Biopharmaceutical Development Program's Progress on USAMRIID Vaccines for Biological Warfare Agents

Recombinant Protective Antigen (rPA)

Current Status of rPA
As of October 2004, 60 subjects have been treated in a NIAID-sponsored Phase I clinical trial being conducted at the University of Maryland. No safety issues were identified. rPA was administered in doses ranging from 5 to 75 micrograms and was very well tolerated. Due to the robust humeral response additional studies are currently being planned using this material. Commercial scale production of rPA is now underway at an outside NIAID contractor.

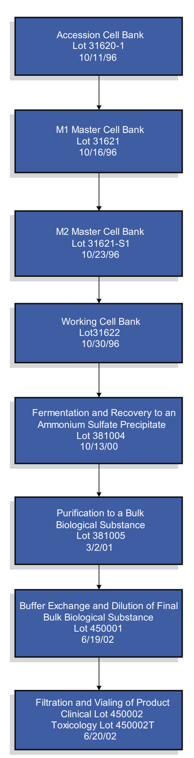
ABSTRACT

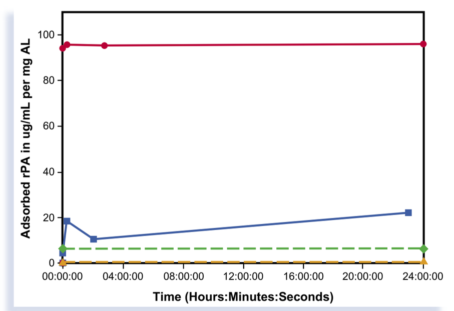
Recombinant Protective Antigen (rPA) is the Active Pharmaceutical Ingredient in a second generation Anthrax Vaccine undergoing clinical investigation. This rPA vaccine differs from the currently licensed vaccine, Anthrax Vaccine Adsorbed (AVA), in that the sole component is a recombinant form of Protective Antigen (PA). Unlike AVA the rPA vaccine contains no components of the two bipartite toxins, Lethal Factor (LF) or Edema Factor (EF), or any of the other Bacillus anthracis-related contaminating proteins that are present in AVA.

rPA was manufactured in an 80-liter fermentor and harvested. The 82 kDa rPA was partially purified from soluble, extracellular protein by tangential flow filtration, liquid chromatography, and ammonium sulfate precipitation. It was further purified using liquid chromatography including hydrophobic interaction, anion-exchange, and size-exclusion matrices. The final product was buffer exchanged and formulated in Dulbecco's Phosphate Buffered Saline (DPBS). The final vialed product was > 95% pure as determined by Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and high pressure-size exclusion chromatography, and passed all safety tests.

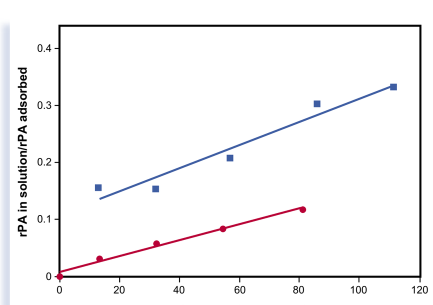
The clinical protocol involves adsorption of the rPA to an aluminum-based adjuvant. The adsorptive characteristics of rPA and two aluminum-containing adjuvants were examined. Based on the pI of rPA (pI 5.6) and the zero charge point of aluminum hydroxide adjuvant (11.5) and aluminum phosphate adjuvant (4.5), it was predicted and demonstrated that rPA bound in a more efficient manner to aluminum hydroxide adjuvant than to aluminum phosphate adjuvant in the physiological buffer.

Summary Production Flowchart for rPA

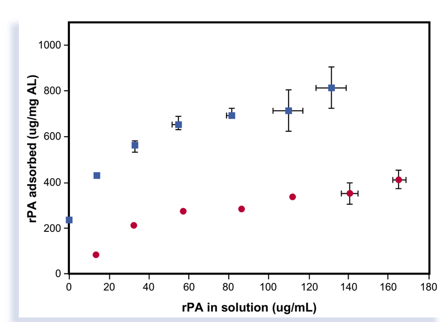




The graph shows recombinant Protective Antigen adsorption study comparing Adju-Phos (■) and Alhydrogel (●) adsorption as a function of time. There are 2 controls, the first being the diluted rPA (▲), the second being rPA mixed with a non-binding resin (◆). The x-axis is time and the y-axis is adsorbed rPA in µg/mL; 100 µg/mL was the maximum amount of rPA available for adsorption.

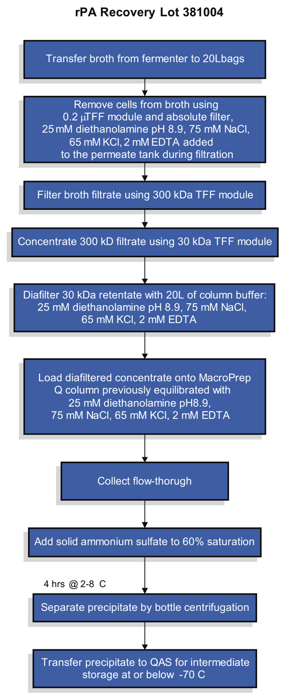


Linear Langmuir plots for the adsorption of rPA onto Alhydrogel as a function of diluent, water (●) and DPBS (■). The average adsorptive capacity for water-diluted Alhydrogel from 4 runs was 0.73 mgs rPA per mg of aluminum with an adsorptive capacity of 100 mLs/mg and an R2 value of 0.9850. The average adsorptive capacity for DPBS-diluted Alhydrogel from 2 runs was 0.50 mgs rPA per mg of aluminum with an adsorptive capacity of 4.6 mLs/mg and an R2 value of 0.9376.

