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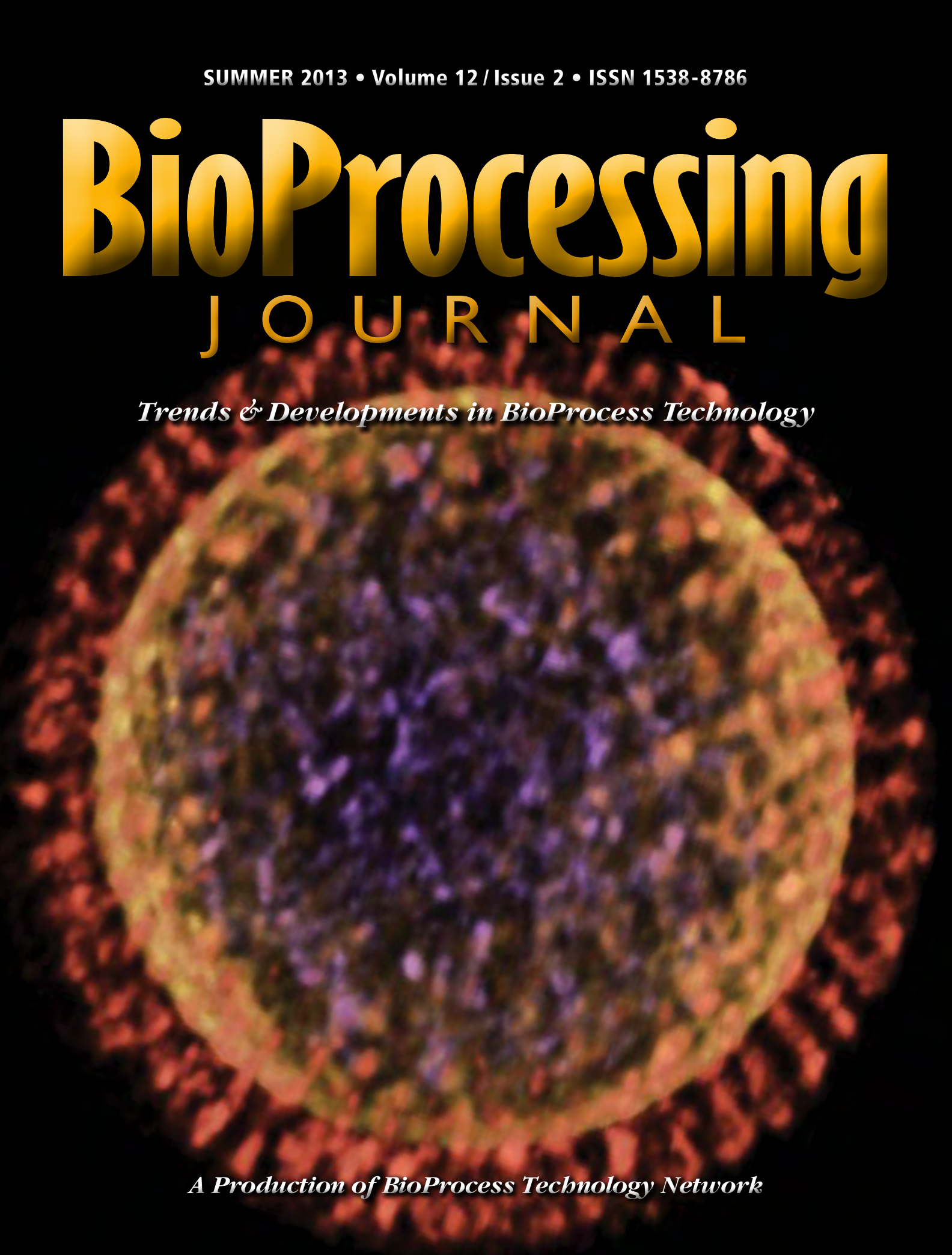
BioProcessing JOURNAL

Trends & Developments in BioProcess Technology



A Production of BioProcess Technology Network

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TRENDS & DEVELOPMENTS IN BIOPROCESS TECHNOLOGY

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Rapid Manufacture and Release of a GMP Batch of Avian Influenza A(H7N9) Virus-Like Particle Vaccine Made Using Recombinant Baculovirus-Sf9 Insect Cell Culture Technology

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Abstract

Novavax reported that, in 28 days following public release of the avian influenza A/Anhui/1/2013 (H7N9) virus gene sequences, its recombinant DNA and baculovirus-*Spodoptera frugiperda* cell culture-based technology was used to produce a virus-like particle vaccine to avian influenza A(H7N9) virus, and murine animal challenge studies were initiated.[1]

This report describes Novavax's manufacturing process and the coordinated timing of critical activities necessary to produce and release a clinical batch of avian influenza A(H7N9) virus virus-like particle vaccine, under current good manufacturing practices, within three months from the time that the virus genomic sequences for this potential pandemic influenza virus were reported. The key enabling factors were:

- A detailed, integrated project plan and daily coordination meetings
- Advanced use of the baculovirus master virus seed to bypass production of the passage 3 virus stock
- Successful functional testing of the master virus seed to establish process parameters
- Drug substance quantitation with an alternate method prior to availability of single radial immunodiffusion assay reagents
- Forward processing of intermediates prior to completion of quality control testing
- A process that uses single-use manufacturing technology

Introduction

Since 1997, avian influenza A viruses of various subtypes, including H5N1, H9N2, H7N7, H5N2, and now H7N9 have infected humans. Unfortunately, there is no method to predict which existing, novel avian, or other influenza A subtypes—for which little or no pre-existing immunity in the human population exists—may emerge as human-to-human transmissible and lead to the next pandemic.

