A publication of
The Williamsburg BioProcessing Foundation

March/April 2005

BioProcessing JOURNAL™

The Most Trusted Source of BioProcess Technology™

Vol. 4 No. 2

www.bioprocessingjournal.com

Large-Scale Chromatographic Purification of an Attenuated Chimeric Poliovirus

By TOM OUELLETTE, STEVEN L. GIARDINA, EARL NELSON, JUDITH POILEY-NELSON, TREVOR BROADT, and SIBYLLE HERZER

Poliovirus is a small (28–30-nm diameter) non-enveloped RNA virus belonging to the family Picornaviridae. The ability of poliovirus to cross the blood–brain barrier and its natural infectivity of central nervous system (CNS) tissue via the CD155 receptor, found exclusively in primates, has promoted the investigation of an attenuated poliovirus for the treatment of malignant gliomas.[1–3] However, use of the virus in clinical testing is limited due to low yields obtained from conventional purification methodologies.

Conventional methods of virus concentration and purification by ultracentrifugation are labor intensive and do not meet production-scale requirements for nonclinical and clinical testing. These procedures are limited in scale due to lengthy processing times and small-volume capacity. Process recoveries are low due to the number of physical manipulations required and shear effects during centrifugation. The chromatographic method described herein offers the advantage of scalability, decreased processing times, and increased recovery of infective virus. Purification of retroviruses using size-exclusion chromatography had been reported previously.[4]

Poliovirus has an isoelectric point of approximately 7.0 and a molecular weight of 20–30 million daltons. The viral capsid is made up of five proteins arranged in an icosahedral geometry (Fig. 1) containing 60 copies each of VP1 (33–35 kDa) and VP3 (24–27 kDa) and an average of 58 to 59 copies each of proteins VP2 (28–31 kDa) and VP4 (6–8 kDa). One to two copies of their precursor VP0 (~41,000 kDa) are also present.[5] Both size and isolelectric point characteristics were utilized in the purification strategy.

The virus was separated from host cell contaminants using a Sepharose 4 Fast Flow column (GE Healthcare-Biosciences, Piscataway, NJ) utilizing size differences. This purification step had the added benefit of minimal shear forces on the virus during processing. The virus was further purified by anion-exchange chromatography using a Q650M (Tosoh Biosciences, Montgomeryville, PA) column based on the reported purification of hepatitis A.[6]

Before final formulation, the virus was concentrated more than 100-fold to meet the 2 x 10^9 pfu/ml titer required for preclinical testing. Early attempts at concentrating the virus by binding to anion-exchange resins, Q650M (Tosoh Biosciences) and Q Sepharose XL (GE Healthcare-Biosciences), were unsuccessful (data not shown). However, the development of prototype "salt-tolerant" amine ligand resins was reported in the literature.[7] Three different anion-exchange resin chemistries were obtained from GE Healthcare-Biosciences (Custom Design Media group) and evaluated with purified poliovirus. Based on the ionic conditions for virus elution and recovery of infectivity, the bis (3-Aminopropyl) amine resin was selected. The final purified virus pool was buffer exchanged over a Sephadex G-25 column (GE Healthcare-Biosciences) into 1X MEM. Human serum albumin was added to a final concentration of 0.2 percent to help stabilize the virus.

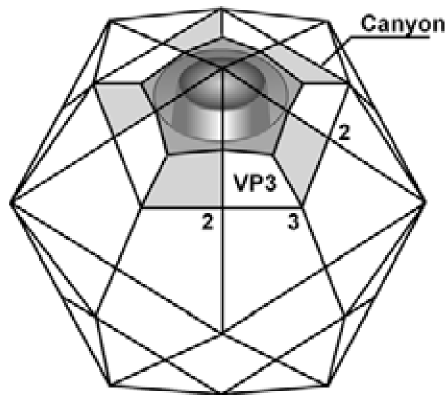

Figure 1: Schematic representation of the poliovirus capsid. The two-, three-, and fivefold axes of symmetry and the positions of capsid proteins VP1, VP2, and VP3 are indicated for one protomer (of sixty total). The depression along the fivefold axis is indicated as well.

In order to evaluate the potential for PVS(RIPO), a nonpathogenic chimera containing the IRES element of human rhinovirus type 2 (HRV2) in a poliovirus type 1 (Sabin) background, in preclinical and clinical studies, it was necessary to develop a robust purification process capable of generating virus of acceptable purity. In this study, we present

Steven L. Giardina, Ph.D. *(giardina@ncifcrf.gov), is director, biopharmaceutical quality control; Earl Nelson is a development associate; Judith Poiley-Nelson, Ph.D., is a QC development scientist; Trevor Broadt is a QC development scientist; and Tom Ouellette is a development associate, biopharmaceutical development program, SAIC-Frederick, Inc., Frederick, MD; Sibylle Herzer, Ph.D., is a staff scientist, GE Healthcare-Biosciences, Piscataway, NJ.*

a robust chromatographic process that meets both qualitative and quantitative requirements.

