, responsiveness to an influenza pandemic threat is limited by the time it takes to generate a Candidate Vaccine Virus (CVV) as reported by the 2015 WHO Informal Consultation report titled "Influenza Vaccine Response during the Start of a Pandemic".

In previous work, we have shown that HEK-293 cell culture in suspension and serum free medium is an efficient production platform for cell culture manufacturing of influenza candidate vaccines. This report, took advantage of, recombinant DNA technology using Reverse Genetics of influenza strains, and advances in the large-scale transfection of suspension cultured HEK-293 cells. We demonstrate the efficient generation of H1N1 with the PR8 backbone reassortant under controlled bioreactor conditions in two sequential steps (transfection/rescue and infection/production). This approach could deliver a CVV for influenza vaccine manufacturing within two-weeks, starting from HA and NA pandemic sequences. Furthermore, the scalability of the transfection technology combined with the HEK-293 platform has been extensively demonstrated at >100 L scale for several biologics, including recombinant viruses.

Thus, this innovative approach is better suited to rationally engineer and mass produce influenza CVV within significantly shorter timelines to enable an effective global response in pandemic situations.
Crown Copyright © 2017 Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

The influenza virus is responsible for a global epidemic every year that infects millions of people and causes serious illness and death worldwide [1]. Vaccination remains the primary and most effective strategy for the prevention and control of influenza. Due to the high mutation rate of the influenza virus, vaccine manufacturers must reformulate their products every year to ensure a good match between the HA and NA present in the vaccine and the circulating strains [2]. The majority of the current licensed influenza vaccines are made using embryonated hen's eggs, a system that has been used for more than 60 years [3]. Increased demand and the threat of a pandemic outbreak have accelerated the introduction of new manufacturing strategies for influenza vaccine production. The US Human Health Services actively supported the development of alternative manufacturing strategies for influenza vaccine. For example, cell-based production technology allows manufacturers to respond to public health needs faster and in shorter production cycles. It also allows for greater surge capacity, greater process control and more reliable and well-characterized products [4–6]. Yet in spite of major advancements and licensing of cell culture manufactured processes [7], responsiveness to an influenza pandemic threat remains limited by the timely availability of the "master viral seed stock" or Candidate Vaccine Virus (CVV) as reported in the last WHO Informal Consultation report 2015 titled "Influenza Vaccine Response during the Start of a Pandemic" [8].

Reverse genetics can be used to streamline the generation of viral seed stocks, which would be vital in case of a pandemic outbreak, thereby avoiding the long and cumbersome reassortant generation process in eggs [2,9]. Reverse genetics is a process to generate live viruses from a set of plasmid-cloned cDNA collectively encoding the influenza viral genome [10,11]. This strategy can be used to generate specific and high-growth 6 + 2 reassortant

* Corresponding author at: Department of Bioengineering, MacDonald Engineering Building, Room 270. 810 Sherbrooke Street West, Montreal, QC H3A 0C3, Canada.
E-mail addresses: amine.kamen@mcgill.ca, amine.kamen@gmail.com (A. Kamen).

http://dx.doi.org/10.1016/j.vaccine.2017.04.065
0264-410X/Crown Copyright © 2017 Published by Elsevier Ltd.
This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

viruses integrating only the HA and NA gene segments of the seasonal strain within the Puerto Rico backbone for further use as viral seeds in egg-based or cell culture technology [12]. However, all current protocols for obtaining influenza viral particles by reverse genetics use a co-culture of adherent cells [13], such as HEK293T, COS-1, Vero, CHOK1 or MDCK [14–16], limiting the scalability of the system. The use of suspension cell lines such as HEK-293 improves the scalability of the process, which is a key element in large-scale cell culture vaccine production [17,18]. Building on extensive developments and expertise from our group and others in large-scale transient transfection using HEK-293 suspension cultures to produce RNA and DNA recombinant viral vectors [19–23], we demonstrate that influenza PR8 can be effectively produced by reverse genetics from transfected suspension HEK-293 cells at both small and large-scale after only one amplification step. Moreover, the characterization of produced virions showed no change of the viral antigen sequences Overall, we propose that HEK-293 suspension cells can be used as a production platform for influenza A viruses as an alternative and rapid system to obtain reassortant viral seed stocks (CVV) for vaccine manufacture.

2. Materials and methods

2.1. Cell lines and culture conditions in small and large scale

The human embryonic kidney (HEK-293SF) cells adapted to suspension and derived from a cGMP master cell bank were cultured in serum-free SFM4Transfx-293™ (HyClone, USA) supplemented with 4 mM of L-glutamine (HyClone, USA) in a humidified incubator at 37 °C with 5% CO_2, at an agitation rate of 100–110 rpm. MDCK cells were cultured in EMEM medium (ATCC, USA) supplemented with 10% (v/v) Fetal Bovine Serum (NorthBio, Canada) in a humidified incubator at 37 °C with 5% CO_2.

For large-scale experiments, a 3-L Chemap type SG bioreactor (Mannedorf, Switzerland) was used with a working volume of 1.8 L at an initial concentration of 0.16×10^6 cells/mL. The bioreactor features were published in previous publication of our group [24]. Cells were grown for 72 h until reaching a density of $\sim 1.0 \times 10^6$ cells/mL for transfection. Samples were taken once a day for cell culture and viral production monitoring.

2.2. Transient transfection

2.2.1. Plasmids

The cDNA obtained from the different genes of the virus A/Puerto Rico/8/34 (H1N1) were cloned into the pHW2000 vector [14]. Briefly, viral RNA was extracted by using QIAmp viral RNA minikit (Qiagen) from infected-MDCK cell culture supernatant, according to the manufacturer's instructions. Two-step RT-PCR was carried out for full-length amplification of each viral RNA gene segment, by using influenza A universal RT primers (Uni-12primer «3′-AGCAAAAGCAGG-5′», Eurogentec, Belgium) as previously described [25].

The eight plasmids [pHW2000-PB1, pHW2000-PB2, pHW2000-PA, pHW2000-NA, pHW2000-M, pHW2000-NS, pHW2000-HA and pHW2000-NP] were amplified using the bacterial strain *E. coli* TOP10 (Invitrogen, USA) and purified using the GigaPrep Extraction Kit (Macherey Nagel, USA). Plasmid DNA quantity was assessed by NanoDrop (Thermo Scientific, USA). The viral sequences within the eight plasmids were controlled and validated by sequencing by MWG Eurofins Company.