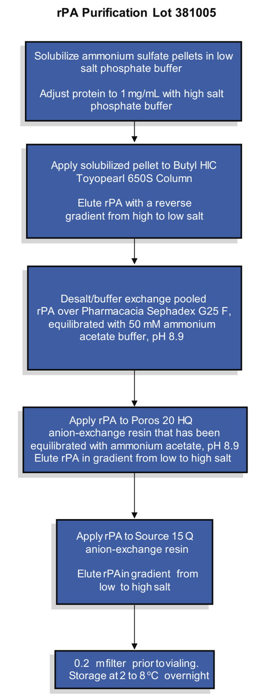


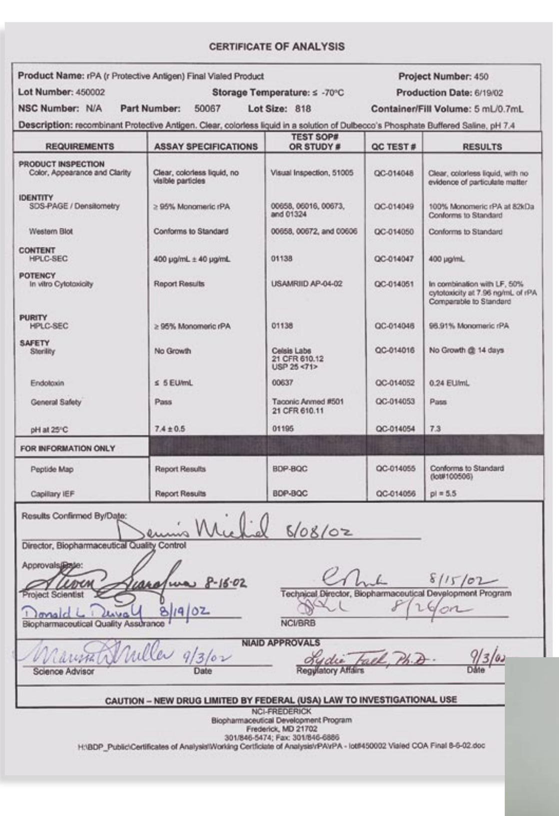
Adsorption Isotherms of rPA onto Alhydrogel by diluent. The graph shows the difference in adsorption to the adjuvant as a function of diluent, water (■) and DPBS (●).

rPA Recovery Lot 381004



rPA Purification Lot 381005





CERTIFICATE OF ANALYSIS

Product Name: rPA (r Protective Antigen) Final Vialed Product — Project Number: 450
Lot Number: 450002 — Storage Temperature: ≤ -70°C — Production Date: 6/19/02
NSC Number: N/A — Part Number: 50067 — Lot Size: 818 — Container/Fill Volume: 5 mL/0.7mL
Description: recombinant Protective Antigen. Clear, colorless liquid in a solution of Dulbecco's Phosphate Buffered Saline, pH 7.4

REQUIREMENTS	ASSAY SPECIFICATIONS	TEST SOP# OR STUDY #	QC TEST #	RESULTS
PRODUCT INSPECTION Color, Appearance and Clarity	Clear, colorless liquid, no visible particles	Visual Inspection, 51005	QC-014048	Clear, colorless liquid, with no evidence of particulate matter
IDENTITY SDS-PAGE / Densitometry	≥ 95% Monomeric rPA	00658, 00016, 00673, and 01324	QC-014049	100% Monomeric rPA at 82kDa Conforms to Standard
Western Blot	Conforms to Standard	00658, 00672, and 00606	QC-014050	Conforms to Standard
CONTENT HPLC-SEC	400 µg/mL ± 40 µg/mL	01138	QC-014047	400 µg/mL
POTENCY In vitro Cytotoxicity	Report Results	USAMRIID AP-04-02	QC-014051	In combination with LF, 50% cytotoxicity at 7.96 ng/mL of rPA Comparable to Standard
PURITY HPLC-SEC	≥ 95% Monomeric rPA	01138	QC-014048	96.91% Monomeric rPA
SAFETY Sterility	No Growth	Celsis Labs 21 CFR 610.12 USP 25 <71>	QC-014016	No Growth @ 14 days
Endotoxin	≤ 5 EU/mL	00637	QC-014052	0.24 EU/mL
General Safety	Pass	Taconic Anmed #501 21 CFR 610.11	QC-014053	Pass
pH at 25°C	7.4 ± 0.5	01195	QC-014054	7.3
FOR INFORMATION ONLY				
Peptide Map	Report Results	BDP-BQC	QC-014055	Conforms to Standard (lot#100506)
Capillary IEF	Report Results	BDP-BQC	QC-014056	pI = 5.5

