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Development and Manufacture of Alphavaccines

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Vaccines represent the most effective means of disease prevention. A variety of vaccines including live-attenuated strains, inactivated organisms, and subunit forms are currently in use.[1,2] However, advances in molecular biology, virology and immunology have made new classes of potential vaccines possible. One such class currently being developed by AlphaVax is based on an alphavirus-derived expression system.[3]

Alphaviruses are members of the *Togaviridae* family and have positive-sense RNA genomes.[4] The RNA genome is surrounded by a capsid composed of 240 copies of a single capsid protein. Alphaviruses are enveloped viruses that mature at the plasma membrane of the host cell and are 50-70 nm in diameter. The viral envelope contains 80 trimers composed of two viral glycoproteins, E1 and E2. These trimers contain three E1-E2 heterodimers and are arranged in the viral envelope as spikes which protrude from the surface of the virus particle. These protein spikes mediate attachment of the virus to susceptible cells and are the targets of neutralizing antibodies.

The plus-stranded, message-sense RNA genome of an alphavirus is sufficient to establish an infection when introduced directly into cells. Thus, development of full-length infectious clones for alphaviruses such as *Sindbis*, *Semliki Forest*, and *Venezuelan equine encephalitis* (VEE) has made the construction of expression vectors from each of these systems possible.[5,6,7] A detailed account of the alphavirus life-cycle has been described by Strauss and Strauss.[8] A brief diagrammatic summary of the alphavirus replication strategy is shown in Figure 1. After the viral RNA enters the target cell, the nonstructural proteins are translated. These proteins direct production of full-length, negative-sense RNA. From the negative-sense RNA, large numbers of copies of two different positive-sense RNA species are produced. First, a subgenomic message is transcribed, and this message directs production of the structural proteins. In addition, full-length, positive-sense progeny genomes are made. These genomes are packaged by the capsid protein and the complete capsid-RNA complex interacts with the cytoplasmic tail of the E2 glycoprotein which has been inserted in the host cell membrane. The capsid-RNA complex is enveloped at the plasmid membrane and finally the virus buds from the cell.

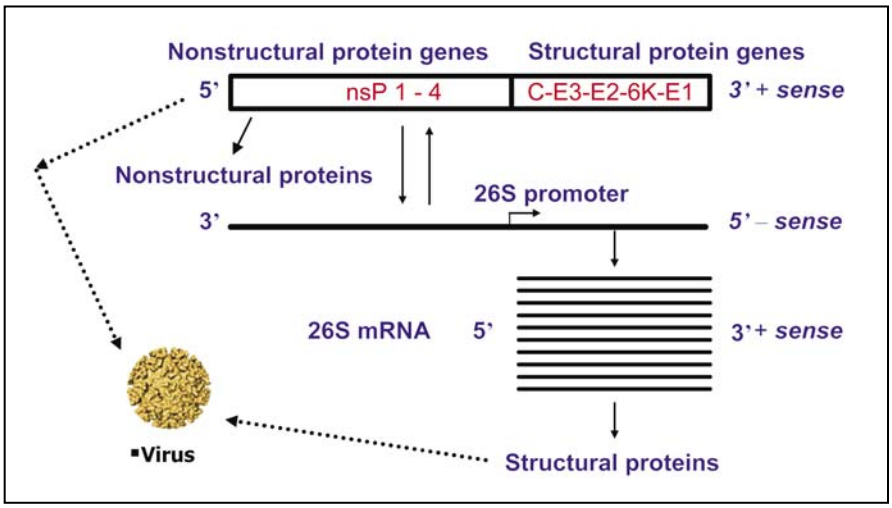

Figure 1. Diagrammatic Summary of the Alphavirus Replication Strategy.