2.2.2. Transfections in shake flasks

Suspension HEK-293 cells at 1×10^6 cells/mL were transfected with 25-kDa linear polyethylenimine (PEIpro) (PolySciences, USA) in 20 mL working volume shake flasks. Complexes were generated by mixing 1 μg of total plasmid DNA/mL cell culture and 2 μg PEIpro/mL cell culture. Mixture was vortexed for 5 s and incubated at room temperature for 10–15 min. After incubation the volume of transfection was adjusted to 10% total volume with medium and transferred drop-by-drop into the shake flasks. The flasks were incubated at 37 °C and 5% CO_2. After 24 h post transfection (hpt), 1 μg/mL TPCK-Trypsin (Affymetrix, USA) was added. The viral particles were harvested at 48 hpt generating the P0 of the influenza A/Puerto Rico/8/34 virus. For the P0 amplification and generation of P1, 10 mL of the supernatant of P0 were used to infect 10 mL of suspension HEK-293 cells at 2×10^6 cells/mL in medium with 1 μg/mL TPCK-Trypsin (final concentration). The infected flasks were incubated for 96 h post-infection (hpi).

2.2.3. Transfection in 3-L bioreactor scale

Cells at 1×10^6 cells/mL (1.8 L) were transfected using 200 mL (10% of the final volume) of medium, PEIpro and plasmid DNA for a final volume of 2L in the bioreactor. Complexes were generated as described above. The mixture was transferred into the bioreactor by gravity using the inoculum bottle. After 24 hpt, 1 μg/mL TPCK-Trypsin (final concentration) was added into the bioreactor. After 48 hpt the bioreactor was harvested (P0) and used to infect 20 mL suspension HEK-293 (2×10^6 cells/mL) in shake flask to generate the P1 following the protocol described above (Fig. 1). Table 1 summarizes the main conditions used in this work for cell transfection in shake flasks and bioreactor.

2.3. Influenza virus quantification and characterization

2.3.1. Tissue culture infectious dose at 50% assay ($TCID_{50}$)

The infectious titer (IVP/ml) of the viruses was quantified by the tissue culture infectious dose at 50% ($TCID_{50}$) assay as previously described [24]. The cytopathic effects were measured using Alamar blue (Life Technologies) [26].

2.3.2. Dot Blot assay

The protocol followed in this study for hemagglutinin (HA) and neuraminidase (NA) has been previously published [27]. Briefly, a calibration curve was generated using a recombinant protein for NA (H1N1/A/USSR/90/77 from Sino Biological Inc., China) and an in-house standard for HA PR8 virus semi-purified by sucrose cushion and previously quantified by SRID. The primary antibody used for the dot-blot assay against HA was an anti-HA monoclonal antibody produced in-house; the membranes were probed with 6 μg/ml anti-HA antibody overnight at 4 °C. For the dot-blot against NA, a universal rabbit anti-NA HCA-2 antibody was kindly provided by Health Canada [28]. Infrared-conjugated secondary antibodies (LI-COR Bioscience, USA) were used to reveal the signal with an Odyssey CLx imaging system (LI-COR Bioscience, USA).

2.3.3. Single radial immunodiffusion assay

A previously published protocol [29] was used. In absence of the final calibration of antigen reagent 14/200 from NIBSC at the time of the study, an in-house reference material using an influenza A PR8 sucrose cushion stock extensively characterized was used for the standard curve. The antibody used to form precipitation rings was a sheep polyclonal antibody (Ref. 03/242, NIBSC, UK).

2.3.4. Hemagglutination assay

The protocol for hemagglutination (HA) assay described previously was used to determine the quantity of Total Viral Particles (VP)/ml [29,30].