Material and Methods

Virus stock

Dr. Matthias Gromier (Duke University) provided the PVS (RIPO) viral plasmid to the National Cancer Institute/BioPharmaceutical Development Program (NCI/BDP). Following fermentation and purification, 15 µg of plasmid DNA was linearized with Sal*I* restriction endonuclease (5–10 u/µg DNA) at 37° C for 1.5 hours. The PVS(RIPO) RNA was synthesized *in vitro* with T7 RNA polymerase using the RiboMax IVT kit (Promega, Madison, WI). The viral RNA was electroporated at a voltage of 0.5 KW (0.25 µuF capacitance) into Vero cells to generate the viral seed stock and transferred to the purification development laboratory.

Virus Production Cell Culture

Vero cells used for virus production and infectivity assays were obtained from a World Health Organization (WHO) master cell bank from Magenta Corporation (Rockville, MD). The working cell bank was prepared at the BDP. Four 10-tier Nunc Cell factories (Nalge Nunc International, Rochester, NY) were used for virus production. Each cell factory was seeded with 3 x 10^4 cells/cm^2 and grown to a cell density of 2.96 x 10^5 cells/cm^2 (1.83 x 10^9 cells total) in three days at 36–37° C. Prior to inoculation, the cell factories were drained and washed with Hams F12/Dulbecco's modified Eagle's media (DMEM) (Invitrogen/Gibco, Carlsbad, CA) to remove serum-containing growth medium. Cell factories were infected at an MOI of 0.1 with virus in Hams F12/DMEM serum-free media (Invitrogen/Gibco). The cell cultures were incubated at 33° C until 90–100% cytopathic effect (CPE) was observed (approximately 72 hours). Medium and lysed cells were harvested and clarified by centrifugation at 640 x g for 20 minutes at 2–8° C. The supernatant was centrifuged at 4225 x g for 20 minutes at 2–8° C. Infectious virus was titered by plaque assay, then 3.2 L of supernatant with an average titer of 1-2 x 10^8 pfu/ml/cm^2 per cell factory was produced. A 1/3-scale production run using 1100 mls of the clarified virus supernatant is described below. The supernatant was treated with 25 u/ml Benzonase (EMD Biosciences-Novagen, Madison, WI) for 16–18 hours at 20–25° C in the presence of 1 mM $MgCl_2$.

Table 1: Step Recovery and Overall Yields for 1/3-scale Production Run

Step	Volume (ml)	Titer (pfu/ml)	Total pfus	Step Recovery (%)	Overall Yield (%)
Harvest Benzonase treated	1100	2.8 x 10^7	3.08 x 10^{10}	100	100
4 Sepharose FF Run #1	1600	1.05 x 10^7	2.43 x 10^{10}	79	79
Run #2	750	1.0 X 10^7			
Q650M/CDM Pool 1	3.0	7.0 x 10^9	2.10 x 10^{10}	86	68
Pool 2	9.0	5.5 x 10^8	4.95 x 10^9	20	16
G-25 Formulation run #1 for Q650M/CDM pool 1	4.1	2.17 x 10^9	8.9 x 10^9	42	29
G-25 Formulation run #2 for Q650M/CDM pool 2	7.2	4 x 10^8	2.9 x 10^9	58	9
Vialed formulated virus G-25 run #1 pool (0.2-µm filtered)	4.1	2.17 x 10^9	8.9 x 10^9	100	29

Size-Exclusion Chromatography: Column 1

A 10 cm (i.d.) bioprocess glass (BPG) column (GE Healthcare-Biosciences) was packed with 3.4 L of Sepharose 4FF resin (GE Healthcare-Biosciences) to a bed height of 44 cm, depyrogenated and then flushed with water for injection (WFI) quality water prior to equilibration with 4.7 mM $NaPO_4$, 25 mM NaCl pH 7.5. The Benzonase-treated harvest was applied in two column injections, one 750 ml (22% column volume) and one 350 ml (10% column volume) at 30 cm/hr. The column eluate was monitored for UV absorbance (OD_{280} nm) and conductivity (mS/cm).

An initial volume of 1100 ml was collected into a 2-liter polyethelene terephthalate copolyester (PETG) bottle (Nalgene, Rochester, NY). UV-absorbing peaks were collected into 2-liter sterile PETG bottles (Nalgene). The virus activity, determined by plaque infectivity assay, was detected in the excluded volume for both column injections with fraction volumes of 1600 ml for the first injection and 750 ml for the second.

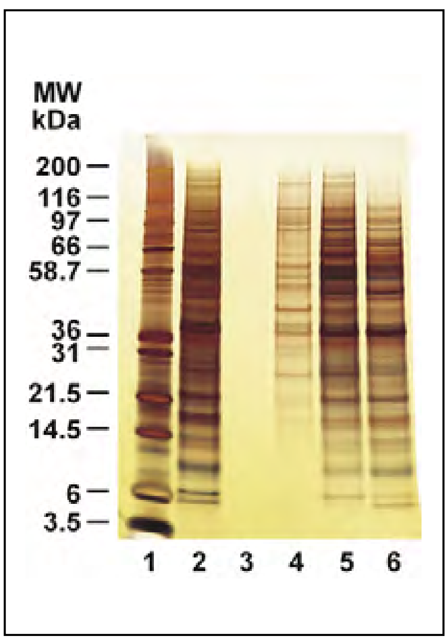

Figure 2: Partial purification of lysate on Sepharose 4FF column. Lane 1: mark 12 molecular weight standards; Lane 2: load; Lane 3: pre-virus pool; Lane 4: virus pool; Lane 5: post-virus pool; Lane 6: salt peak.