One of the best methods of protecting society from the health, social, and economic risks of an influenza pandemic is rapid immunization of the population with a potent vaccine prior to the major wave of infections that come with the onset of a pandemic. In the past decade, manufacturers have improved the response rate and timelines for influenza vaccine production through the use of cell culture and recombinant DNA technology—as compared to the traditional production method that uses embryonated chicken eggs with reassortant viruses adapted for high growth.[2] The industry is now preparing vaccines for avian influenza A(H7N9) virus through various novel manufacturing process technologies[3-7] in response to the recent human H7N9 infections resulting in severe respiratory symptoms, extended hospitalizations, and a high mortality rate, especially in the elderly.

On March 31, 2013, the Chinese Health and Family Planning Commission notified the World Health Organization (WHO) of the first confirmed human infections with avian influenza A(H7N9) virus.[8] Although the H7N9 virus is a low pathogen in avian species, three confirmed human avian influenza A(H7N9) virus-associated infections had occurred in Shanghai and Anhui.[9,10] During the first weeks of April, the incidence of confirmed cases rose rapidly, spreading to eight provinces of eastern China.[11] Patients were admitted to the hospital displaying severe

respiratory symptoms and requiring oxygen or mechanically assisted breathing.[11, 12] Surviving patients were hospitalized an average of three weeks from the onset of illness to discharge.[12] As of July 4, 2013, the WHO reported that it has been informed of 133 laboratory-confirmed cases of avian influenza A(H7N9) virus infection resulting in 43 fatalities, a 32% mortality rate.[13]

Chinese authorities responded to this pandemic threat by closing live animal markets and culling poultry, which resulted in an apparent drastic reduction in the incidence of new cases at the end of April.[11, 14] Although the initial outbreak of the H7N9 virus infections appears to be quelled, the threat of a potential avian influenza A(H7N9) virus pandemic still exists. At this time, it is unknown whether the decrease in newly identified cases could be attributable to the normal seasonal fluctuation of incidence of influenza infection, and if this is the case, whether the avian influenza A(H7N9) virus infection and associated serious disease could return with the onset of colder weather. More troubling is the knowledge that, while there is no clear evidence of human-to-human transmission, avian influenza A(H7N9) virus has acquired genetic characteristics shown to be adaptive for transmission of disease in humans and ferrets,[12] the most relevant animal model for human influenza infection. The fear is that further adaptation to a human host could occur in asymptomatic or less severely infected subjects.

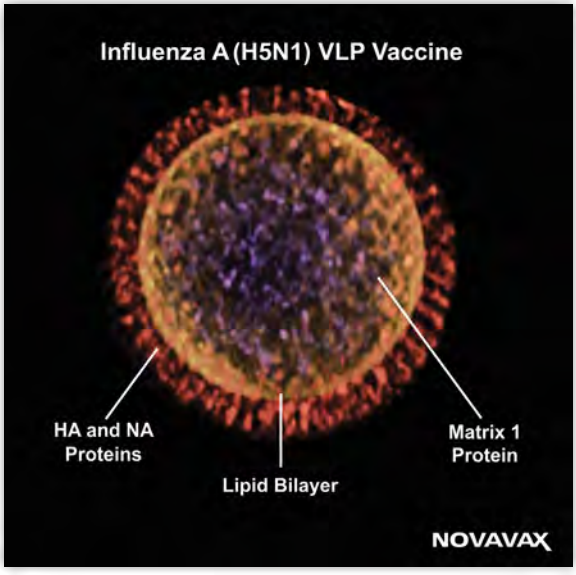

FIGURE 1. Novavax's influenza A(H5N1) VLP vaccine determined by transmission electron microscopy tomography analysis. Colors were manually applied (NanoImaging Services, Inc.).

Given a rising concern over the pandemic potential of this avian influenza A(H7N9) virus, Novavax responded with a high-priority effort to develop a monovalent avian influenza A/Anhui/1/2013 (H7N9) virus virus-like particle (VLP) vaccine. Regardless of whether this virus becomes a declared pandemic, this endeavor serves as a test case of Novavax's production method for the rapid development and release of a pandemic influenza vaccine.