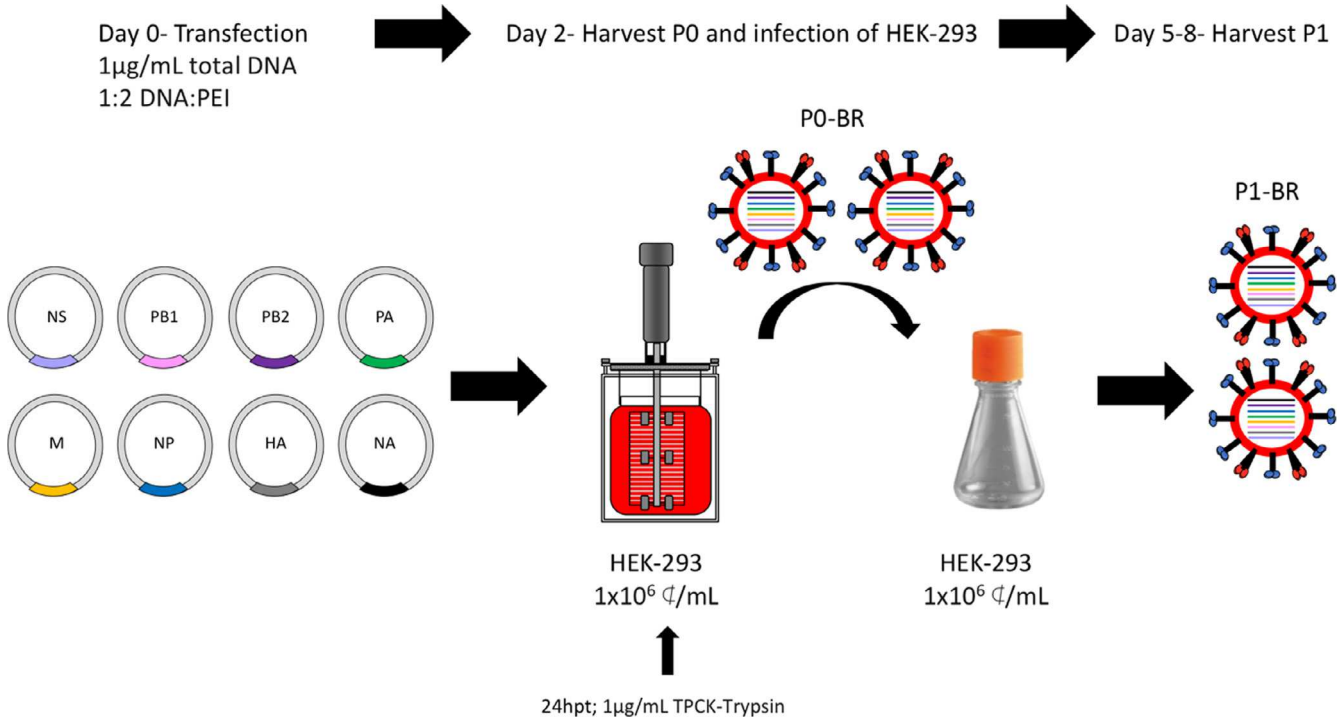

Fig. 1. Schematic representation of the protocol carried out for reverse genetics generation of influenza A/Puerto Rico/8/34 virus in 3L-bioreactor. Suspension HEK-293 cells were cultured in a 3L-bioreactor until the cell density reached 1×10^6 cells/mL. Then, cells were transiently transfected with 8 plasmids and the supernatant was harvested after 48 hpt. The viral particles produced in the supernatant (P0-BR) were amplified by infecting a new culture of suspension HEK-293 cells in shake flask and after 72–96 hpi the supernatant was harvested for viral titration (P1-BR).

Table 1
Summary of the key parameters used for cell transfection.

Condition	Total plasmid DNA (µg)	Mass ratio DNA-reagent	Vol. transfected (mL)	Cell density @ time of transfection (cell/mL)	Cell-DNA ratio (cell/µg)
Shake flasks	1	1:2	20	1×10^6	1×10^6
Bioreactor	1	1:2	2000	1.3×10^6	1.3×10^6

2.3.5. Viral titration of total particles (VP) with Tunable Resistive Pulse Sensor

Total virus particle production was quantified by Tunable Resistive Pulse Sensor (TRPS). This technology is based on a size-tunable pore measuring nanoparticle size by resistive pulse sensing [31]. This technology measures the increased electrical resistance induced by the passage of nanoparticles through a pore filled with a conductive liquid. All measurements were made using an Izon qNano (New Zealand). Polystyrene calibration particles with a concentration of 1.0×10^{13} particles/mL and a mode size of 114 nm were purchased from Thermo Fisher Laboratories [32]. All samples were dispersed in phosphate buffered saline (Solution Q: PBS 137 mM & 0.03% Tween from Izon Science) for analysis. Particle concentration and size were calculated using the Izon Control Suite Software V3.3 on a minimum of 500 particle events on a Nanopore Np 100.

2.3.6. Negative stain electron microscopy

Sucrose cushion purified samples containing influenza virus were obtained using a protocol described elsewhere [33] and analyzed by Negative Stain Electron Microscopy (NSEM) at Institut Armand-Frappier (Laval, Canada) according to a method described previously [34]. Samples were concentrated 25× for the P0-BR 48 hpt, 20× for the P1-BR 96 hpi and 10× for the reference PR8. Total viral particles concentration expressed as viral particles/mL (VP/mL) was quantified by NSEM as: VP/mL = (virus particle count/latex beads count) × (latex beads concentration ∗ virus dilution).

2.3.7. HA influenza A/Puerto Rico/8/34 sequencing

Viral RNA (vRNA) was extracted from viral particles collected in 140 µl of cell culture surpernatant with QIAamp Viral RNA Mini Kit (250) (Qiagen, ref 52906). RNA extraction was performed according to manufacturer protocol and eluted in 60 µl AVE Buffer. vRNA of HA and NA proteins were amplified in cDNA by RT-PCR with optimized protocol based on universal primer Uni-12 and AMV RT enzyme. HA and NA cDNAs were then amplified by PCR with optimized protocol using Pfu polymerase, as previously described [25]. Sequencing was then performed by MWG Eurofins Company on amplified cDNA with these specific primers. Alignment was realized on Bioedit software with PR8 sequence obtained from NCBI database.

3. Results and discussion

3.1. Generation of H1N1 influenza A/Puerto Rico/8/34 (PR8) by reverse genetics in shake flasks

A feasibility study was first performed in 6-well plates and is presented in supplementary material: Suspension HEK-293 cells were transfected with 8 plasmids (8 µg DNA) using TransIT-LT1 (Mirus, USA) as a transfection reagent. The resulting virus was amplified in MDCK adherent cells as well as suspension HEK-293 cells, demonstrating that HEK-293 cells can generate influenza virus from plasmid transfection (Supplementary Table 1). These preliminary results provided evidence of influenza PR8 generation

Table 2
Influenza A/Puerto Rico/8/34 production in suspension HEK-293 cells in shake flasks. N.D. = No Determined.

Samples	Infectious titer (IVP/mL)	Dot-Blot (μg/ml)	
		HA	NA
P0-SF 48 hpt	1.5×10^5	N.D.	N.D.
P1-SF 96 hpi	1.0×10^7	1.6 ± 0.6	0.25 ± 0.04

from transfection of suspension HEK-293 cells and amplified in the same cell line. With the goal to assess process scalability, influenza A/Puerto Rico/8/34 was also produced in shake flasks using the 25-kDa linear transfection reagent PEIPro (Polyplus). PEI is more suitable for large-scale transfection due to its high transfection efficiency in suspension-growing HEK-293 cells and cost-effectiveness [35]. As PEIPro has shown some toxicity effect on suspension HEK-293 cells [36], shake flasks experiments were performed to determine the optimal DNA and PEIPro concentration. Results (Table 2) showed that 1 μg/mL concentration of total plasmid DNA was sufficient to generate higher virus titers after only one amplification step (1.0×10^7 IVP/ml) in suspension HEK-293 cells over the 8 μg/mL DNA concentration used in 6-well plates (6.2×10^6 IVP/ml) (Supplementary Table 1). This DNA: PEI mass ratio was also found to be optimal in previous productions using suspension HEK-293 cells [37–39]. After 48 h post-transfection (hpt) the supernatant was harvested and constituted the P0-SF viral passage. The P0-SF viral stock was used to infect suspension HEK-293 cells and the supernatant (P1-SF) was collected after 96 h post-infection (hpi). Presence of HA and NA antigens were detected by dot-blot only in P1 samples since the amount of viral particles allowed their detection [40]. Viral titer generated after 48 hpt (1.5×10^5 IVP/ml) were slightly lower than those obtained previously by reverse genetics in co-cultured 293T-MDCK by Hoffmann et al. [11] with 2×10^6 IVP/mL measured at 48 hpt, but significantly higher than titers obtained in Vero-MDCK co-cultured cells (1×10^4 IVP/mL) [13]. Influenza A values obtained at P1-SF were also comparable to values previously described in suspension-growing HEK-293 cells (2.7×10^6 IVP/mL) [24]. The MOI used for infection was unknown at the time of amplification since infection was performed right away with fresh supernatant, but a MOI of 0.08 could be back-calculated. This value is similar to MOI values used to produce influenza virus using conventional methods (MOI of 0.001–0.01) [24].