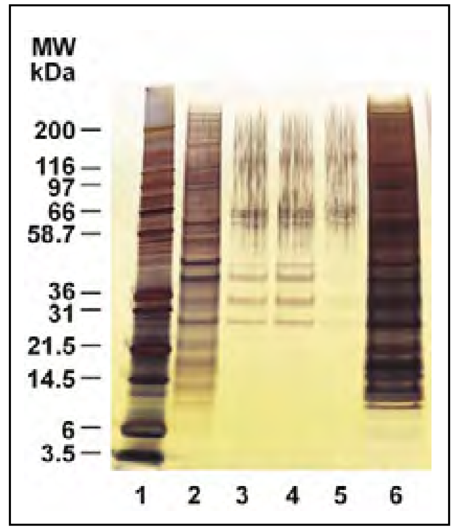

Figure 3: Purification of PVSRIPO during Q650M flow-through column. Lane1: Mark 12 molecular weight standards; Lane 2: load; Lane 3: virus peak; Lane 4: virus peak (0.2 um filtered); Lane 5: post-virus peak; Lane 6: column strip with 1 M NaCl buffer.

Anion-Exchange: Column 2/Column 3 (Tandem Columns)

Column 2 (Virus Non-Binding)

A 2.6-cm (i.d.) XK column (GE Healthcare-Biosciences) was packed with 24 mls of Q650M media to a final bed height of 4.5 cm. The packed column was depyrogenated, flushed with WFI-quality water prior to equilibration with 4.7 mM NaPO4, 25 mM NaCl, pH 7.5, at 20–25 cm/hr for 10 column volumes.

Column 3 (Virus Binding)

A 1.6-cm diameter XK column (GE Healthcare-Biosciences) was packed with 19 mls of CDM resin, which is a "salt-tolerant" prototype multimodal anion-exchanger (GE Healthcare-Biosciences). The column was equilibrated with 4.7 mM $NaPO_4$, 25 mM NaCl, pH 7.5 at 20–25 cm/hr for 10 column volumes. This column was connected in series and downstream of the Q650M column. The pooled virus from the Sepharose 4FF column (2350 mls) was applied to the tandem Q650M/CDM columns at a flow rate of 20–25 cm/hr. The tandem columns were washed with three combined column volumes of 4.7 mM $NaPO_4$, 25 mM NaCl, pH 7.5, at 20–25 cm/hr. The CDM column was uncoupled from the Q650M column and eluted in reverse flow at 20–25 cm/hr with 4.7 mM $NaPO_4$, 1 M NaCl, pH 7.5, collecting 1-ml fractions. Two pools were made based on silver stain SDS-PAGE gel analysis of the elution peak. Enriched fractions were assayed for infectivity by plaque assay (described below), silver stain SDS-PAGE, and Western blot using a poliovirus type I antibody from rabbit antiserum (Access Biomedical, San Diego, CA).

Purified Virus Formulation: Column 4

Poliovirus was found to lose infectivity upon prolonged exposure to high salt. The concentrated virus was formulated into 1X MEM without phenol red (Invitrogen/Gibco), using Sephadex G-25. A 1.6-cm diameter XK column (GE Healthcare-Biosciences) was packed with 10 ml of media to a final bed height of 5 cm, and a XK 2.6 column (GE Healthcare-Biosciences) was packed with 50 ml of media to a final bed height of 9.4 cm. The columns were equilibrated with 3–5 column volumes of 1X MEM. The 10-ml Sephadex G-25 column was loaded at 26% column volume with the high-titer virus pool (7–8 x 10^9 pfu/ml) from the concentration column at a linear flow rate of 30 cm/hr, collecting the void volume in 2 ml fractions. The 50-ml Sephadex G-25 column was loaded at 15% column volume with the second concentration column pool at a linear flow rate of 30 cm/hr. The void volume was collected in 2-ml fraction volumes and pooled based on silver stain SDS-PAGE gel analysis. Human serum albumin (25% v/v, Bayer HealthCare, West Haven, CT) was added to the formulated pools to a final concentration of 0.2% (v/v). The activity of the final formulated purified virus was analyzed for infectivity by plaque assay.

Testing for Potency, Purity, and Safety

Infectivity by Plaque Assay

Sixty-mm dishes (BD Falcon, Franklin Lakes, NJ) were seeded with 4 mls of Vero cells at 7.7 x 10^4 –1.5 x 10^5 cells/ml. The seeded 60-mm dishes were incubated at 35–37° C for 2–3 days (80–100% confluency). The media was aspirated and the dishes dosed in triplicate with 0.2 mls of serially diluted virus to yield 10–100 plaques per dish. The virus was adsorbed to the cells for 1 hour at 33° C with gentle rocking of replicate dishes every 15–20 minutes. The viral inoculum was removed and the dishes overlaid with a 50:50 mixture of 2X essential modified Eagle's media (EMEM; Invitrogen/Gibco) and 1.5% SeaPlaque agarose. The agarose was allowed to gel, then incubated at 33° C for 2–3 days until plaques were fully formed. The plaques were overlayed in subdued light with the 50:50 mixture of media and agarose, described above, containing 33 µg/ml neutral red, and allowed to gel. Exposure of dishes with neutral red overlay to light was avoided to prevent cell toxicity. Dishes were incubated for 18–24 hours then the plaques were counted on a light box. Infectivity as determined by plaque assay has an experimental variability of 0.5 logs.