The avian influenza A(H7N9) virus VLP vaccine candidate described in this report demonstrated immunogenicity and protection in mice challenged with a lethal dose of avian influenza A/Anhui/1/2013 (H7N9) virus.[1] Preclinical and clinical studies previously have shown the safety and immunogenicity of Novavax's influenza VLP monovalent and multivalent vaccines toward influenza A(H1N1), influenza A(H3N2), influenza A(H5N1), influenza A(H2N3), influenza A(H7N2), and influenza B.[15-21]

This report describes the successful manufacture and release of a GMP batch of avian influenza A(H7N9) virus VLP vaccine. This demonstrates Novavax's current capability to manufacture and release a recombinant influenza VLP pandemic vaccine within three months from the time the genetic sequences of an emerging threat are identified to the release of the first doses of vaccine.

Process Description

Novavax's influenza VLP vaccine manufacturing technology is designed for rapid response in the event of an emerging pandemic. Overall, the manufacturing process is a platform format for VLP vaccine production and requires only a few virus strain-dependent process modifications, including the generation of a specific baculovirus master virus seed (MVS), verification of specific key process parameters, and generation of strain-specific assay reagents. The processing begins with MVS production, continues with drug substance manufacturing, and concludes with drug product filling and release.

The core of Novavax's technology is the genetically engineered baculovirus MVS which is designed to express strain-specific influenza hemagglutinin (HA) and neuraminidase (NA) proteins and a non-strain-specific matrix 1 (M1) protein. When *Spodoptera frugiperda* (Sf9) insect cells are infected with the MVS, the HA, NA, and M1 proteins are expressed and self-assemble into roughly spherical structures approximately 150 nm in diameter that bud from the cell with a lipid bilayer envelope containing external surface HA and NA glycoprotein spikes. VLPs closely resemble mature influenza viruses displaying conformational-dependent antigenic epitopes of the viral surface glycoproteins but in a non-infectious form lacking influenza genetic material (Figure 1).

A specific sequence of events is required for the generation of a baculovirus MVS. First, the HA and NA gene sequences derived from the novel virus are codon-optimized for high-level expression in insect cells and then biochemically synthesized. These genes are cloned into a pFastBac™ 1 baculovirus transfer vector (Life Technologies™)

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and transposed into a baculovirus genome using the Bac-to-Bac® Baculovirus Expression System (Life Technologies) using *Escherichia coli* host cells. Recombinant bacmid DNA is purified from the bacteria and transfected into Sf9 insect cells from which a single recombinant baculovirus expressing HA, NA, and M1 is identified, plaque-purified, and amplified as passage 1 (P1). The P1 virus is used to produce MVS at passage 2, which is frozen for future use in the manufacture of the influenza VLP during the production campaign.

The upstream current good manufacturing practices (GMP) drug substance manufacturing process begins with the thaw of a frozen vial of Sf9 cells (Figure 2). The culture is continuously passaged in larger flasks (Corning)