Results Confirmed By/Date: Dennis Michiel 6/08/02
Director, Biopharmaceutical Quality Control
Approvals/Date: 8-16-02 — 8/15/02
Project Scientist — Technical Director, Biopharmaceutical Development Program
Donald L. 8/19/02 — 8/24/02
Biopharmaceutical Quality Assurance — NCI/BRB
NIAID APPROVALS
9/3/02 — 9/3/02
Science Advisor — Date — Regulatory Affairs — Date
CAUTION – NEW DRUG LIMITED BY FEDERAL (USA) LAW TO INVESTIGATIONAL USE
NCI-FREDERICK
Biopharmaceutical Development Program
Frederick, MD 21702
301/846-5474; Fax: 301/846-6886

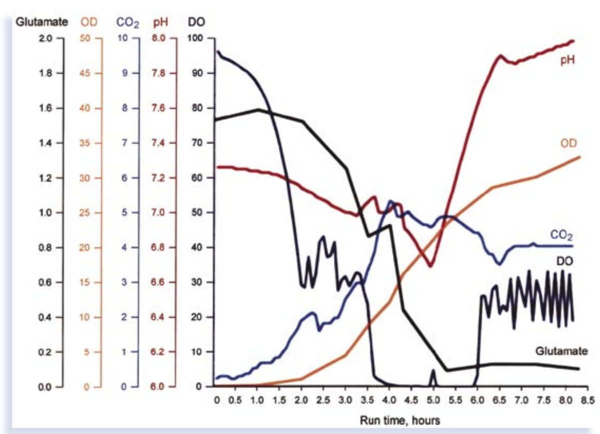
recombinant Protective Antigen (rPA) 413 mcg/mL
289 mcg/vial — Vol. 0.7 mL
Lot #450002 — Sterile — Store at ≤ -70°C
Formulated in Dulbecco's PBS pH 7.4 — Mfg Date 6-19-02
Contains No Preservatives
Caution: New Drug Limited by Federal (USA) Law to Investigational Use
BDP, NCI-Frederick, Frederick, MD 21702

Staphylococcal Enterotoxin B (rSEB)

Current Status of rSEB
A high-yield process was developed for the production and purification of rSEB. In May of 2000 over one gram of clinical-grade material was delivered to the Department of Defense. A drug master file has been submitted to the FDA.

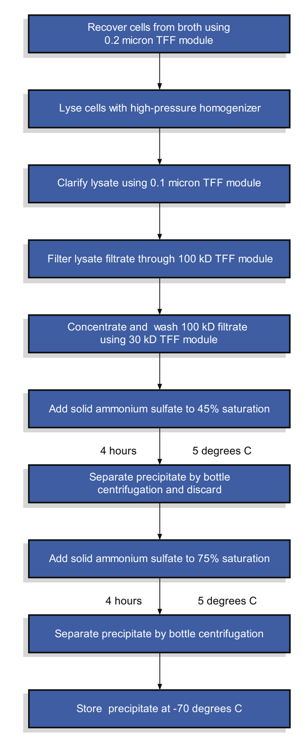
ABSTRACT

An attenuated, recombinant form of Staphylococcal Enterotoxin B (rSEB) was overexpressed in *E. coli* under transcriptional control of the T7 promoter. The 28-kDa rSEB was partially purified from soluble, intracellular protein by tangential flow filtration and differential ammon-ium sulfate precipitation. The intermediate product was then further purified using low-pressure liquid chromatography including hydrophobic interaction, cation-exchange, and size exclusion matrices. The final vialed product was >95% pure as determined by Coomassie blue-stained sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), high pressure-size exclusion chromatography (HP-SEC), and capillary zonal electrophoresis (CZE). The endotoxin level was <0.6 EU/mg. Final estimated yield of purified rSEB was 147 mg/L of starting culture. Purified rSEB was stable, elicited an immune response in mice, and protected mice against a lethal challenge with the native toxin.

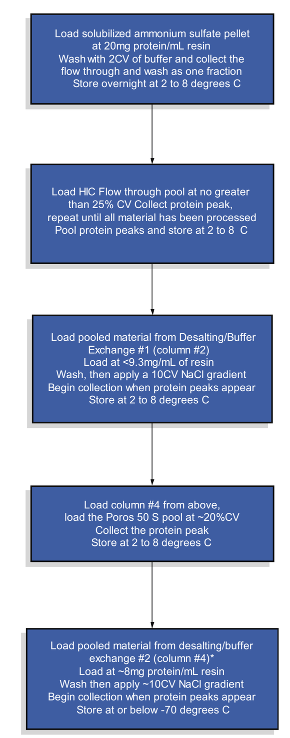


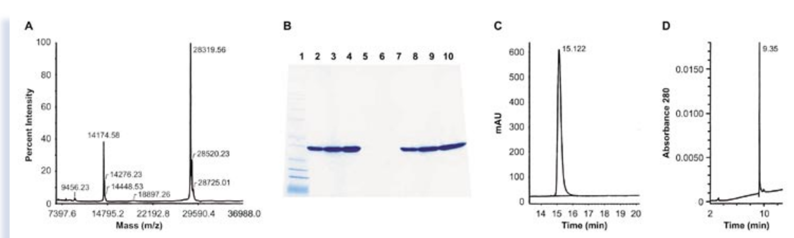
Physical-chemical parameters from an 80-liter fermentation. Glutamate, OD_{600}, pH, DO and CO_2 were monitored throughout the campaign. The temperature range was 36-38°C. The agitation rate was increased from 185 to 500 rpm by the 3.5-h time point.