Alphaviruses in general, and VEE virus in particular, offer an exceptional system for gene delivery and expression in mammalian cells based on three key features. First, there exists very little anti-VEE immunity in the human population.[9] Second, the VEE virus appears to target dendritic cells which, after infection, migrate to the lymphoid tissue and generate a robust humoral and cellular immune response in animals for most expressed antigens.[10] And third, if the structural genes for VEE virus are removed from the RNA genome and replaced with a foreign gene, a self-

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replicating RNA (replicon) is generated, driving high levels of foreign gene expression in infected cells.[11]

Because the VEE structural genes have been deleted, the replicon RNA is a single cycle, propagation-defective RNA and replicates only within the cell into which it is introduced. The replicon RNA can be packaged *in vitro* into virus-like replicon particles (VRP) by supplying the structural protein genes of VEE in trans, as shown in Figure 2. Resulting VRP can efficiently infect cells and express the gene of interest, but they cannot form new particles in the vaccine recipient. The protein specified by the gene of interest on the replicon RNA is produced in the infected cells of the vaccine recipient, and a robust immune response is generated. An additional level of safety is introduced in the vaccine by the inclusion of attenuating mutations in the nucleic acid.

For use as a vaccine, the efficacy of this system has been demonstrated in a number of diverse animal-based challenge models, including *influenza, Lassa, Ebola* and *Marburg viruses,* and *C. botulinum, S. aureus and B. anthracis* bacteria.[12-19]

The development of a vaccine candidate requires the involvement of multiple disciplines including vector development (molecular biology), process development, manufacturing, quality assurance and control, and pre-clinical and clinical testing. The involvement of each discipline is critical in bringing a vaccine product to market. This article will describe the development and production of VEE-based alphavaccines at AlphaVax. An alphavaccine, as described here, is a propagation-defective viral particle containing a replicon RNA derived from the VEE virus that is formulated in a suitable excipient matrix. In order to provide experimental data as an example of the development process, data generated during clinical manufacture of a multigene HIV alphavaccine candidate will be presented as a case study.

Production of Alphavaccines

In order to produce clinical grade alphavaccines, a multi-step, robust, controlled process is required, as is the case for all biopharmaceuticals. A simplified flow diagram for the production of alphavaccines is shown in Figure 3. As a brief introduction, a generalized process will be described here and a more detailed description of the current manufacturing process will be presented in the following sections.

First, the replicon RNA containing the gene of interest must be produced in an *in vitro* transcription system. Since the replicon lacks the structural genes, production of replicon particles requires a method to provide the production cells with the structural proteins. To accomplish this, structural proteins are supplied by a separate helper function.

After RNA has been generated, a suitable cell line must be expanded to provide a host for alphavaccine production. Once the cells are expanded in flasks and roller bottles at the current scale, they are harvested, washed and electroporated with the purified RNA species. The transfected cells are re-seeded to roller bottles in a serum-free medium and incubated to allow the production of VRP. The VRP is separated from the cells, purified, and formulated. Formulated bulk vaccine is stored frozen at or below -70º C until it is diluted and filled to the final container closure system.

The product and process intermediate pools are analyzed to provide information on purity, safety, process consistency, and quality. Critical assays are qualified prior to use in manufacture of clinical material. Critical processing operations are also qualified (*e.g.,* filling operations and sterilizing grade filtration operations) prior to performing manufacturing operations. Production operation documentation and methods are finalized and approved by technical personnel and Quality Assurance prior to technology transfer from development to manufacturing. Manufacturing personnel are trained prior to the initiation of manufacturing operations to assure they possess the appropriate knowledge and skill required for production of the specific alphavaccine.