3.2. Production and characterization of influenza virus A/Puerto Rico/8/34 (H1N1) in bioreactor scale

The feasibility of the production of influenza virus generated by reverse genetics at large-scale was further assessed in a fully instrumented bioreactor (3-L scale). The bioreactor was inoculated at 0.15×10^6 cells/mL and transfected at 1×10^6 cells/mL (Fig. 2A). Temperature, pH and dissolved oxygen were maintained constant and monitored during the run (Fig. 2B). As was done in the shake flasks, cells were transfected using 1 μg/mL total plasmid DNA and a DNA:PEI ratio of 1:2. At 24 hpt, 1 μg/mL TPCK-Trypsin was added into the bioreactor. The supernatant was harvested after 48 hpt and used to infect suspension HEK-293 cells shake flasks (20 mL working volume) for virus amplification (P1-BR) that was harvested after 72 and 96 hpi. Harvested influenza PR8 generated in the bioreactor (P0-BR) and the amplified virus (P1-BR) were quantified by $TCID_{50}$, HA assay, SRID, NSEM and Dot-Blot assays (Table 3). Infectious titers measured in the 3-L bioreactor transfection were comparable to the titers obtained in shake flasks (P0-SF and P1-SF) with a maximum value of infectious particles of 3.5×10^7 IVP/mL. No increase in the production titers of infectious particles was detected between 72 hpi and 96 hpi, although an increase of HA protein was measured by Dot-Blot and SRID assay. This effect was previously observed for infection kinetics [24,41]. Hemagglutination (HA) assay did not show differences between the P1 samples harvested at 72 and 96 hpi, likely because this assay detects the amount of total HA protein present in the sample, including the HA on the infectious particles, on empty influenza particles, on fragments of cell membrane carrying HA and the free HA. Instead, $TCID_{50}$ detects only viral particles with infectious capacity. The variability of the techniques such as the detection of trimeric HA for SRID and monomeric HA for Dot-Blot may explain the variation between the different viral kinetic trends [42].

It is important to highlight that the overall process only takes 9 days from the inoculation of the bioreactor to the harvest of the amplified virus (P1). Interestingly, with such a bioreactor production level a new batch of 100L could be infected for amplification in manufacturing process. The short production time contrasts with the overall 6 months and the 3–6 weeks required for production of High Growth Virus for egg-based influenza vaccine production [12]. Characterization of viral particles obtained from bioreactor transfection and subsequent amplification showed that

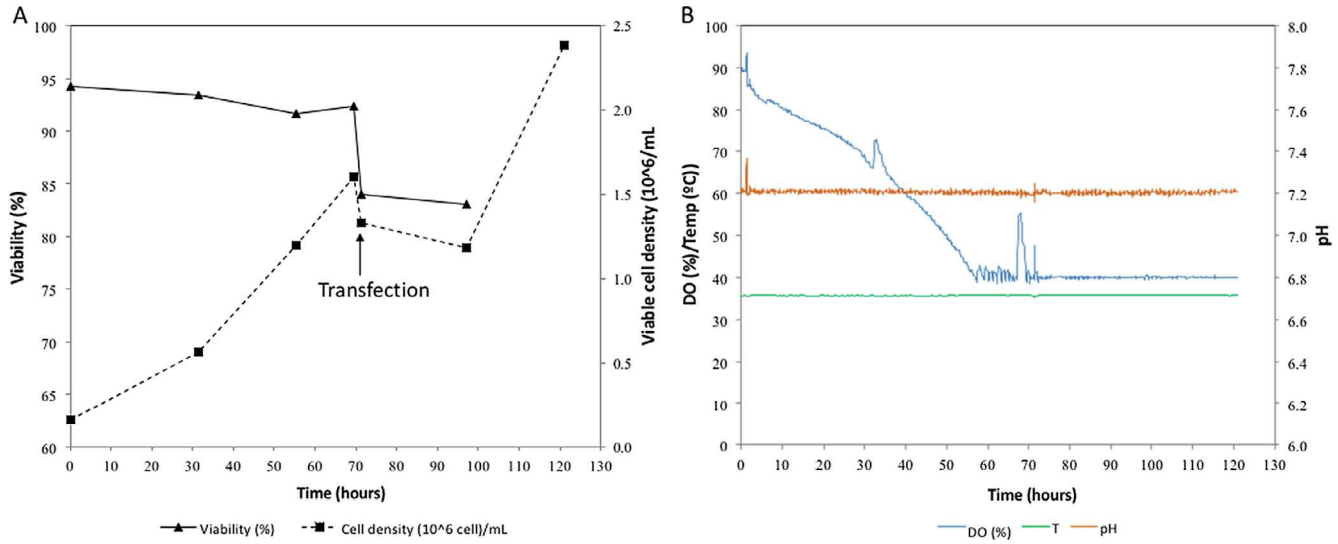

Fig. 2. Influenza A/Puerto Rico/8/34 production in 3L-bioreactor by reverse genetics. (A) Cell density and viability. (B) Bioreactor monitoring data.

Table 3
3-L Bioreactor Influenza A/Puerto Rico/8/34 production in suspension HEK-293 cells. N.D. = No Determined.