rSEB Recovery Lot 100702

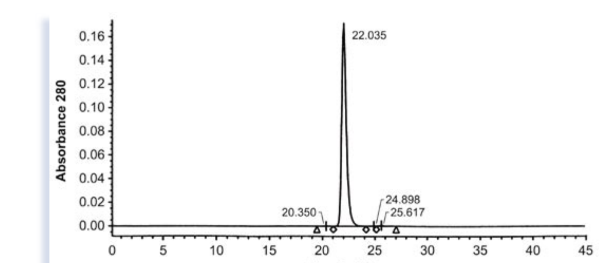


rSEB Intermediate Purification Lot 2490002

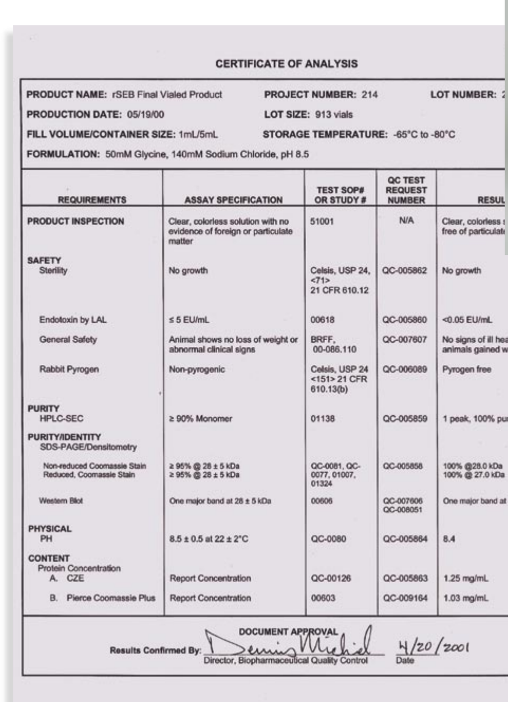




Purity of final purified bulk rSEB following polishing on Superdex-75. (**A**) MALDI; (**B**) SDS-PAGE. Lanes 1, molecular weight standards; 2-4, 5 µg,10 µg, 20 µg under reducing conditions; 8-10, 5 µg,10 µg, 20 µg under non-reducing conditions. (**C**) Reverse phase HPLC; (**D**) CZE.



Purity of final, purified rSEB bulk product by SEC-HPLC. Twenty-five µg of purified rSEB was analyzed on a Tosohaas G3000SW HPLC column. Detection was at OD280 nm. Peak purity was >99%.

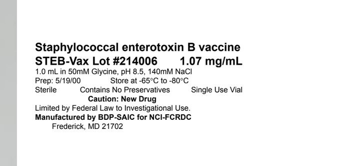


CERTIFICATE OF ANALYSIS

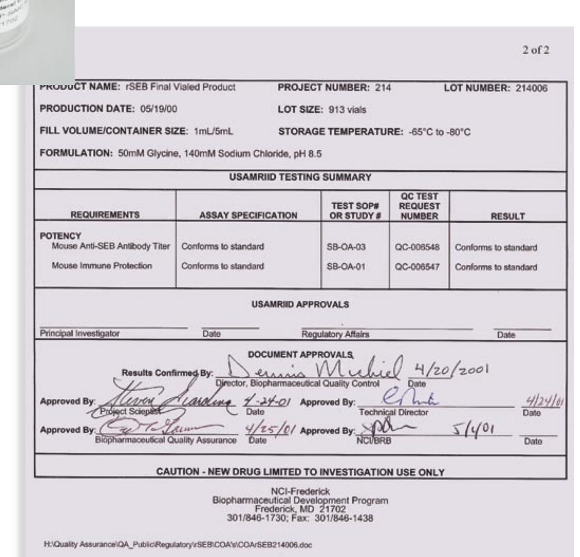
PRODUCT NAME: rSEB Final Vialed Product — PROJECT NUMBER: 214 — LOT NUMBER: 214006
PRODUCTION DATE: 05/19/00 — LOT SIZE: 913 vials
FILL VOLUME/CONTAINER SIZE: 1mL/5mL — STORAGE TEMPERATURE: -65°C to -80°C
FORMULATION: 50mM Glycine, 140mM Sodium Chloride, pH 8.5

REQUIREMENTS	ASSAY SPECIFICATION	TEST SOP# OR STUDY #	QC TEST REQUEST NUMBER	RESULT
PRODUCT INSPECTION	Clear, colorless solution with no evidence of foreign or particulate matter	51001	N/A	Clear, colorless solution, free of particulate matter
SAFETY Sterility	No growth	Celsis, USP 24, <71> 21 CFR 610.12	QC-005862	No growth
Endotoxin by LAL	≤ 5 EU/mL	00618	QC-005860	<0.05 EU/mL
General Safety	Animal shows no loss of weight or abnormal clinical signs	BRFF, 00-086.110	QC-007607	No signs of ill health, all animals gained weight
Rabbit Pyrogen	Non-pyrogenic	Celsis, USP 24 <151> 21 CFR 610.13(b)	QC-006089	Pyrogen free
PURITY HPLC-SEC	≥ 90% Monomer	01138	QC-005859	1 peak, 100% purity
PURITY/IDENTITY SDS-PAGE/Densitometry Non-reduced Coomassie Stain Reduced, Coomassie Stain	≥ 95% @ 28 ± 5 kDa ≥ 95% @ 28 ± 5 kDa	QC-0081, QC-0077, 01007, 01324	QC-005858	100% @28.0 kDa 100% @ 27.0 kDa
Western Blot	One major band at 28 ± 5 kDa	00606	QC-007606 QC-008051	One major band at 31.3 kDa
PHYSICAL pH	8.5 ± 0.5 at 22 ± 2°C	QC-0080	QC-005864	8.4
CONTENT Protein Concentration A. CZE	Report Concentration	QC-00126	QC-005863	1.25 mg/mL
B. Pierce Coomassie Plus	Report Concentration	00603	QC-009164	1.03 mg/mL