Silver Stain SDS-PAGE

Samples taken at various stages of purification and stored at 2–8° C were analyzed by silver stain SDS-PAGE using 4–12% Bis-Tris polyacrylamide gels (Invitrogen, Carlsbad, CA). Samples were diluted 1:4 with NuPage sample buffer with 10% β-mercaptoethanol (reducing conditions) and electrophoresed at 200 V (constant voltage) for 30 minutes. Gels were silver stained according to Invitrogen's procedure.

Western Blot

Gels were loaded with 10, 20, and 40 µL of purified poliovirus (2 x 10^8 pfu/ml). Protein bands were transferred from a 4–12% NuPage gel to a poly(vinylidine difluoride) (PVDF) membrane at 25 V (constant voltage). The membrane was blocked with immunoblot buffer (50 mM Tris, 0.5 M NaCl, pH 7.4) with 3% (w/v) BSA for four hours with gentle agitation. Following blocking, the membrane was incubated with a 1/500 dilution of primary antibody (poliovirus type 1 Sabin rabbit antiserum) (Access Biochemical, San Diego, CA) in immunoblot buffer with 1% (w/v) BSA for 14 hours. The membrane was washed with immunoblot buffer with 0.05% Tween 20 for 40 minutes (three washes total), then washed for 20 minutes with immunoblot buffer. The membrane was incubated with a 1/2500 dilution of goat anti-rabbit alkaline phosphatase-conjugated secondary antibody (Chemicon International, Temecula, CA), with agitation for three hours at ambient temperature. Unbound secondary antibody was removed by washing as above. The membrane was washed with a buffer of 20 ml 0.1 M Tris, 0.1 M NaCl, 5 mM $MgCL_2$, pH 9.5 for 20 minutes, drained, then incubated with 20 ml nitro blue tetrazolium chloride (NBT)/ 5-bromo-4-chloro-3-indolyl phosphate (BCIP) reagent (Invitrogen/Gibco) for 20 minutes. Due to the low protein concentration of the purified poliovirus, the color development of the bands was slow.

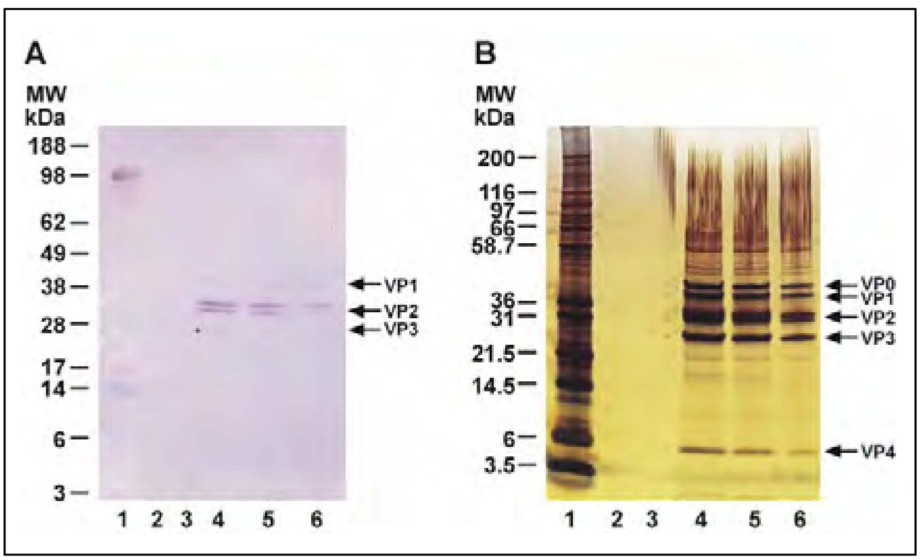

Figure 4: Western Blot and silver stain SDS-PAGE gel for purified PVSRIPO **(A)** Western blot. Lane 1: See Blue Plus molecular weight standards; Lane 2: blank; Lane 3: blank; Lane 4: 2 x 10^7pfus PVSRIPO; Lane 5: 1.2 x 10^7pfus PVSRIPO; Lane 6: 6 x 10^6 pfus PVSRIPO; **(B)** Corresponding silver stain gel (reduced). Lane 1: Mark 12 molecular weight standards; Lane 2: blank; Lane 3: blank; Lane 4: 2 x 10^7pfus (PVS)RIPO; Lane 5: 1.2 x 10^7pfus (PVS)RIPO; Lane 6: 6 x 10^6 pfus (PVS)RIPO.