Samples	Volume (mL)	Infectious titer (IVP/mL)	Dot-Blot (μg/ml)		SRID (μg/ml)	HA-assay (HAU/mL)	NSEM (VP/mL)	Based on HA-Assay	Total Particle Count (VP)/mL			
			HA	NA	HA	HA		Total VP/mL	50–600 nm	50–100 nm	100–200 nm	>200 nm
P0-BR 48 hpt	2000	5.0×10^4	N.D.	N.D.	N.D.	67.8	1.3×10^8	1.4×10^8	7.3×10^9	2.9×10^9	3.4×10^9	1.1×10^9
P1-BR 72 hpi	20	3.5×10^7	2.0 ± 0.1	0.2 ± 0.02	6.3 ± 0.6	3548.1	N.D.	7.5×10^9	3.7×10^{10}	6.4×10^9	2.1×10^9	9.3×10^9
P1-BR 96 hpi	20	2.8×10^7	3.1 ± 0.43	1.2 ± 0.06	10.2 ± 0.3	3548.1	8.0×10^8	7.5×10^9	2.2×10^{10}	3.8×10^9	1.3×10^{10}	5.8×10^9

on P0-BR sample much of the total particles were non-infectious or exosomes. Two orthogonal populations were observable on TRPS plots (Fig. 3A). The exosomes are particles with size range below 100 nm with a longer bioclade baseline duration (duration through the pore). These parameters also refer to the charge carried by the particles counted. In contrast, the infectious particles generated a more homogeneously charged population demonstrated by the bioclade baseline duration of within 5 ms for P1-BR (96 hpi) and reference viral particles produced by infection (Fig. 3B). The morphology observed by NSEM of the PR8 virus generated by reverse genetics (Fig. 4A and B) was identical to the reference influenza PR8 (Fig. 4C). Moreover, the NSEM images confirm the presence of endosomes in the P0-BR sample, making it less accurate in the NSEM quantification, therefore the concentration of P0-BR sample obtained by NSEM was superior to the value obtained with $TCID_{50}$. The P1-BR (96 hpi) sample showed a more homogenous population. Taken together, these results indicate the generation of biologically active influenza virus. Viral genome extracted from production at P0 and P1 were sequenced to confirm that reverse genetics at large-scale did not induce variation of the viral population, especially on the two major antigens, HA and NA. PR8 HA and NA sequences were aligned using Bioedit software (IBIS Bioscience, Carlsbad, USA) with the original plasmid sequence and with one of the reference sequences obtained from NCBI database for PR8. Sequences from P0 and P1 demonstrate 100% homology with the original reverse genetics plasmid sequences (Supplementary material for HA and data not shown for NA) demonstrating no change of the viral antigen sequences PR8. These results indicate that transfection with eight plasmids for influenza A/Puerto Rico/8/34 can be performed at bioreactor scale using suspension HEK-293 cells generating high amounts of viral particles after only one amplification step.

4. Conclusion

In their preparedness plans to respond to influenza pandemic threats, National and International Public Health Organizations emphasized the need to build capacities and adopt advanced technologies to deliver pandemic influenza vaccines in a timely fashion. Cell culture-based production processes have the potential to address these needs [43]. Combining the use of reverse genetics and advances in large-scale transient transfection of mammalian suspension cell cultures provides a strategic advantage in generating reassortant virus seed stocks as a Candidate Vaccine Virus within timelines compatible with a pandemic situation. Overall, adoption of this approach could be of great interest to WHO influenza reference centers and industrial vaccine manufacturers as it would significantly speed up the early phase of the process. Furthermore, limiting the rescue and production of the influenza CVV to two steps in a human cell line would minimize the mismatch of the viral strain in the vaccine formulation and limit the amino acid substitutions in the HA glycoprotein due to the avian-host or egg-host propagation [44]. Results from the present work provide solid evidence to support the use of mammalian cell culture production processes and recombinant technologies to further accelerate the delivery of the influenza vaccines. Here, we demonstrate for the first time that suspension HEK-293 cells can be efficiently transfected not only at small-scale but also at bioreactor scale with eight reverse genetically engineered plasmids. We demonstrated that this methodology followed by infection amplification allowed the generation of high titers of influenza PR8 strain in two steps. Virus yield as high as 10^7 IVP/mL were achieved for both shake flasks and bioreactor production, indicating that HEK-293 cell culture is a robust system to obtain viral seed stock for influenza production within a very short period (9 days). This is comparable to the 7 days