DOCUMENT APPROVAL
Results Confirmed By: Dennis Michiel 4/20/2001
Director, Biopharmaceutical Quality Control — Date



Staphylococcal enterotoxin B vaccine
STEB-Vax Lot #214006 1.07 mg/mL
1.0 mL in 50mM Glycine, pH 8.5, 140mM NaCl
Prep: 5/19/00 — Store at -65°C to -80°C
Sterile — Contains No Preservatives — Single Use Vial
Caution: New Drug
Limited by Federal Law to Investigational Use.
Manufactured by BDP-SAIC for NCI-FCRDC
Frederick, MD 21702



2 of 2

PRODUCT NAME: rSEB Final Vialed Product — PROJECT NUMBER: 214 — LOT NUMBER: 214006
PRODUCTION DATE: 05/19/00 — LOT SIZE: 913 vials
FILL VOLUME/CONTAINER SIZE: 1mL/5mL — STORAGE TEMPERATURE: -65°C to -80°C
FORMULATION: 50mM Glycine, 140mM Sodium Chloride, pH 8.5

USAMRIID TESTING SUMMARY

REQUIREMENTS	ASSAY SPECIFICATION	TEST SOP# OR STUDY #	QC TEST REQUEST NUMBER	RESULT
POTENCY Mouse Anti-SEB Antibody Titer	Conforms to standard	SB-OA-03	QC-006548	Conforms to standard
Mouse Immune Protection	Conforms to standard	SB-OA-01	QC-006547	Conforms to standard

USAMRIID APPROVALS
Principal Investigator — Date — Regulatory Affairs — Date

DOCUMENT APPROVALS
Results Confirmed By: Dennis Michiel 4/20/2001
Director, Biopharmaceutical Quality Control — Date
Approved By: 4-24-01 — Approved By: 4/24/01
Project Scientist — Date — Technical Director — Date
Approved By: 4/25/01 — Approved By: 5/4/01
Biopharmaceutical Quality Assurance — Date — NCI/BRB — Date

CAUTION - NEW DRUG LIMITED TO INVESTIGATION USE ONLY
NCI-Frederick
Biopharmaceutical Development Program
Frederick, MD 21702
301/846-1730; Fax: 301/846-1438

Venezuelan Equine Encephalitis (VEE)

Current Status of VEE
In July 2003, over 800 ml of bulk material was delivered for use in preclinical safety and efficacy studies. The product was used in non-clinical toxicology studies and was found to be protective in equine challenge studies.

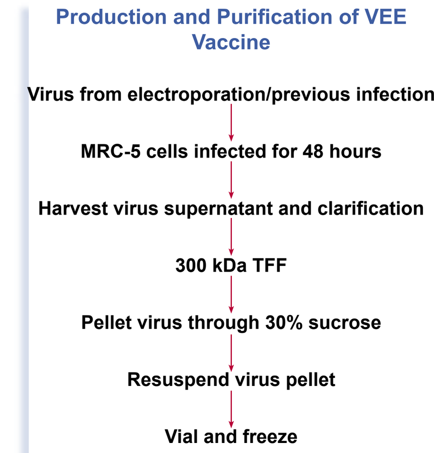
ABSTRACT

Venezuelan Equine Encephalitis (VEE) Virus can cause severe and often deadly encephalitis in horses and humans. VEE is naturally transmitted via the bite of mosquitoes, but it may also be transmitted via the aerosol route and constitute an effective biological warfare agent. Using recombinant DNA technology a new VEE vaccine candidate, V3526, has been identified.

A production process was developed for the manufacture of the V3526 vaccine. The process started with the fermentation of bacteria harboring the plasmid pV3526. Plasmid DNA was then purified from these bacteria. Plasmid pV3526 DNA was transcribed into RNA, which was subsequently introduced into MRC-5 cells to produce VEE virus. Through several rounds of amplification and purification, 10^9 to 10^{10} infectious units of bulk virus materials were produced and formulated.

The project was jointly managed by JVAP, DVC, NCI and SAIC. Bulk material was vialed and is being used for preclinical evaluation.