Determination of PVS(RIPO) Viral RNA Copies by Reverse-Transcription Quantitative PCR (RT-qPCR)

Samples from the PVS(RIPO) recombinant polio virus project were tested for the presence of viral RNA using a quantitative TaqMan® RT-qPCR assay using an ABI 7900HT 96-well sequence detection system. The PVS(RIPO) viral RNA RT-qPCR amplicon is a 71-bp RT-PCR product capable of accurate quantitation of the 7332-nucleotide PVS(RIPO) RNA virus and 9945-bp plasmid DNA constructs. The amplicon was targeted to the human rhinovirus type 2 (HRV-2)–derived internal ribosomal entry site (IRES) sequence present in PVS(RIPO), and was able to amplify both viral and plasmid sequences. Reverse transcription with viral RNA was achieved using the PCR reverse primer. The PVS(RIPO) amplicon TaqMan probe utilizes a 5' 6-carboxyfluorescein (6-FAM) reporter dye and a 3' quencher dye, 6-carboxytetramethylrhodamine (TAMRA). The 22-base probe sequence utilizes CAATTGCGGGATGGGACCAACT; forward primer (27 bases), AAC CCAATGTGTATCTAGTCGTAA TGA; and reverse primer (20 bases), TGAAACACGGACACCCAAAG. Primer concentrations were 10 pmol with a typical probe concentration of 5 pmol. Assays were performed using ABI (Foster City, CA) TaqMan EZ RT-PCR using standard qPCR amplification conditions: 30-minute reverse transcriptase (RT) at 48° C, 15s strand denaturation at 95° C, and 60s primer anneal and extension at 60° C. Reverse transcriptase efficiency was found to be typically 100% ± 10%. The assay exhibited a 7-log linear range (r2 >0.95), from 100 pg/rxn (2.43 x 10^7 viral copies/rxn) to <100 pg/rxn (<24 viral copies/rxn), using PVS(RIPO) plasmid DNA or PVS(RIPO) viral RNA. In addition to the standard curve and test sample reactions, each assay contained controls for sample inhibition of polymerase chain reaction (PCR), RT efficiency, sample extraction efficiency, buffer blank, and no test sample controls (NTCs) to demonstrate assay validity. The standard Vero plasmid DNA and viral RNA utilized in the assay standard curve reactions were column purified from cell culture, guanidinium inactivated, extracted using the Qiagen Viral RNA method, verified for concentration and purity via A_{260}, log diluted, and stored at –80° C prior to use.

Quantitation of Residual Vero Host Cell Genomic DNA by qPCR

Samples of PVS(RIPO) recombi-

Table 2: CDM Prototype Resin Screening

Sample	Volume (ml)	Titer (pfu/ml)	Total pfus	Step Recovery (%)
Q650M purified virus (Load)	10 ml	6.65×10^6	6.65×10^7	100
1,3-diaminopropane (108 µmol/ml)				
Flow-through/Wash	18 ml	1.65×10^4	2.97×10^5	0.4
150 mM NaCL	8 ml	1.5×10^6	1.2×10^7	18
0.5 M NaCL	8 ml	0 @ 10^{-5} diln ($\leq 1.66 \times 10^5$)	$\leq 1.33 \times 10^6$	≤ 2.0
1.0 M NaCL	9 ml	2.58×10^6	2.3×10^7	34
bis (3-Aminopropyl) amine (105 µmol/ml)				
Flow-through/Wash	18 ml	0 @10^{-4} diln ($\leq 1.66 \times 10^4$)	$\leq 2.99 \times 10^5$	≤ 0.4
150 mM NaCL	8 ml	2.34×10^6	1.9×10^7	28
0.5 M NaCL	8 ml	6.65×10^5	5.3×10^6	8
1.0 M NaCL	9 ml	0 @ 10^{-5} diln ($\leq 1.66 \times 10^5$)	$\leq 1.49 \times 10^6$	≤ 2.2
bis (3-Aminopropyl) amine (72 µmol/ml)				
Flow-through/Wash	18 ml	0 @ 10^{-4} diln ($\leq 1.66 \times 10^4$)	$\leq 2.99 \times 10^5$	≤ 0.4
150 mM NaCL	8 ml	0 @ 10^{-5} diln ($\leq 1.66 \times 10^5$)	$\leq 1.33 \times 10^6$	≤ 2.0
0.5 M NaCL	8 ml	0 @ 10^{-5}diln ($\leq 1.66 \times 10^5$)	$\leq 1.33 \times 10^6$	≤ 2.0
1.0 M NaCL	9 ml	1.67×10^6	1.5×10^7	23
1,2-diaminoethane (108 µmol/ml)				
Flow-through/Wash	18 ml	6.65×10^4	1.2×10^6	1.8
150 mM NaCL	8 ml	3.35×10^5	2.7×10^6	4
0.5 M NaCL	8 ml	3.93×10^6	3.1×10^7	47
1.0 M NaCL	9 ml	1.5×10^6	1.3×10^7	19