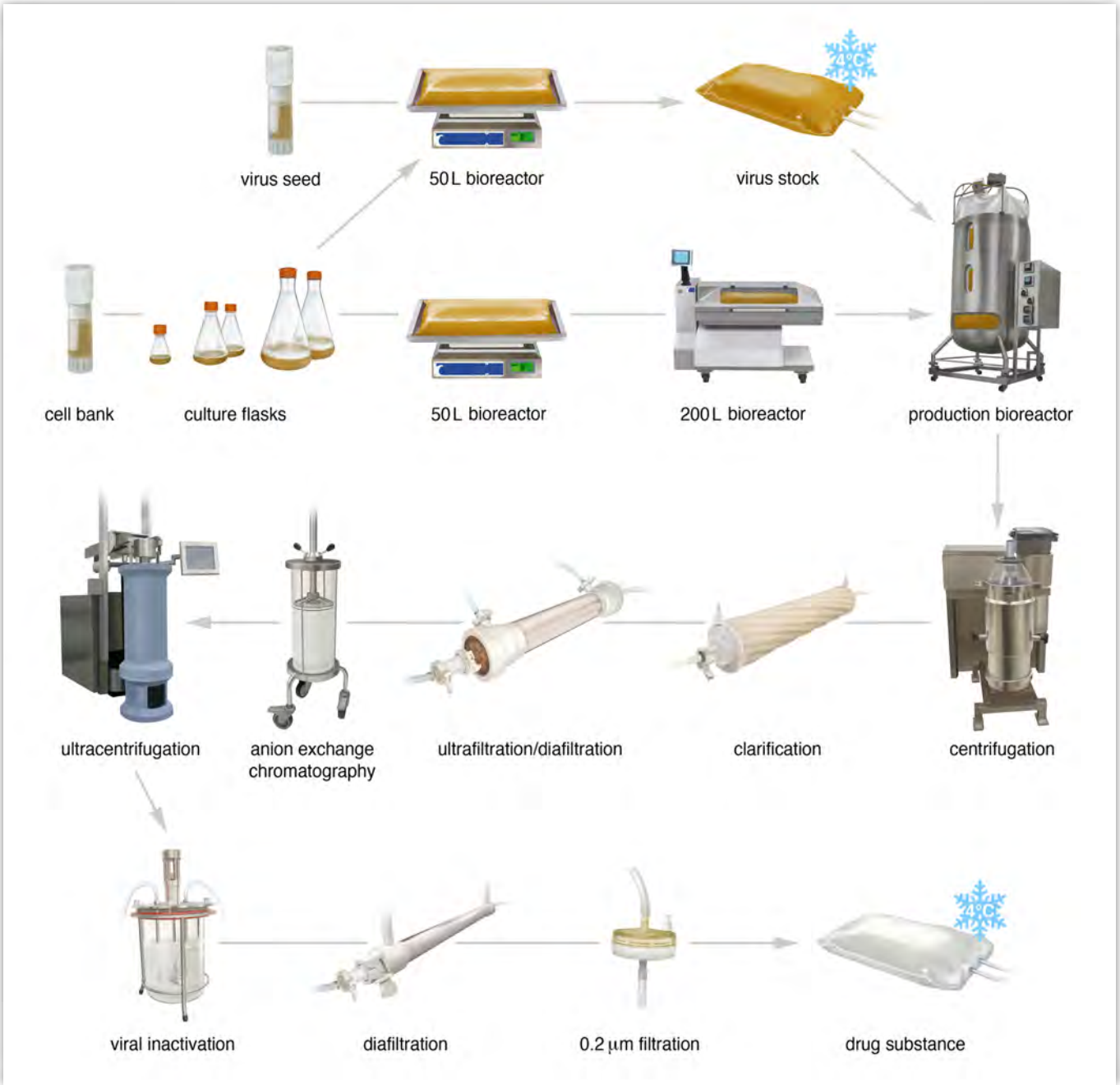

FIGURE 2. The Novavax Influenza VLP Vaccine production process flow diagram. A master cell bank vial is thawed and progresses through cell culture in flasks, WAVE bioreactors, and an Xcellerex 1000 L production bioreactor. The production bioreactor culture is infected with clarified virus stock that is held at 4°C after being prepared by infecting a 50 L WAVE cell culture with virus seed. VLP vaccine produced in the production bioreactor is harvested, filtered, and purified by a sequence of steps including ultrafiltration/diafiltration, anion exchange chromatography, sucrose gradient ultracentrifugation, viral inactivation, diafiltration, and 0.2 µm filtration. The drug substance is stored at 4°C.

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to achieve the number of cells needed for inoculation of two 50 L WAVE bioreactors (GE Healthcare Life Sciences). Conventionally, one 50 L WAVE bioreactor is infected with MVS to produce P3 recombinant baculovirus stock. Upon harvest of the infected WAVE bioreactor media, the P3 stock virus is clarified, titered, and retained at 2–8 °C for use in the pending bioreactor infection processes. The other 50 L WAVE bioreactor is used to inoculate a 200 L WAVE bioreactor expansion culture which, in turn, is used to inoculate the 1000 L production bioreactor (XDR-1000, Xcellerex, GE Healthcare Life Sciences). Following several days of cell growth, the bioreactor is infected with the P3 recombinant baculovirus stock to produce influenza VLP which are secreted into the culture media during the infection.

For the platform influenza process, standard operating ranges have been established for the multiplicity of infection (MOI) and VLP harvest time (post-baculovirus infection) for the production bioreactor process. Each MVS used to make a new strain of influenza VLP vaccine is evaluated to establish the specific optimal MOI and harvest time within the operating range. For these evaluation studies, Sf9 insect cells are infected at different MOI and analyzed over a time course from early to late infection for cell density and viability, HA protein productivity, and percentage of infected cells. Cell density and viability are measured by standard methods (trypan blue staining and either manual or automated enumeration). The HA protein productivity is estimated by SDS-PAGE or other protein quantification methods. The percentage of infected cells is assessed by fluorescence-activated cell sorting (FACS) using a monoclonal antibody to gp64, a major baculovirus envelope glycoprotein expressed on the surface of baculovirus-infected insect cells, and polyclonal antibodies (if available) to the strain-specific HA. An example of the progression of infection of a representative Sf9 cell culture infected with the influenza A(H7N9) baculovirus, as measured by FACS using a monoclonal antibody against gp64, is provided in Figure 3. These data show the temporal progression of an increasing percentage of infected Sf9 cells. Confirmation of expression of the influenza A(H7N9) virus HA glycoprotein from the same study is shown in Figure 4. Based on a thorough analysis of the laboratory data, the optimal parameters (MOI and harvest time) are established for the production process.