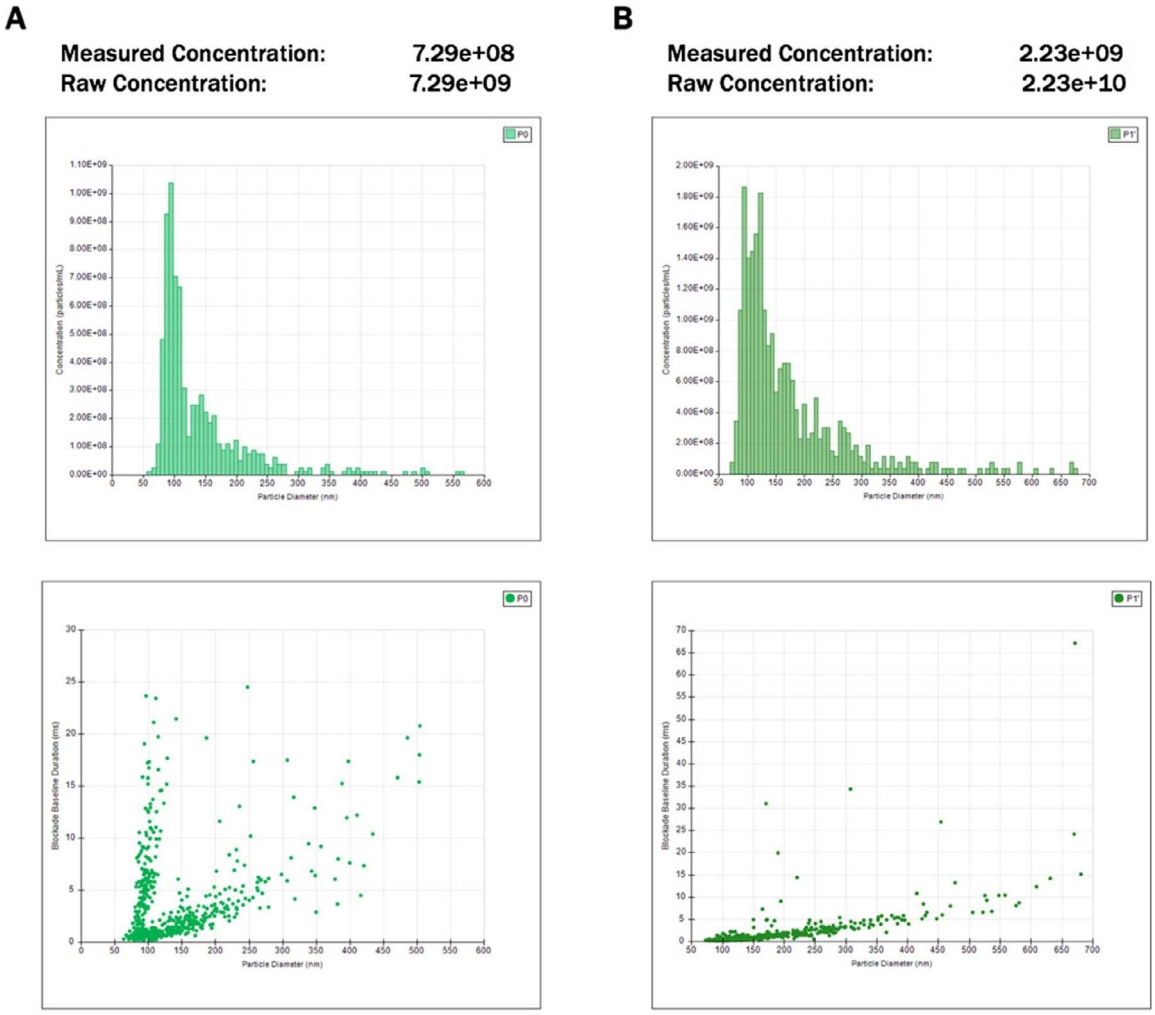

Fig. 3. Characterization of influenza A/Puerto Rico/8/34 produced in 3L-bioreactor by reverse genetics. (A) Tunable Resistive Pulse Sensor (TRPS) plot for P0-BR sample and (B) P1-BR (96 hpi) sample.

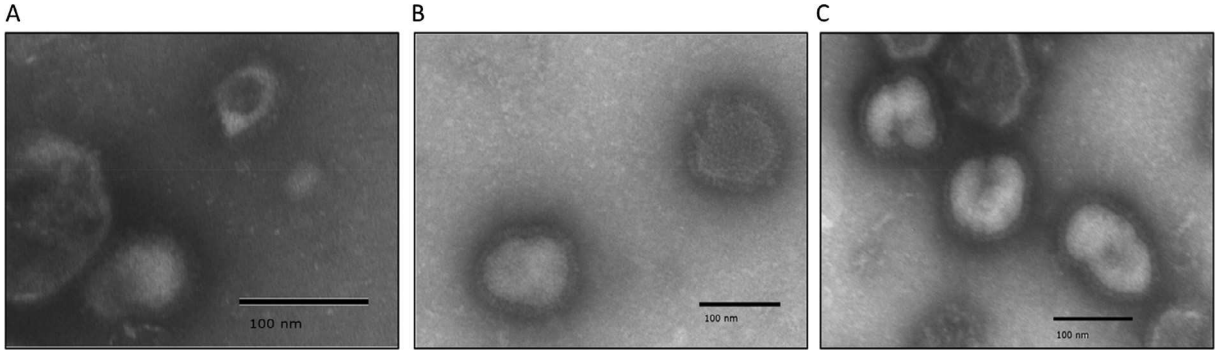

Fig. 4. Negative stain electron microscopy (NSEM) image of PR8 virions obtained in 3L-bioreacor by reverse genetics. (A) P0-BR sample (B) P1-BR (96 hpi) sample and (C) PR8 reference material. Each virion presented a sharp fringe of evident spikes outside likewise to PR8 virus obtained in previous work [24,33]. Bar represents 100 nm. Picture magnification: 40,000×.

required for the HA-based VLP production in plants reported by Medicago Inc. [45,46] or recombinant HA vaccine produced in insect cell culture [47,48]. Importantly, it is significantly shorter than the 3–6 weeks currently needed for egg-based technology to generate reassortant for the CVV [49]. The influenza virus produced by the method described in this report was functional and had all the critical quality attributes for a CVV. Consequently, results from this work and previously published work [24] contribute to establish the cGMP-compliant HEK-293 expression platform as a suitable system for industrial manufacturing of influenza vaccines starting from identified HA and NA sequences of dominantly circulating strains or pandemic strains.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

The authors would like to thank Danielle Jacob for the bioreactor operations, Shu Zhuang for the plasmid purification, Melanie Simoneau for plasmid sequencing and Rénald Gilbert and his team at NRC for valuable advises. Also, the authors would like to thank Micheline Letarte from Institut Armand-Frappier (Laval, Canada) for the electron microscopy analyses and Camille Roesch from IZON company for the TRPS samples analyses. This work was partially funded through Natural Sciences and Engineering Research Council Grants RGPIN-2015-132; STPGP-462995-14 and Canada Research Chair CRC-240394.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2017.04.065.