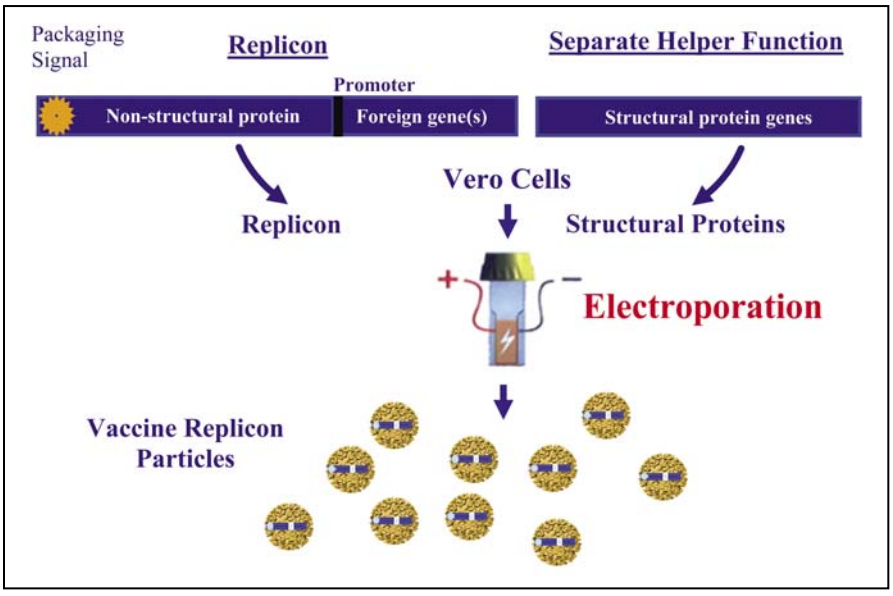

Figure 2. Resultant Virus-Like Replicon Particles.

Development and Manufacturing Process Evolution

As the technology to produce alphavaccines has been developed within AlphaVax, a variety of process improvements have been implemented. These improvements have been focused on increasing yield, increasing product purity, increasing particle stability, and improving the physical parameters surrounding the process (closing unit operations and increasing process consistency). AlphaVax has multiple

Table 1. Vaccine Products in Development.

Disease Target	VRP Components	Stage of Development
HIV	Gag	Phase I clinical trials
HIV	Gag/Nef/Pol and gp160 Clade C	Clinical material manufactured, IND ready for Submission
HIV	Gag/Nef/Pol and gp160 Clade B	Clinical material manufactured
Marburg	gp (Musoke strain)	Clinical material manufactured
Botulism	BoNT/A Hc and BoNT/B Hc	Clinical material manufactured
CMV	gB and pp65/IE1	Clinical material manufactured
Flu	HA and NA	Pilot lots complete
SARS	SARS-CoV S, M, N & E	Research lots complete
VEE/EEE/WEE	glycoproteins (E1 & E2)	Research lots complete
Smallpox	vaccinia envelope proteins (A33R, B5R, A27L & L1R)	Replicons under construction

vaccine products in development, some of which are multi-component vaccines and have required production of two or more independent VRP preparations. A summary of some of those products is shown in Table 1.

Since there has been a succession of vaccine candidates produced at AlphaVax, it has been possible to monitor the impact that the process improvements have had on the purity, quality and consistency of the vaccine. The production campaigns for our first vaccine to enter the clinic, a human immunodeficiency virus (HIV) *gag* alphavaccine, utilized a simple process. The process incorporated a single VRP capture step on fast flow heparin resin. After elution from the resin, VRP were concentrated, formulated in storage buffer, and frozen. This bulk material was later diluted to make drug product, sterile filtered, and filled to vials. The material was tested in a GLP rabbit toxicology study and was found to have only mild to moderate local reactogenicity and no systemic toxicity. An IND was filed and a Phase 1 clinical trial was initiated in the United States and South Africa.

Following the production of the HIV *gag* alphavaccine, a "second generation process" was introduced in which the VRP yields were increased by a process modification, and the heparin affinity purification step was replaced with an anion-exchange based operation. The result was a process capable of generating a vaccine with a higher concentration of VRP and increased purity. In an effort to further increase product purity, with respect to process residuals such as host cell protein and DNA, additional process modifications were introduced. Downstream operations were modified to include a TFF operation containing a Benzonase treatment and an affinity chromatography purification unit operation. This process is the basis for the current production process and has been used for production of material for the multigene HIV, botulinum, cytomegalovirus (CMV) and influenza vaccines. The process continues to be optimized to increase product purity, product safety, and process consistency.