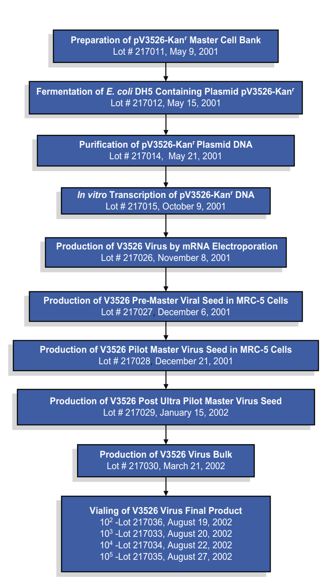
Production and Purification of VEE Vaccine



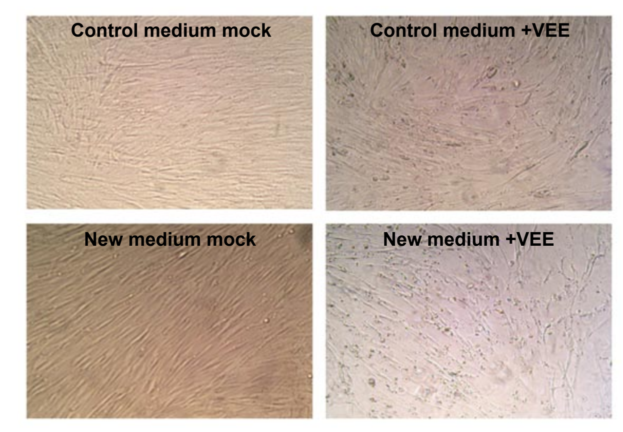
Recovery of VEE Vaccine During Purification Process

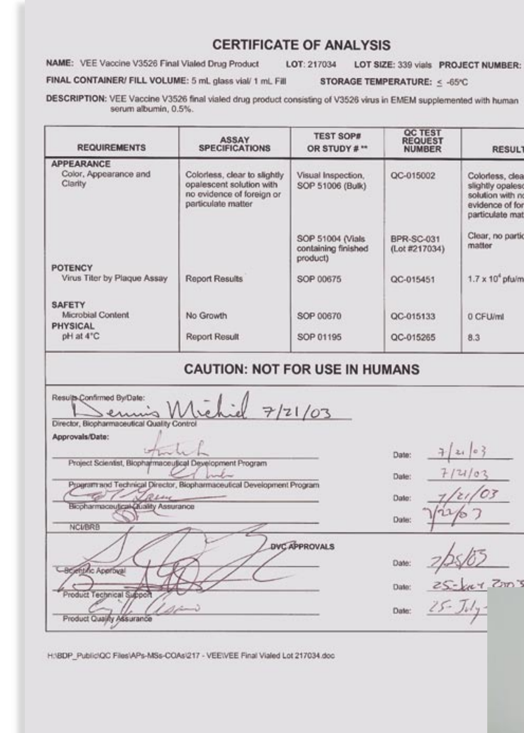
Samples	Titers (PFU/ml)	Volume (ml)	Total Virus	Recovery
Pre-TFF	7.0×10^5	1250	8.8×10^8	100%
Post-TFF	1.2×10^6	275	3.3×10^8	37.7%
Post-Ultracentrifuge	7.5×10^5	330	2.5×10^8	28.3%
Post-Filtration	9.4×10^5	330	3.1×10^8	35.5%

Production Summary of V3526 Virus Vaccine Product



A new infection process was developed. The below photos indicate that higher levels of virus infection were achieved by using a new cell culture medium.





CERTIFICATE OF ANALYSIS

NAME: VEE Vaccine V3526 Final Vialed Drug Product — LOT: 217034 — LOT SIZE: 339 vials — PROJECT NUMBER: 217
FINAL CONTAINER/ FILL VOLUME: 5 mL glass vial/ 1 mL Fill — STORAGE TEMPERATURE: ≤ -65°C
DESCRIPTION: VEE Vaccine V3526 final vialed drug product consisting of V3526 virus in EMEM supplemented with human serum albumin, 0.5%.

REQUIREMENTS	ASSAY SPECIFICATIONS	TEST SOP# OR STUDY # **	QC TEST REQUEST NUMBER	RESULT
APPEARANCE Color, Appearance and Clarity	Colorless, clear to slightly opalescent solution with no evidence of foreign or particulate matter	Visual Inspection, SOP 51006 (Bulk)	QC-015002	Colorless, clear to slightly opalescent solution with no evidence of foreign or particulate matter
		SOP 51004 (Vials containing finished product)	BPR-SC-031 (Lot #217034)	Clear, no particulate matter
POTENCY Virus Titer by Plaque Assay	Report Results	SOP 00675	QC-015451	1.7×10^4 pfu/ml
SAFETY Microbial Content	No Growth	SOP 00670	QC-015133	0 CFU/ml
PHYSICAL pH at 4°C	Report Result	SOP 01195	QC-015265	8.3

CAUTION: NOT FOR USE IN HUMANS

Results Confirmed By/Date: Dennis Michiel 7/21/03
Director, Biopharmaceutical Quality Control
Approvals/Date:
Project Scientist, Biopharmaceutical Development Program — Date: 7/21/03
Program and Technical Director, Biopharmaceutical Development Program — Date: 7/21/03
Biopharmaceutical Quality Assurance — Date: 7/21/03
NCI/BRB — Date:
DVC APPROVALS
Scientific Approval — Date: 7/25/03
Product Technical Support — Date: 25-July-2003
Product Quality Assurance — Date: 25-July-

Venezuelan Equine Encephalitis V3526 Vaccine
Lot # 217034 — DVC Part # 0019-0003 — **2.5x10⁴ pfu/ml***
1.0 ml — Diluent: EMEM with 0.5% HSA
Mfg. Date: 8-22-02 — Store at ≤ -65°C
Contains No Preservatives — Sterile
To Investigational Use
Caution: New Drug Limited By Federal (USA) Law
Manufactured for **DynPort Vaccine Company LLC**
By BDP, NCI-Frederick, Frederick, MD 21702 ***See COA for Actual Titer**

Yersinia pestis F1 and V-antigen (F1-V)

Current Status of F1-V
As of December 2004, a purification process has been developed capable of producing gram amounts of highly-purified product. Over 800 mgs are now available and are being formulated for the evaluation of administration strategies by NIAID.