References

[1] Rolfes MA, Foppa IM, Garg S, Flannery B, Brammer L, Singleton JA et al. Estimated Influenza Illnesses, Medical Visits, Hospitalizations, and Deaths Averted by Vaccination in the United States, n.d. <https://www.cdc.gov/flu/about/disease/2015-16.htm>.
[2] Minor PD, Engelhardt OG, Wood JM, Robertson JS, Blayer S, Colegate T, et al. Current challenges in implementing cell-derived influenza vaccines: implications for production and regulation, July 2007, NIBSC, Potters Bar, UK. Vaccine 2009;27:2907–13. http://dx.doi.org/10.1016/j.vaccine.2009.02.064.
[3] Partridge J, Kieny MP. Global production capacity of seasonal influenza vaccine in 2011. Vaccine 2013;31:728–31. http://dx.doi.org/10.1016/j.vaccine.2012.10.111.
[4] Wong S-S, Webby RJ. Traditional and new influenza vaccines. Clin Microbiol Rev 2013;26:476–92. http://dx.doi.org/10.1128/CMR.00097-12.
[5] Dormitzer PR, Tsai TF, Del Giudice G. New technologies for influenza vaccines. Hum Vaccin Immunother 2012;8:45–58.
[6] Hilleringmann M, Jobst B, Baudner BC. Influenza cell-culture vaccine production. In: Giese M, editor. Mol. Vaccines, vol. 2. Springer; 2014. p. 823–37. http://dx.doi.org/10.1007/978-3-319-00978-0.
[7] Milián E, Kamen AA. Current and emerging cell culture manufacturing technologies for influenza vaccines. Biomed Res Int 2015. http://dx.doi.org/10.1155/2015/504831.
[8] WHO Global Influenza Programme. Report of a WHO Informal Consultation held in Geneva, Switzerland Influenza Vaccine Response during the Start of a Pandemic Influenza Vaccine Response during the Start of a Pandemic; 2015.
[9] Ulmer JB, Valley U, Rappuoli R. Vaccine manufacturing: challenges and solutions. Nat Biotechnol 2006;24:1377–83. http://dx.doi.org/10.1038/nbt1261.
[10] Neumann G, Watanabe T, Ito H, Watanabe S, Goto H, Gao P, et al. Generation of influenza A viruses entirely from cloned cDNAs. Proc Natl Acad Sci 1999;96:9345–50. http://dx.doi.org/10.1073/pnas.96.16.9345.
[11] Hoffmann E, Neumann G, Kawaoka Y, Hobom G, Webster RG. A DNA transfection system for generation of influenza A virus from eight plasmids. Proc Natl Acad Sci USA 2000;97:6108–13. http://dx.doi.org/10.1073/pnas.100133697.
[12] WHO. Process of influenza vaccine virus selection and development. World Heal Organ; 2007. p. 1–11.
[13] Hoffmann E, Krauss S, Perez D, Webby R, Webster RG. Eight-plasmid system for rapid generation of influenza virus vaccines. Vaccine 2002;20:3165–70. http://dx.doi.org/10.1016/S0264-410X(02)00268-2.
[14] Hoffmann E, Webster RG. Unidirectional RNA polymerase I-polymerase II transcription system for the generation of influenza A virus from eight plasmids. J Gen Virol 2000;81:2843–7.
[15] Medina J, Guillot V, Totain E, Rouleau M, Sodoyer R, Moste C, et al. Vero/CHOK1, a novel mixture of cell lines that is optimal for the rescue of influenza A vaccine seeds. J Virol Methods 2014;196:25–31. http://dx.doi.org/10.1016/j.jviromet.2013.09.007.
[16] De Wit E, Spronken MIJ, Bestebroer TM, Rimmelzwaan GF, Osterhaus ADME, Fouchier RaM. Efficient generation and growth of influenza virus A/PR/8/34 from eight cDNA fragments. Virus Res 2004;103:155–61. http://dx.doi.org/10.1016/j.virusres.2004.02.028.
[17] Lohr V, Genzel Y, Behrendt I, Scharfenberg K, Reichl U. A new MDCK suspension line cultivated in a fully defined medium in stirred-tank and wave bioreactor. Vaccine 2010;28:6256–64. http://dx.doi.org/10.1016/j.vaccine.2010.07.004.
[18] Aubrit F, Perugi F, Léon A, Guéhenneux F, Champion-Arnaud P, Lahmar M, et al. Cell substrates for the production of viral vaccines. Vaccine 2015;33:5905–12. http://dx.doi.org/10.1016/j.vaccine.2015.06.110.
[19] Durocher Y, Pham PL, St-Laurent G, Jacob D, Cass B, Chahal P, et al. Scalable serum-free production of recombinant adeno-associated virus type 2 by transfection of 293 suspension cells. J Virol Methods 2007;144:32–40. http://dx.doi.org/10.1016/j.jviromet.2007.03.014. S0166-0934(07)00124-3 [pii]/r.
[20] Chahal PS, Schulze E, Tran R, Montes J, Kamen AA. Production of adeno-associated virus (AAV) serotypes by transient transfection of HEK293 cell suspension cultures for gene delivery. J Virol Methods 2014;196:163–73. http://dx.doi.org/10.1016/j.jviromet.2013.10.038.
[21] Segura MM, Garnier A, Durocher Y, Coelho H, Kamen A. Production of lentiviral vectors by large-scale transient transfection of suspension cultures and affinity chromatography purification. Biotechnol Bioeng 2007;98:789–99. http://dx.doi.org/10.1002/bit.21467.
[22] Meneses-Acosta A, Dormond E, Jacob D, Tom R, Bernier A, Perret S, et al. Development of a suspension serum-free helper-dependent adenovirus production system and assessment of co-infection conditions. J Virol Methods 2008;148:106–14. http://dx.doi.org/10.1016/j.jviromet.2007.10.017.
[23] Dormond E, Meneses-Acosta A, Jacob D, Durocher Y, Gilbert R, Perrier M, et al. An efficient and scalable process for helper-dependent adenoviral vector production using polyethylenimine-adenofection. Biotechnol Bioeng 2009;102:800–10. http://dx.doi.org/10.1002/bit.22113.
[24] Le Ru A, Jacob D, Transfiguracion J, Ansorge S, Henry O, Kamen AA. Scalable production of influenza virus in HEK-293 cells for efficient vaccine manufacturing. Vaccine 2010;28:3661–71. http://dx.doi.org/10.1016/j.vaccine.2010.03.029.
[25] Moulès V, Terrier O, Yver M, Riteau B, Moriscot C, Ferraris O, et al. Importance of viral genomic composition in modulating glycoprotein content on the surface of influenza virus particles. Virology 2011;414:51–62. http://dx.doi.org/10.1016/j.virol.2011.03.011.
[26] Mo C, Yamagata R, Pan A, Reddy J, Hazari N, Duke G. Development of a high-throughput Alamar blue assay for the determination of influenza virus infectious dose, serum antivirus neutralization titer and virus ca/ts phenotype. J Virol Methods 2008;150:63–9. http://dx.doi.org/10.1016/j.jviromet.2008.03.006.
[27] Li C, Jaentschke B, Song Y, Wang J, Cyr TD, Van Domselaar G, et al. A simple slot blot for the detection of virtually all subtypes of the influenza A viral hemagglutinins using universal antibodies targeting the fusion peptide. Nat Protoc 2010;5:14–9. http://dx.doi.org/10.1038/nprot.2009.200.
[28] Gravel C, Li C, Wang J, Hashem AM, Jaentschke B, Van Domselaar G, et al. Quantitative analyses of all influenza type A viral hemagglutinins and neuraminidases using universal antibodies in simple slot blot assays. J Vis Exp 2011:5–11. http://dx.doi.org/10.3791/2784.
[29] Thompson CM, Petiot E, Mullick A, Aucoin MG, Henry O, Kamen AA. Critical assessment of influenza VLP production in Sf9 and HEK293 expression systems. BMC Biotechnol 2015;15:31. http://dx.doi.org/10.1186/s12896-015-0152-x.
[30] Venereo-sanchez A, Gilbert R, Simoneau M, Caron A, Chahal P, Chen W, et al. Hemagglutinin and neuraminidase containing virus-like particles produced in HEK-293 suspension culture: an effective influenza vaccine candidate. Vaccine 2016;34:3371–80. http://dx.doi.org/10.1016/j.vaccine.2016.04.089.
[31] Kozak D, Anderson W, Grevett M, Trau M. Modeling elastic pore sensors for quantitative single particle sizing. J Phys Chem C 2012;116:8554–61. http://dx.doi.org/10.1021/jp211845t.
[32] Vogel R, Willmott G, Kozak D, Roberts GS, Anderson W, Groenewegen L, et al. Quantitative sizing of nano/microparticles with a tunable elastomeric pore sensor. Anal Chem 2011;83:3499–506. http://dx.doi.org/10.1021/ac200195n.
[33] Transfiguracion J, Manceur AP, Petiot E, Thompson CM, Kamen Aa. Particle quantification of influenza viruses by high performance liquid chromatography. Vaccine 2015;33:78–84. http://dx.doi.org/10.1016/j.vaccine.2014.11.027.
[34] Alain R, Nadon F, Séguin C, Payment P, Trudel M. Rapid virus subunit visualization by direct sedimentation of samples on electron microscope grids. J Virol Methods 1987;16:209–16. http://dx.doi.org/10.1016/0166-0934(87)90005-X.
[35] Schlaeger EJ, Christensen K. Transient gene expression in mammalian cells grown in serum-free suspension culture. Cytotechnology 1999;30:71–83. http://dx.doi.org/10.1023/A:1008000327766.
[36] Cervera L, Gutiérrez-Granados S, Martínez M, Blanco J, Gòdia F, Segura MM. Generation of HIV-1 Gag VLPs by transient transfection of HEK 293 suspension cell cultures using an optimized animal-derived component free medium. J Biotechnol 2013;166:152–65. http://dx.doi.org/10.1016/j.jbiotec.2013.05.001.
[37] Durocher Y, Perret S, Kamen A. High-level and high-throughput recombinant protein production by transient transfection of suspension-growing human 293-EBNA1 cells. Nucl Acids Res 2002;30. e9–e9.
[38] Pham PL, Perret S, Doan HC, Cass B, St-Laurent G, Kamen A, et al. Large-scale transient transfection of serum-free suspension-growing HEK293 EBNA1 cells: peptone additives improve cell growth and transfection efficiency. Biotechnol Bioeng 2003;84:332–42. http://dx.doi.org/10.1002/bit.10774.
[39] Ansorge S, Lanthier S, Transfiguracion J, Durocher Y, Henry O, Kamen A. Development of a scalable process for high-yield lentiviral vector production by transient transfection of HEK293 suspension cultures. J Gene Med 2009;11:868–76. http://dx.doi.org/10.1002/jgm.1370.
[40] Thompson CM, Petiot E, Lennaertz A, Henry O, Kamen AA. Analytical technologies for influenza virus-like particle candidate vaccines: challenges and emerging approaches. Virol J 2013;10:141. http://dx.doi.org/10.1186/1743-422X-10-141.
[41] Petiot E, Jacob D, Lanthier S, Lohr V, Ansorge S, Kamen AA. Metabolic and kinetic analyses of influenza production in perfusion HEK293 cell culture. BMC Biotechnol 2011;11:84. http://dx.doi.org/10.1186/1472-6750-11-84.