Case Study: Multigene HIV Vaccine Production

The multigene HIV alphavaccine is composed of two VRP preparations and

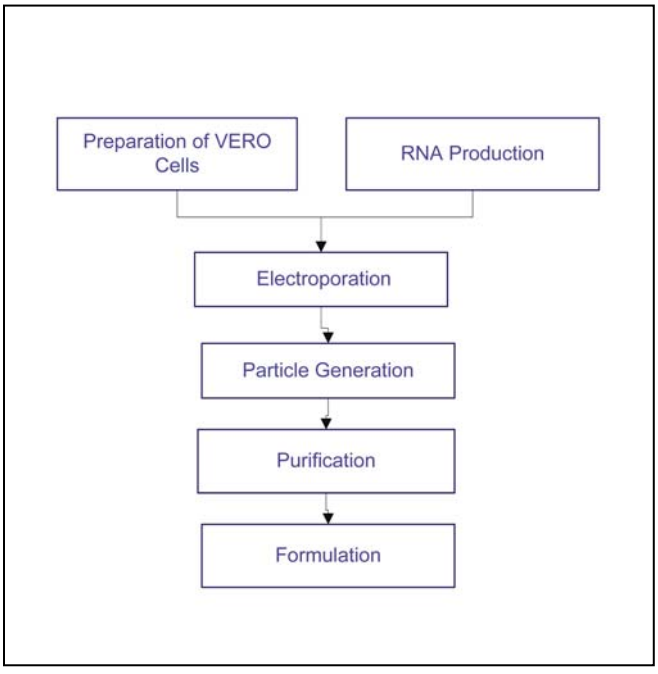

Figure 3. Flow Diagram for the Production of Alphavaccines.

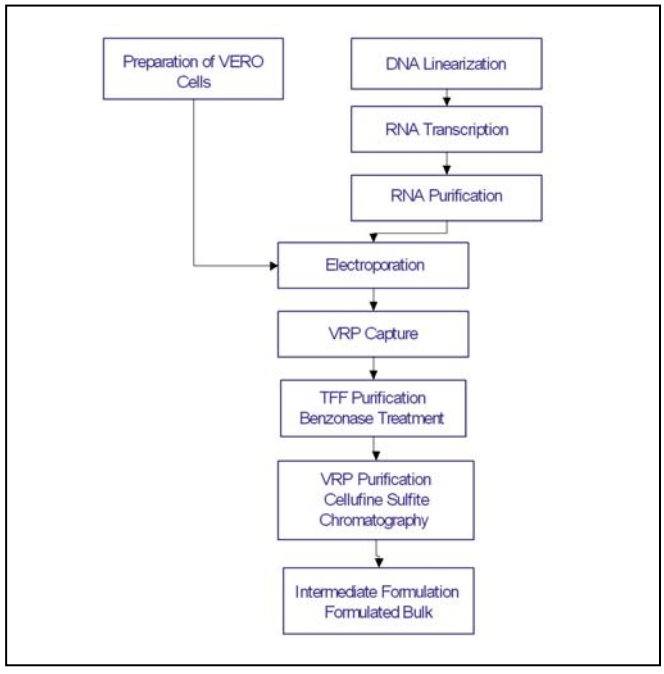

Figure 4. Production Process for Vaccine Components.

each was manufactured in a separate production lot. The first component, gp160 VRP, expresses the gp160 envelope glycoprotein from HIV-1, subtype C. The second component, *gag/nef/pol* VRP, expresses a *gag/nef/pol* fusion protein whose *gag, nef* and *pol* genes were derived from subtype C HIV-1 isolates.

The development cycle for the vaccine included molecular optimization of the constructs for VRP yield and immunogenicity. Once a construct type was chosen, the conditions of production were optimized with respect to the RNA levels required for Vero cell transfection, and electroporation and growth conditions. After a set of conditions was chosen, a small-scale batch of vaccine was produced and analyzed. Material for each component was produced in each of two pilot lots. The pilot lots were produced at one-third of the clinical scale under batch record. The material generated from the pilot lots was used in stability studies, immunogenicity studies, assay development, and as a reference standard. After review of the pilot lot production data and analytical results, it was determined that the data supported a decision to take the process forward and begin manufacturing clinical material.