ABSTRACT

Yersinia pestis F1- and V-antigen protein subunit vaccines are protective in animal models of pneumonic and bubonic plague. The US Army Medical Research Institute of Infectious Disease (USARMRID) has sought robust protective immunity by engineering a two-subunit antigen termed 'F1-V' (Figure 1).

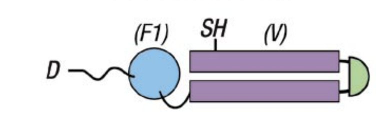
The National Institute of Allergic and Infectious Disease (NIAID) has interest in F1-V for investigating new vaccination methods and has asked the National Cancer Institute-Developmental Therapeutics Program Biopharmaceutical Development Program (NCI-DTP, BDP) to manufacture pre-clinical supplies.

Critically, F1-V associates both with itself and with host cell proteins to form mixed-multimers that complicate high yield F1-V purification. To meet this challenge, the BDP Fermentation Development Laboratory has optimised F1-V expression yield and the BDP Purification Development Laboratory has minimized multimer formation and redesigned the purification process to produce purified, monomeric F1-V.

Purified F1-V forms multimers were identified (Figure 2). During processing these multimer species were dispersed by maintaining moderately basic solution conditions, preventing oxidation using argon gas sparging, cleaving disulfide cross-links with reducing agents, and disrupting pi-cation interactions using buffer additives, (Figure 3).

These dispersion methods have been incorporated into a novel and scaleable process (Figure 4) permitting manufacture of 850 mg of monomeric F1-V having sufficient purity for pre-clinical studies (Figure 5 and Figure 6).

Figure 1 – Representation of the F1-V Fusion Protein



Schematic of the 53.2 kDa F1-V fusion protein consisting of the globular F1-subunit (16.1 kDa) connected by a short inserted linker to the coiled-coil V-subunit (36.8 kDa). Note the F1-subunit N-terminal, 'fibril-forming', donor-strand 'D' and the V-subunit exposed, free-thiol provided by Cysteine-274.

Figure 2 – F1-V Forms

Illustrations of F1-V association through (A) Free Thiol form; (B) Globulin-like, charge-complementation at low pH; (C) covalent, V-subunit disulfide-bonding at Cysteine 274; (D) non-covalent, F1-domain pi-cation interactions or open coiled-coil interactions. Covalent and non-covalent interactions together permit formation of (E) trimer; (F) multimers and (G) mixed covalent linkage to contaminant; and (H) non-covalent linkage to contaminant."

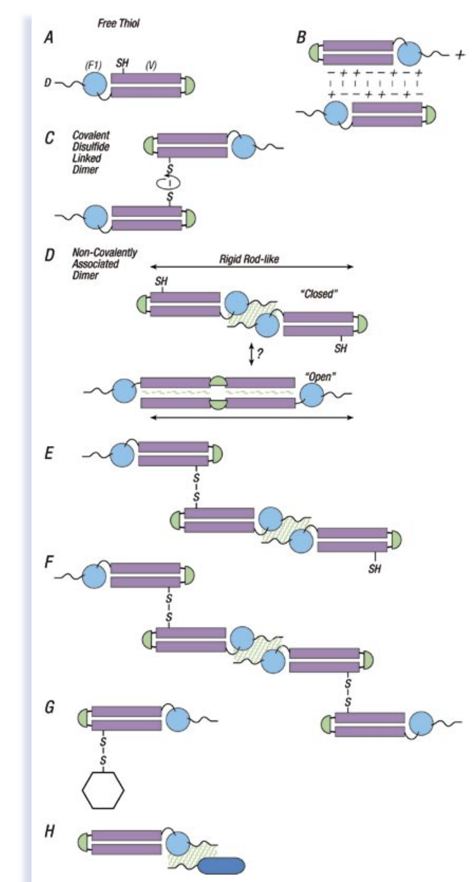
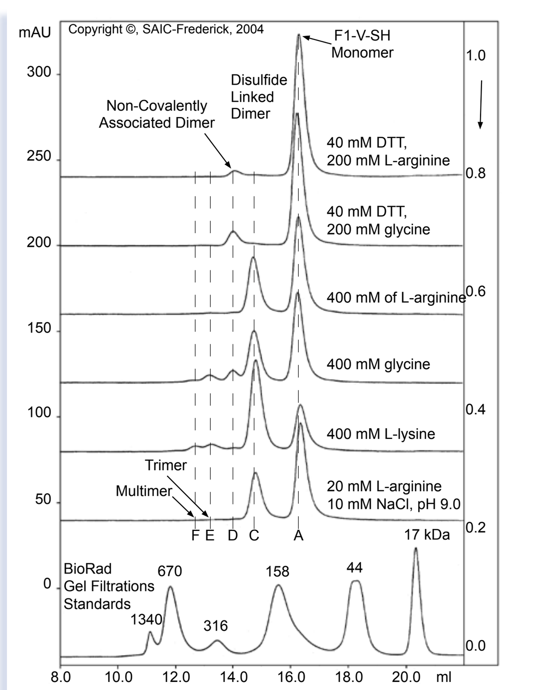


Figure 3 – Examples of F1-V Multimer Profiles under Test Conditions



HPLC-SEC (Tandem G3000SWxl columns) analysis of the current F1-V lots exposed to extended room temperature incubation (19-26 hours) in the presence of the additives noted above. Peak Identifications match those in the legend for Figure 2. Note that based upon refractive index and QUELS analysis, F1-V multimers have varying degrees of rod-like geometry (data not shown), leading to anomalously larger apparent molecular sizes by HPLC-SEC.