[42] Manceur AP, Kamen AA. Critical review of current and emerging quantification methods for the development of influenza vaccine candidates. Vaccine 2015;33:5913–9. http://dx.doi.org/10.1016/j.vaccine.2015.07.104.
[43] Ampofo WK, Azziz-Baumgartner E, Bashir U, Cox NJ, Fasce R, Giovanni M, et al. Strengthening the influenza vaccine virus selection and development process: report of the 3rd WHO Informal Consultation for Improving Influenza Vaccine Virus Selection held at WHO headquarters, Geneva, Switzerland, 1–3 April 2014. Vaccine 2015;33:4368–82. http://dx.doi.org/10.1016/j.vaccine.2015.06.090.
[44] Parker L, Wharton SA, Martin SR, Cross K, Lin Y, Liu Y, et al. Effects of egg-adaptation on receptor-binding and antigenic properties of recent influenza A (H3N2) vaccine viruses. J Gen Virol 2016;97:1333–44. http://dx.doi.org/10.1099/jgv.0.000457.
[45] Aoust D, Couture MM, Charland N, Tre S, Landry N. The production of hemagglutinin-based virus-like particles in plants : a rapid, efficient and safe response to pandemic influenza. Plant Biotechnol J 2010;8:607–19. http://dx.doi.org/10.1111/j.1467-7652.2009.00496.x.
[46] Landry N, Ward BJ, Trépanier S, Montomoli E, Dargis M, Lapini G, et al. Preclinical and clinical development of plant-made virus-like particle vaccine against avian H5N1 influenza. PLoS ONE 2010;5:e15559. http://dx.doi.org/10.1371/journal.pone.0015559.
[47] Cox MMJ, Karl Anderson D. Production of a novel influenza vaccine using insect cells: protection against drifted strains. Influenza Other Respi Viruses 2007;1:35–40. http://dx.doi.org/10.1111/j.1750-2659.2006.00007.x.
[48] Cox MMJ, Hashimoto Y. A fast track influenza virus vaccine produced in insect cells. J Invertebr Pathol 2011:107. http://dx.doi.org/10.1016/j.jip.2011.05.003.
[49] Gerdil C. The annual production cycle for influenza vaccine. Vaccine 2003;21:1776–9. http://dx.doi.org/10.1016/S0264-410X(03)00071-9.