A flow diagram depicting the production process used for each component of the vaccine is shown in Figure 4. The production process required ten days from Vero cell breakout until the formulated bulk vaccine was placed in the freezer for storage. During the first week, cell expansion operations and production of RNA occurred. The cell expansion occurred with breakout into flatstock, followed by seeding to roller bottles. The cells were expanded in a serum-containing medium. The expansion process required seven days and resulted in a 45-fold increase in cell number. After three expansions, cells were trypsinized, collected, washed by centrifugation, and electroporated. The RNA required for electroporation was produced in an *in vitro* transcription reaction using linearized plasmid DNA as the template. After production, RNA was purified by size exclusion chromatography and stored at -70° C or below until the time of electroporation. Both the cell expansion and RNA production/purification operations took place in the first week of a production campaign.

During the second week of the campaign, Vero cells were electroporated and seeded into serum-free media in roller bottles. The cells were incubated for 18 to 24 hours and the alphavaccine particles were harvested in a capture-type affinity step. VRP were concentrated and washed on a hollow fiber ultrafiltration membrane and treated with Benzonase to degrade host cell DNA. Following an additional wash, the VRP were purified by Cellufine Sulfate (Chisso Corp.) affinity chromatography. Elution of VRP was achieved by an increasing salt step gradient. The VRP eluted from Cellufine Sulfate was formulated with protein and a disaccharide in a buffered solution and frozen at -70° C or below until final formulation, and fill operations commenced.

Analysis of formulated bulk and process intermediates was conducted to assure process consistency and product quality, safety and potency. Both the VRP-containing and waste process streams were analyzed to obtain a mass balance for product and contaminant species. Table 2 summarizes the analytical and biosafety testing performed at each stage of the process. Microbiological testing of the environment, process pools and product was also performed. Process expectations for microbial contamination were established based on past experience. For example, spent cell culture medium sampled after cell splits had a bioburden of 0.0 CFU/mL while a process stream such as the Cellufine Sulfate elution pool had a bioburden of less than 0.1 CFU/mL.

Adventitious agent testing was performed by a contract research organization. An assay for replication-competent virus was performed at AlphaVax—a key safety assay conducted to demonstrate that the alphavaccine produced did not contain replication-competent alphavirus. The assay was performed by infecting cultured Vero cells with VRP at a low multiplicity of infection. The infected cells were incubated overnight and the media was transferred to a second set of Vero cultures. After incubation of the cells and medium for a pre-determined period, the medium was discarded and replaced with fresh

Table 2. Analytical and Biosafety Testing Performed at Each Stage of Production.

Process Step	Testing	
Unprocessed Bulk Pool	·*In Vitro* detection of adventitious virus ·*In Vivo* detection of adventitious virus ·Mycoplasma ·PCR based reverse transcriptase ·Potency by immunofluoresence	
Capture Pool	·Potency by immunofluoresence ·Replication competent virus assay ·Protein	·BSA ·DNA ·Residual Vero protein
Tangential Flow Filtration Pool	·Benzonase ·Potency by immunofluoresence ·Protein ·DNA size	·BSA ·DNA ·Residual Vero protein ·Yield
Cellufine Sulfate Elution Pool	·Benzonase ·Potency by immunofluoresence ·Protein ·DNA size	·BSA ·DNA ·Residual Vero protein ·Yield
Formulated Bulk	·Potency/Identity by immunofluoresence ·Protein ·DNA size ·Residual Vero protein ·Appearance ·Disaccharides	·DNA · pH ·Yield ·Endotoxin ·Immunogenicity

media. The passage 2 cultures were examined for cytopathic effect each day for three days and when no cytopathic effect was observed at 60 to 72 hours, the production lot was judged to be free of replication-competent virus. Once the preparation passed the replication-competent virus assay, the VRP and all process pools were released from the containment facility for further analysis.

The yield of VRP at each step in the production process, for a clinical lot of gp160 VRP and *gag/nef/pol* VRP, is shown in Table 3. For the gp160 VRP lot, the VRP production per cell was approximately 180 and for the *gag/nef/pol* VRP lot, the VRP production per cell was approximately 75. The process yields for gp160 VRP and *gag/nef/pol* VRP processes were 42% and 64% respectively. Most of the unrecovered VRP was lost during the TFF unit operation.