Figure 4 – Process Summary

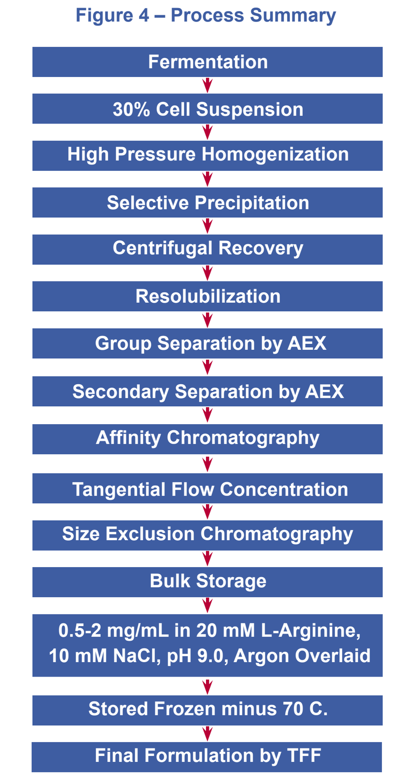
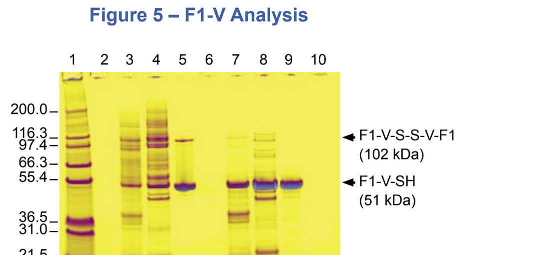
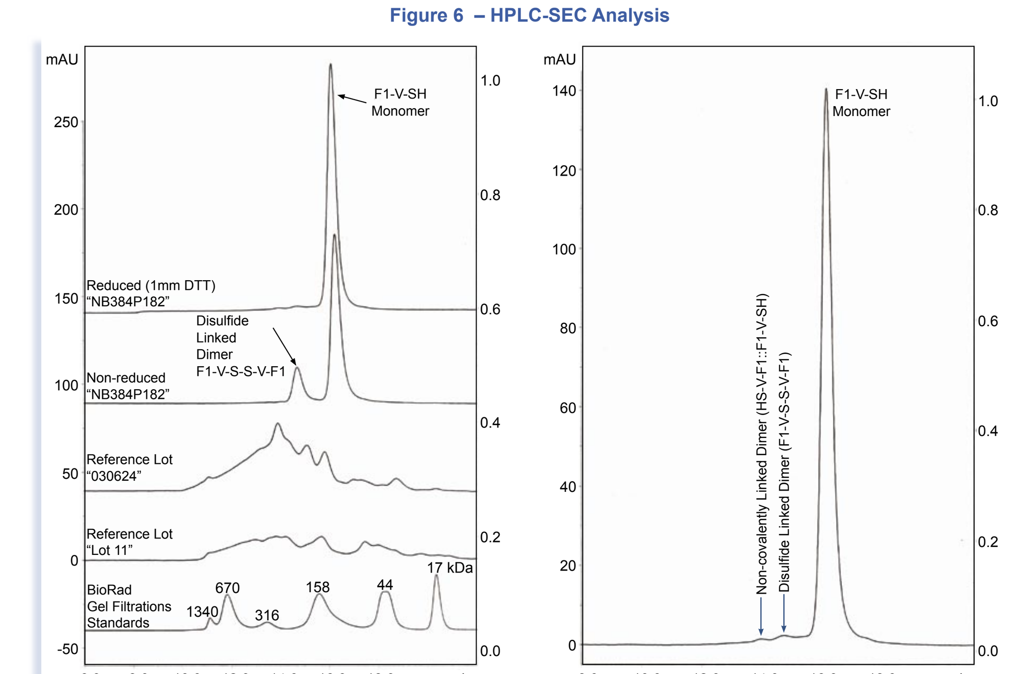


Figure 5 – F1-V Analysis



Sypro Ruby-Stained, 4-12% SDS-PAGE analysis of three F1-V lots with added 10 mM iodoacetamide for non-reducing (lanes 3-5) or added 5% 2-mercaptoethanol for reducing conditions (lanes 7-9). Two micrograms total protein were loaded per lane. Invitrogen Mark 12 Molecular Weight Marker (20 uL) was loaded (lane 1). Samples were prior lot Reference 'Lot 11' (lanes 2,7), prior lot Reference Lot '030624' (lanes 3,8); and the current lot BDP 'NB384P182' (lanes 4,9)."

Figure 6 – HPLC-SEC Analysis



HPLC-SEC (Tandem G3000SWxl Columns) analysis of three F1-V lots under reducing (trace 1) and non-reducing (traces 2-5) conditions normalized to 20 micrograms of total protein injection. Trace identifications are noted above. Apparent molecular weight assignments were made versus BioRad Gel Filtrations Standards.