nant polio virus were tested for residual host-cell Vero (African Green Monkey, *Cercopithecus aethiops*) genomic DNA using a novel Vero-specific Nectin-1 alpha-gene targeted TaqMan qPCR assay using an ABI 7900HT 96-well sequence detection system. A major consideration in the development of a Vero-specific TaqMan amplicon was the reduction or elimination of unintended primer and probe annealing to highly homologous *Homo sapiens* genomic sequences that might be present in the analytical laboratory and that might lead to inaccurate results. The Vero Nectin-1 qPCR amplicon is a 111-bp PCR product capable of accurate quantitation of the single-copy VERO Nectin-1 gene. Discrimination between human and Vero sequences is provided by the presence of a 9-bp duplication that is unique to *Cercopithecus aethiops* Nectin-1, including Vero cell cultures.[8,9] The 5' end of the TaqMan probe was designed to partially anneal within the duplicated sequence present in Vero reducing by more than 3 logs the level of sensitivity to human genomic nectin-1 DNA. The Vero Nectin-1 gene amplicon TaqMan probe utilizes a 5' 6-FAM reporter dye and a 3' TAMRA quencher dye. The 24-base probe sequence utilizes CACCCAAGCCACC AATGGCTCCAA; forward primer (18 bases), CCTCTGCCCAGCGTGAAG; and reverse primer (19 bases), CACAGACACGCCCATGGAT. Primer concentrations were 10 pmol, with a typical probe concentration of 5 pmol. Assays were performed using ABI Universal TaqMan Maser Mix containing UNG using standard qPCR amplification conditions: 15s strand denaturation at 95° C and 60s primer anneal and extension at 60° C. The assay exhibited a 5-log linear range (r^2 >0.97), from 1000 ng/rxn to less than 100 pg/rxn, using Vero total genomic DNA. In addition to the standard curve and test sample reactions, each assay contained controls for sample inhibition of PCR, extraction efficiency, buffer blank, and NTCs to demonstrate assay validity. The standard Vero genomic DNA utilized in the assay standard curve reactions was purified from cell culture, verified for concentration and purity via A_{260}, log diluted, and stored at –80° C prior to use.

Results and Discussion

Cell Culture

By 72 hours post-infection, 90–100% CPE was observed microscopically. The total production of virus from the four 10-tier cell factories following clarification was 5.36 x 10^{11} pfus in a final volume of 3200 ml. The viral harvest was stored at 2–8° C until further processing.

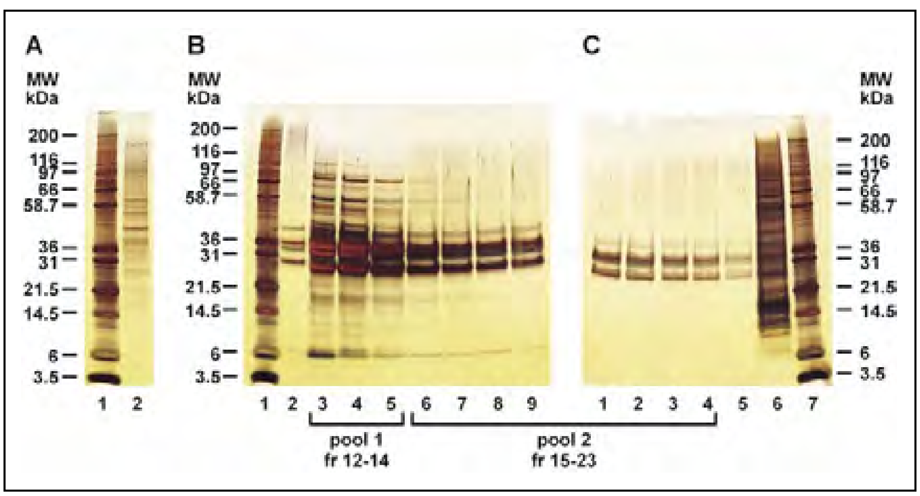

Figure 5: Silver stain SDS-PAGE gel for Qbelix column elution fractions **(A)** Lane 1: Mark 12 molecular weight standards; Lane 2: load; **(B)** Lane1: molecular weight standard; Lane 2: fraction 11; Lane 3: fraction 12; Lane 4: fraction 13; Lane 5: fraction 14; Lane 6: fraction 15; Lane 7: fraction16; Lane 8: fraction 17; Lane 9: fraction 18; **(C)** Lane 1: fraction 19; Lane 2: fraction 20; Lane 3: fraction 22; Lane 4: fraction 23; Lane 5: fraction 24; Lane 6: 1 M NaCl strip Q650M column; Lane 7: Mark 12 molecular weight standard.

Benzonase Treatment

The first step in the purification method was to remove host cell genomic DNA from the viral lysate. Purification methods within the literature have reported the use of nucleases (i.e., benzonase) for removal of host DNA in adenoviral cell lysates and picornavirus.[6,10] Based on this information, Benzonase was evaluated in the purification process. The quantity of host genomic DNA in the viral lysate determined by qPCR was reduced from 6500 ng/ml to less than 5 ng/ml (limit of detection) following Benzonase digestion. A comparison of pre-Benzonase (7.9 x 10^7 pfu/ml) and post-Benzonase (1.2 x 10^8 pfu/ml) samples showed that infectivity by poliovirus plaque assay was not affected by Benzonase treatment.

Column Chromatography

Individual step recoveries as well as overall yields are summarized in Table 1.

Size Exclusion

Size-exclusion chromatography was considered to be the best option as an initial purification and buffer-exchange step due to minimal shear forces and acceptable capacity for processing large volumes of lysate. A procedure for the separation of a murine retrovirus using a Sepharose-based resin was described by McGrath *et al.*[4] Sepharose 4FF, a later generation of this resin, was selected for evaluation. The highly cross-linked agarose beads (45–65 μm diameter) allowed for process-scale flow rates and load capacities up to 30% with good recovery of virus activity. The virus activity was detected in the column void volume and collected in a total volume of 1600 mls (0.47 column volumes). The efficiency of the Sepharose 4FF is shown in Figure 2. The bulk of the viral contaminants (lane 5) are well separated from the poliovirus-enriched fraction (lane 4).