The testing performed on process pools is summarized in Table 2. Table 4 contains data derived from the capture pool and the purified VRP eluted from Cellufine Sulfate resin. VRP is concentrated during the process with concentrations increasing from 10^8 infectious units (IU) per mL to 10^9–10^{10} IU/mL. The DNA levels, as measured by the PicoGreen assay (Molecular Probes), drop dramatically during the purification and the concentration of residual Vero DNA was 1.2–3.3 ng per 10^8 IU of VRP. This is below the 10 ng residual Vero DNA per dose specification suggested by the World Health Organization.[20] The amount of residual protein per dose was also reduced by the process. These levels were monitored by the BCA (bicinchoninic acid) Protein Assays (Pierce Biotechnology, Inc.) and by ELISA assay using antibodies generated against a Vero protein panel.[21] Since bovine serum albumin (BSA) was used during cell expansion in the process, the residual levels of this protein were also monitored by ELISA (Bethyl Laboratories, Inc.) The residual BSA levels in all process solutions were below the limit of quantitation of the assay.

An aliquot of various pools sampled during production of gp160 VRP was reduced, denatured, separated by SDS-PAGE and visualized by silver staining as shown in Figure 5. Samples were loaded such that the gel lanes contained the maximum amount of pool in order to maximize detection of low-abundance protein species. Bands corresponding to the VEE E1 (top) and E2 (middle) glycoproteins and the VEE capsid (bottom) protein from the Cellufine Sulfate elution pool are indicated by arrows. These species are not readily detected until the concentrated VRP is eluted from the affinity column. Approximately 8 x 10^7 IU of gp160 VRP was loaded in the Cellufine Sulfate purified material lane.

Purified VRP eluted from this resin were sampled and immediately formulated in a stabilization matrix containing protein, disaccharide, and buffer components, and was frozen at -70° C. Data indicate that the material is stable for periods in excess of two years. Table 5 lists the properties of the formulated bulks for the gp160 and *gag/nef/pol* VRP lots. The material was analyzed after a freeze-thaw cycle and the residual levels of Benzonase and BSA were at or near the limit of quantitation for the assays. For these samples, the residual DNA level was measured by a Vero DNA hybridization assay. The endotoxin content was measured by a gel clot assay and bioburden was determined by a membrane filtration method. Protein determination was performed using the BCA method, and disaccharide concentration was determined by the anthrone method.[21,22]

Formulated bulk VRP was used to make final drug product, material for use in toxicity studies, material for immunogenicity samples, and was used as part of a stability study. Toxicology studies in rabbits demonstrated that the VRP produced by this process resulted in mild to moderate local reactogenic-

Table 3. The Yield of VRP at Each Step in the Production Process.

	Total VRP (IU)	
Sample Process Pool	gp160 VRP	Gag/Nef/Pol VRP
Capture	5.5E+11	2.2E+11
TFF End	3.2E+11	1.3E+11
TFF Break Thru	0	0
Cellufine Sulfate Break Thru	5.3E+08	5.4E+08
Cellufine Sulfate Elution	2.4E+11	1.3E+11
Formulated Bulk Pre-Filter	2.3E+11	1.7E+11
Formulated Bulk post-filter	2.3E+11	1.4E+11

Table 4. Data Derived From the Capture Pool and the Purified VRP Eluted from Cellufine Sulfate Resin.

Test Method	gp160 VRP	Gag/Nef/Pol VRP
Capture Pool		
IFA IU/mL	4.7 x 10^8	1.9 x 10^8
Total Protein µg/mL	81.7	98.8
Bioburden CFU/mL	0	0
Residual DNA ng/mL	1955	2083
BSA ng/mL	<12.5	<12.5
Vero Protein µg/mL	80	155
Cellufine Sulfate Elution Pool		
IFA IU/mL	1.2 x 10^{10}	6.4 x 10^9
Total Protein µg/mL	180	211
Residual DNA ng/mL	149	280
Benzonase ng/mL	0.7	1.9
BSA ng/mL	<12.5	<12.5
Vero Protein µg/mL	43	150

Table 5. Properties of the Formulated Bulks for the gp160 and *gag/nef/pol* VRP Lots.