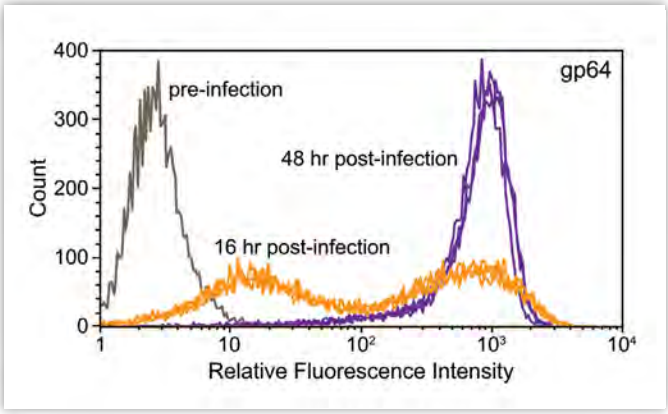

FIGURE 3. FACS analysis of gp64 expression in Sf9 cells infected with recombinant baculovirus for influenza A(H7N9) VLP at laboratory-scale using a monoclonal antibody to gp64 (Expression Systems, Inc.). Comparison of the gp64-positive FACS profiles between pre-infection and 16 and 48 hours post-infection are presented.

The downstream GMP drug substance manufacturing process begins with the harvest

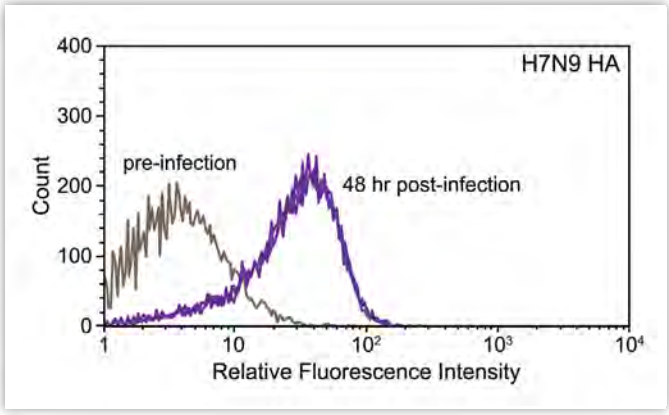

FIGURE 4. FACS analysis of H7 HA expression in Sf9 cells infected with recombinant baculovirus for influenza A(H7N9) VLP at laboratory-scale using a polyclonal antibody to H7 HA (generated by Novavax). Detection of HA on the surface of infected cells confirms effective progression of infection and VLP production.

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of the bioreactor culture fluid using a semi-continuous single-use centrifuge (UniFuge, PneumaticScaleAngelus) (Figure 2). The clarified supernatant is passed through a depth filter (Sartopure®, Sartorius) and then concentrated and diafiltered (KrosFlo® Max, Spectrum Labs) in preparation for anion exchange (AEX) chromatography (Capto Q™, GE Healthcare Life Sciences). The AEX chromatography resin mainly binds baculovirus and nucleic acids thereby reducing their content from the VLP fraction which flows through the chromatography column (Figure 5).

The AEX chromatography-purified process material is further purified by sucrose gradient ultracentrifugation using a continuous flow ultracentrifuge (KII Ultracentrifuge, Alfa Wassermann Separation Technologies). Sucrose solutions of varying volumes and sucrose concentrations are used to create a sucrose density gradient which is optimized to separate VLP from residual baculovirus and low density particles such as contaminating proteins. The performance of a representative sucrose gradient (Figure 6A) generated at laboratory-scale using a scaled-down ultracentrifuge (Promatix 1000™, Alfa Wassermann Separation Technologies) shows the potential to separate the influenza VLP from baculovirus and host cell proteins based on their differing buoyant densities (Figure 6B).

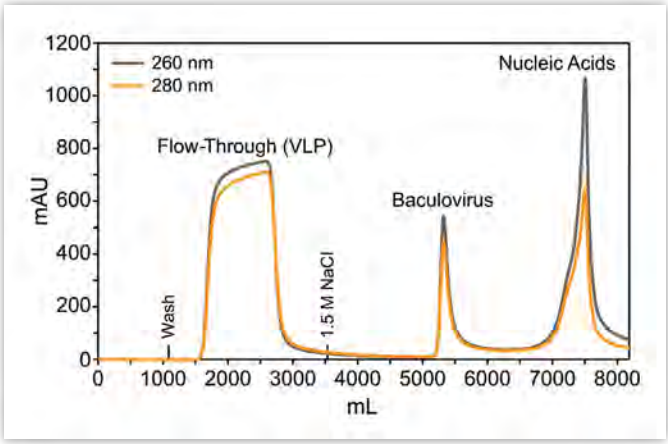

FIGURE 5. AEX chromatogram showing the purification of influenza A (H5N1) VLP at the laboratory scale. The VLP product is in the flow-through fraction. Baculovirus and nucleic acids bound to the column are removed by introducing a step change to 1.5 M NaCl.