Anion-Exchange, Virus Non-Binding (Flow-Through) Column

The selection of chromatography media for poliovirus purification was limited by the virus' tendency to form aggregates under acidic conditions, neutral isoelectric point (7–7.2), and sensitiv-

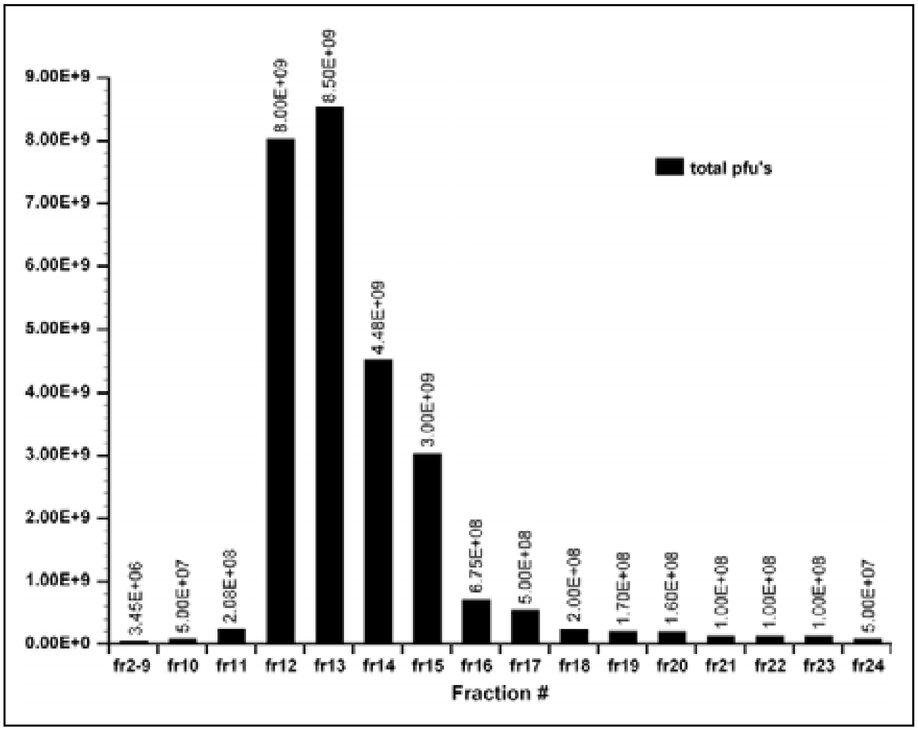

Figure 6: The elution of purified virus from the Obelix concentration column. Samples from each fraction were assayed for infectivity by plaque assay. The total pfus for each fraction are represented in the bar graph.

ity to basic pH (pH 9 or greater). Anion-exchange chromatography was selected as the best option to meet the physical and biological requirements needed for purification. Toyopearl Super Q650M media was selected based on the purification of another picornavirus, hepatitis A.[6] The Q650M column under low conductivity (25 mM NaCL) and physiological pH conditions was very effective in removing host cell contaminants (based on silver stain SDS-PAGE gels). A comparison by silver stain SDS-PAGE (Fig. 3) of the Q650M column load (lane 2), virus pool (lane 3), 0.2-µm filtered virus pool (lane 4) and high-salt strip (lane 6) illustrates the removal of contaminants during this step. The recovery of infective virus was 106%, with an overall yield of 84% at this stage of purification (Table 1). The virus pool had five major bands by silver stain SDS-PAGE. The apparent molecular weights of the bands were consistent with the molecular weights of viral coat proteins VP1–VP4 (Fig. 4). VP1 and VP2 appear as doublets, a condition that has been mentioned in relation to SDS-PAGE of purified poliovirus and has been attributed to possible deamidation of glutamine or asparagines.[11] The other minor bands that appear as a smear above 36 kDa could be due to incomplete reduction of the viral coat proteins or heating effects during electrophoresis. The results from Western blot transfer and immunoblot detection indicated that three SDS-PAGE gel bands with apparent molecular weights consistent to VP1, VP2, and VP3 are reactive with a Sabin poliovirus type I antibody (Fig. 4).

Prototype Anion-Exchange Resin Scouting

A prototype resin based on multimodal amine ligands bound to epoxy-activated Sepharose 6 FF media had been reported.[7] These multimodal resins use ionic, hydrophobic, and hydrogen bonding to allow the separation of proteins at higher ionic conditions. Three resins with ligands 1,3 Diaminopropane (108 µmol/ml), bis (3-Aminopropyl) amine (105 and 72 µmol/ml), and 1,2 Diaminoethane (108 µmol/ml) were tested. Q650M-purified virus (10 ml) from an earlier scale-up run at 6 x 10^6 pfu/ml was applied to 1-ml columns for each resin, washed with four column volumes of 4.7 mM $NaPO_4$, 25 mM NaCL, pH 7.5, and step eluted with four column volumes each of 4.7 mM $NaPO_4$, pH 7.5, buffer with 0.15 M NaCL, 0.5 M NaCL, and 1 M NaCL, collected as separate fractions. The fractions were analyzed by poliovirus plaque assay to determine the elution conditions and recovery of virus from each resin (Table 2). At pH 7.5, the virus bound to these ligands at higher ionic conditions than currently available commercial resins. The bis (3-Aminopropyl) amine resin with a ligand density of 105 µmol/ml was of particular interest as a capture and concentration step due to the elution of virus at ionic conditions less than 0.5 M NaCL.