Parameter	gp160 VRP	Gag/Nef/Pol VRP
Titer (IU/mL) Post-Freeze	1.3×10^9	7.4×10^8
pH	7.2	7.3
Benzonase (ng/mL)	<0.125	0.191
Protein (mg/mL)	9.9	10.2
Vero Protein (µg/mL)	18.4	8.1
BSA (ng/mL)	< 6.25	< 6.25
Disaccharide (mg/mL)	31	30
Vero DNA (ng/mL)	2	50
Bioburden (CFU/mL)	0	0
Endotoxin (EU/mL)	0.13	0.25
Appearance	Clear, colorless solution	Clear, colorless solution

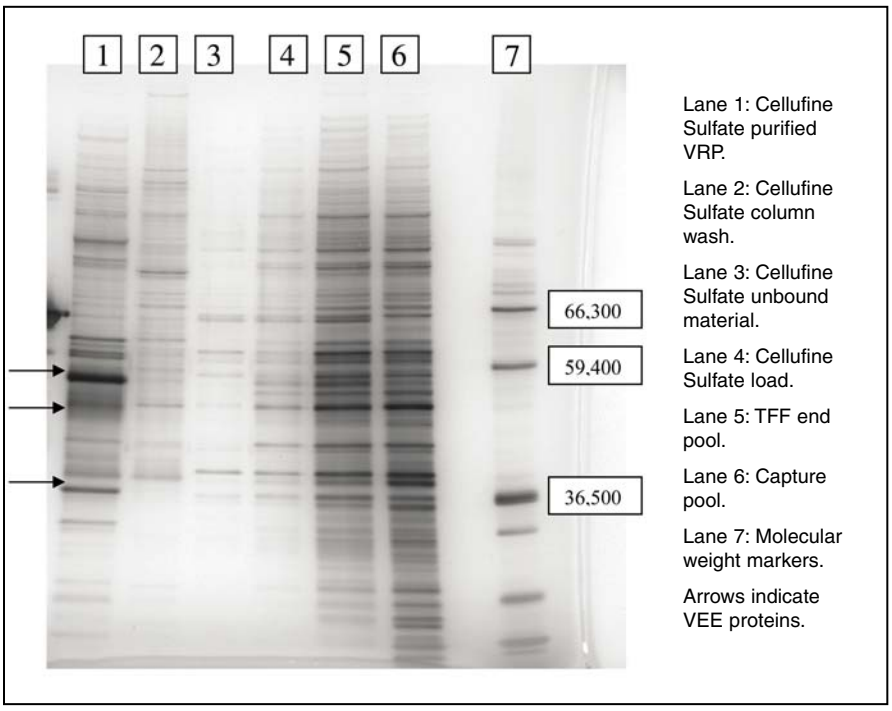

Figure 5. Pools sampled During Production of gp160 VRP.

ity and no systemic toxicity. Material from pilot lots and clinical lots induced comparable levels of antigen-specific humoral and cellular responses in mice.

Summary

Alphavaccines have great potential for use in the human vaccine market as a safe and efficacious alphavirus-based vaccine vector system. The replicon vector system has been engineered to express the gene of interest to high levels in the host cell following VRP infection. However, the VRP are not capable of spreading infection since they do not encode the structural proteins necessary to produce progeny virus particles. The alphavaccine technology developed by AlphaVax has been shown to be amenable for scalable production in a controlled, robust manufacturing process. The manufacturing process was effectively scaled from research and pilot lots. The results obtained from the clinical lots were equivalent to those obtained from pilot lots in all aspects, except scale. The process has been utilized to produce a number of potential vaccine candidates, many of which have two or more components.

The material produced for use in a two-component HIV vaccine was analyzed as described in this report. The material contained no detectable bioburden, had low levels of endotoxin, displayed physical attributes that met expectations, and proved stable when stored frozen below -70° C. The material was used in a GLP rabbit toxicity study and did not cause systemic toxicity. In addition, the material induced both cellular and humoral immune responses in mice after two inoculations.

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