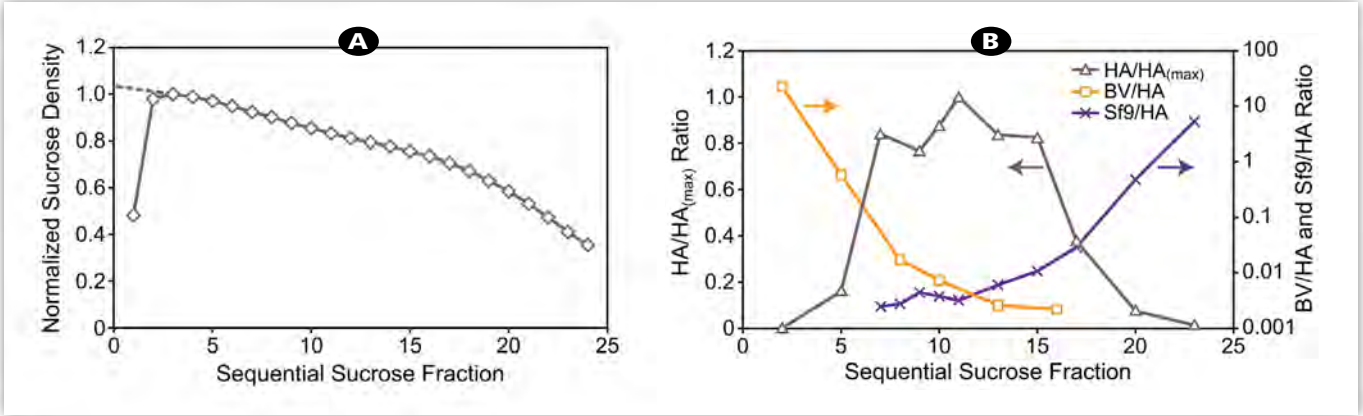

FIGURE 6. (A) Laboratory-scale sucrose density gradient ultracentrifugation study for an H5N1 VLP preparation. The sample was loaded, centrifuged, and fractions (24 fractions of approximately 10 mL) were collected from the bottom of the centrifuge rotor. Sucrose density was measured by refractometer (w/w %) and reported as a normalized value. The relatively lower density for fractions 1 and 2 are an artifact of the sampling procedure. The theoretical density is depicted by the manually generated dotted line. (B) The $HA/HA_{(max)}$ data are the ratio of the fraction's HA value relative to the maximum HA value (fraction 11) as measured by single radial immunodiffusion (SRID). The baculovirus/HA data are the ratio of each antigen concentration of infectious baculovirus divided by the associated (or nearest) corresponding HA concentration. Infectious baculovirus was measured by a rapid titer assay and converted to concentration using a conversion factor. The Sf9/HA data are the ratio of Sf9 protein concentration, as determined by ELISA, divided by the associated (or nearest) corresponding HA concentration.

The VLP fraction, as represented by the normalized HA distribution, is concentrated in the center of the density gradient. Infectious baculovirus, as represented by the BV/HA distribution, is mainly concentrated in the higher density region of the gradient. Other contaminating proteins, as represented by the Sf9/HA purity distribution, are concentrated in the lower density region of the gradient. Overall, the level of infectious baculovirus and contaminating proteins ranges between one and three orders of magnitude less than the amount of HA protein in the VLP fractions. After the fractions are collected from the centrifuge, the subset of fractions high in HA protein concentration and low in infectious baculovirus and host cell proteins are pooled for further processing.

The pooled product is then treated chemically as a viral inactivation step. After viral inactivation, the product is formulated by diafiltration (MiniKros, Spectrum Labs) into formulation buffer and filtered with a 0.2 micron membrane filter (Millipak, EMD Millipore). The resultant drug substance is stored at 2–8˚C. Upon determination of the HA content, the drug substance is diluted to the target dosage in formulation buffer and filled into glass vials to produce the drug product.

Results and Discussion

The avian influenza A/Anhui/1/2013 (H7N9) HA and NA gene sequences were published on the GISAID database[22] on March 31, 2013. Novavax initiated a plan on April 8, 2013 to manufacture and release a GMP batch of vaccine. The key activities for this project are presented in Figure 7. The four main areas of focus were: (1) HA and NA gene processing to produce the P1 virus; (2) production of MVS, drug substance, and drug product; (3) quality control (QC) testing; and (4) quality assurance (QA) review. In addition to activities listed in Figure 7, other project activities included the preparation and qualification of SRID assay reagents, materials ordering and delivery, contracting and coordinating with external QC test labs, and general project administration activities.

The project's primary critical path began on March 31 with the public release of the gene sequences. The HA and NA genes were then codon-optimized for the Sf9 insect cells, and an order was placed for gene synthesis with a vendor on April 8. The plasmids with the synthesized HA and NA genes were delivered from the vendor to Novavax on April 17. The HA, NA, and M1 genes were integrated, cloned, amplified, and prepared as a bacmid for Sf9 cell transfection. Baculovirus were produced, plaque-purified, and P1 virus was harvested and titered on May 8. MVS was prepared on May 11, and samples were submitted for MVS release testing. The MVS release testing was the gating activity leading to product release, based upon the on-test time of the *in vivo* adventitious agent testing.

Other activities, which were offset from the primary critical path by a few days, were designated as the secondary critical path because they could have become

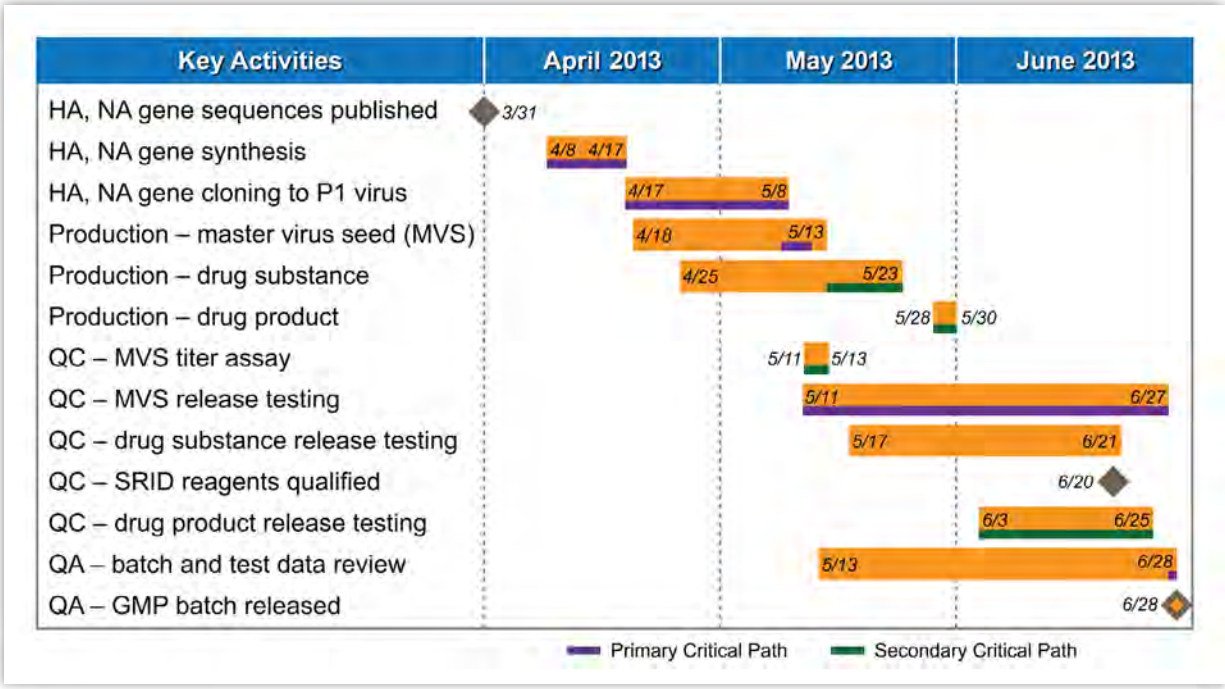

FIGURE 7. Timeline with primary and secondary critical path for the production and release of a GMP batch of avian influenza A (H7N9) virus VLP vaccine within three months from gene sequence publication.

gating events for release had they extended beyond the primary critical path. The secondary critical path began with the generation of the MVS on May 11. In advance, Novavax began the expansion of the cell bank through to the production bioreactor in order to synchronize inoculation of the production bioreactor with the availability of the MVS. MVS was manufactured in sufficient quantity to infect the production bioreactor and to freeze a suitably sized master virus bank. As soon as the MVS was prepared and titered, the production bioreactor was infected with the master virus, thus bypassing the need to generate the conventional P3 virus stock. VLP product was then harvested, purified, and formulated to produce drug substance on May 23. After formulation, the drug product was filled, inspected, labeled, and packaged with a completion date of May 30. The final gating activity for the secondary critical path was drug product release testing which was completed on June 21.

After the final QC test result for MVS was reported on June 27, the QA department finalized all release documentation. The product was released with a certificate of analysis on June 28, three months from the publication of the HA and NA gene sequences. This success was achieved through the following key factors:

- A detailed, integrated project plan and daily coordination meetings
- Advanced use of the MVS to bypass production of the P3 virus stock
- Successful functional testing of the MVS to establish process parameters
- Drug substance quantitation with an alternate method prior to availability of SRID assay reagents
- Forward processing of intermediates prior to completion of QC testing
- A process that uses single-use manufacturing technology

A Detailed, Integrated Project Plan and Daily Coordination Meetings

Project management best practices were applied to the project from its initiation to ensure consistent planning, monitoring, and control for real-time assessment of risks and status. A detailed project plan with dependencies within and across departments and owners was developed and updated throughout the project to manage the multiple overlapping and integrated tasks. Several important intermediate milestone completion dates were given a priority and then activities began (particularly cell expansion prior to bioreactor infection) under the assumption that all required prerequisites would be completed as scheduled.

A critical chain approach was applied to help manage the project to a pre-defined "must complete no later than" constraint. The critical chain approach is based on an

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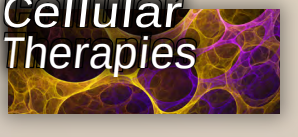

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assumption that the duration of each activity, whether or not it is on the critical path, must be completed no later than planned. In this way, an activity which is not on the critical path will not be extended longer than the contemporaneously occurring critical path task(s).

A cross-functional integrated project team of department leads was empowered to make real-time decisions to mitigate technical, regulatory, and schedule risks. Subject matter experts were added to the team as needed on a rolling basis to support troubleshooting and avoid communication gaps. A communications plan was established with an escalation pathway through the integrated team and upwards to the company's CEO.

Advanced Use of the MVS to Bypass Production of the P3 Virus Stock

The standard production process includes the generation of a baculovirus P3 virus stock from the MVS, which is used to infect the production bioreactor. For the first lot of a production campaign (when the MVS is first manufactured), in place of the P3 culture, the process allows the use of the MVS culture to infect the production bioreactor, with the remainder of the MVS culture being frozen for use in future batches. The avian influenza A(H7N9) virus vaccine batch was manufactured using this approach, thereby reducing the secondary critical path timeline by about one week.

Successful Functional Testing of the MVS to Establish Process Parameters

The technical staff had pre-established timelines that had to be met in order to complete laboratory studies ahead of the need for decisions in the manufacturing facility. Because MVS was not yet titered, the technical staff based their experiments on historical titer yields. In addition, expanded laboratory infection studies were performed at multiple ratios of virus-to-cells to ensure testing of an MOI within the operating range. Based on the study results and the determination of the MVS titer, the appropriate MOI was established prior to the infection of the bioreactor in the manufacturing facility.

Drug Substance Quantitation with an Alternative Method Prior to SRID Reagent Availability

The industry standard quantitation methodology for influenza vaccines is a SRID assay. This assay requires the preparation of purified HA protein, generation of polyclonal antibodies against the purified HA, and calibration of those reagents.[23] Depending on the seroconversion rate of the animal immunized, it may take in excess of seven weeks to generate sufficient polyclonal antibodies after the HA-purified standard is prepared. For the avian influenza A(H7N9) virus vaccine production, the SRID reagents were not available in time to quantify the drug substance to determine the dilution factor to formulate the drug product. Instead, an alternative assay was used for avian influenza A(H7N9) virus HA quantitation.

At the time of this project, a quantitative capillary gel electrophoresis method to measure HA was being evaluated in Novavax's analytical development department. Given the critical need, the program was accelerated, and the assay was demonstrated to be suitable for use just in advance of formulation of drug product. As a result, the drug substance HA level for formulation was based on total protein content by the bicinchoninic acid (BCA) protein assay and percent HA by capillary gel electrophoresis. This HA value was later used to calibrate the SRID reagents.

Forward Processing of Intermediates Prior to Completion of Quality Control Testing

Forward processing of intermediate process material was an essential part of completing the production and release of the GMP batch of avian influenza A(H7N9) virus VLP vaccine within three months. The MVS was forward-processed to the drug substance manufacturing process prior to completion of the MVS QC testing. Likewise, drug substance was forward-processed to drug product prior to completion of QC testing. This strategy enabled QC testing of MVS, drug substance, and drug product to proceed simultaneously. The last QC test completed was *in vivo* adventitious agent testing on the MVS. All other QC testing was completed within the timeframe defined by the guinea pig portion of the *in vivo* adventitious agent assay.

Forward-processing followed approved quality system procedures with documentation of variances to procedures, if applicable. Batch records and test results were reviewed in real time wherever possible. As such, any manufacturing or testing deviation was rapidly identified, investigated, and resolved. The continuous review of batch records and QC test results prepared the QA department for a one-day cycle time for batch release after the final test result was received.

A Process That Uses Single-Use Manufacturing Technology

Novavax is a clinical stage vaccine company with a robust pipeline that often demands full utilization of the clinical vaccine manufacturing facility. The opportunity to schedule an unplanned GMP batch of a novel influenza vaccine would not likely have been possible with conventional stainless steel process equipment due to cleaning qualification and change-over procedures that are typically necessary between product campaigns. By utilizing single-use technology from culture flasks through the first ultrafiltration/diafiltration step, including single-

use bioreactors and a single-use centrifuge, our facility turn-over procedures allowed us to manufacture the GMP batch of avian influenza A (H7N9) virus vaccine and maintain the production schedule for our other clinical products.

Overall, the success of this project at Novavax was rooted in the expertise and dedication of the people who executed the work. The lessons learned during this experience give us confidence that Novavax can further improve its manufacturing, quality, and management systems to drive release of vaccine product from the demonstrated three month cycle time to the theoretical minimum cycle time of 2½ months.

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Competing Interests

All authors are employees of Novavax, Inc. The findings and conclusion in the report are those of the authors and do not necessarily represent the views of the U.S. Department of Health and Human Services or its components.

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