Column Concentration of Virus

Based on these observations, the bis (3-Aminopropyl) amine resin was placed in series to the Q650M flow-through column. The virus eluted from the concentration column after 0.52 column volumes of high-salt buffer (1 M NaCl) in a total volume of 12 mls. Due to the purity of the virus and instability under high-salt conditions, the virus was pooled by silver stain SDS-PAGE. Two pools were made based on the qualitative assessment of virus concentration using band intensity on the silver stain SDS-PAGE gels (Fig. 5). Pool #1 was made from three fractions at the beginning of the elution peak with the highest relative band intensity. The remaining nine fractions were pooled together as pool #2. The infectivity data for the elution of virus from the column show an identical elution profile (Fig. 6). The comparison of this data suggests that the virus can

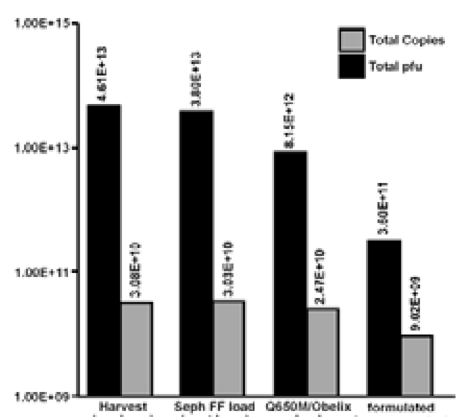

Figure 7: Total copies for (PVS)RIPO RNA and total pfus (determined by plaque assay) for the major steps during the purification process.

be detected using silver stain SDS-PAGE gel analysis at this stage of purification. The virus activity was concentrated 2.95 logs to a final titer of 8-9 x 10^9 pfu/ml. A total of 2.10 x 10^{10} pfus (86%) of the 2.43 x 10^{10} pfus applied was recovered in 0.2 column volumes.

Final Formulation of Virus

The concentrated virus pool was formulated in 1X MEM with 0.2% human serum albumin. The formulation step required a resin with a fractionation range that would exclude virus and offered minimal risk of nonspecific adsorption of virus. Based on these needs, Sephadex G-25 was selected as the best choice. The two virus pools from the concentration column were processed in two runs. The high-titer virus pool (pool #1) was processed over the 10-ml G-25 column (Run #1), and pool #2 was applied to the 50-ml G-25 column (Run #2). Load volumes of 0.25–0.30 column volumes were used to minimize dilution effects. Pool #1 from the concentration column was injected at 0.25 column volumes with the void volume collected in 2-ml fractions. The fractions were analyzed by silver stain SDS-PAGE and two fractions with the highest relative band intensity were pooled (data not shown). The final volume for the formulated virus pool was 4.1 mls. Human serum albumin was added to the formulated pool to a final concentration of 0.2% (v/v) and titered by plaque assay.

The final titer for the formulated virus from Sephadex G-25 run #1 was 2 x 10^9 pfu/ml. The remaining virus (pool #2) from the concentration column was run over G-25 in the same manner (Run #2). Three fractions were pooled, with a final volume of 7.2 mls and a final titer of 4 x 10^8 pfu/ml. Prior to vialing, the formulated virus was sterile filtered through a 0.22-μm filter. The sterile filtration of the purified, formulated virus resulted in no apparent reduction of titer (Table 1). A total of 9 x 10^9 pfus virus (29% yield) with a titer of 2 x 10^9 pfu/ml was recovered. The step recoveries and overall yields for the one-third-scale production run are listed in Table 1.

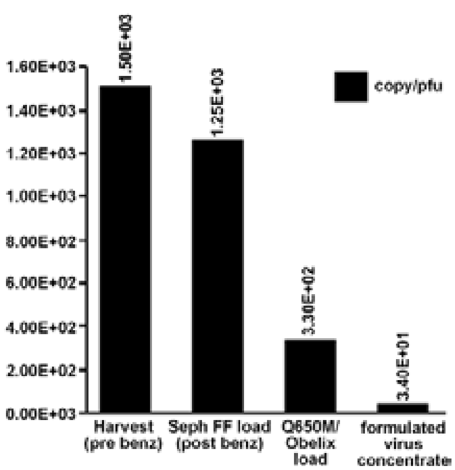

Figure 8: Ratio of (PVS)RIPO viral RNA copies to titer (pfus) for the major steps of the purification process. The reduction in the ratio of viral RNA copies to pfus through the purification process indicates the removal of non-infective virus from infective virus.

Conclusion

The total recovery of virus activity for each purification step was greater than 79%, and the overall recoveries were comparable up to the formulation step. The drop in yield during formulation could be associated with poliovirus aggregation or inactivation. The total number of poliovirus genomes was tracked by qPCR throughout the process. The total RNA copies were unaffected after Benzonase treatment, an RNA/DNA nuclease, and significant reduction (2 logs) occurred after the anion-exchange step (Fig. 7). The comparison of RNA copies to infectivity indicates a reduction from 1500 copies per pfu in the lysate to 34 copies per pfu in the final purified virus (Fig. 8). The increase in titer for purified virus combined with the reduction in RNA copies per pfu suggests that the method is capable of purifying non-infectious virus from infectious virus.

Summary

A total of 9 x 10^9 pfus was produced with a final titer of 2 x 10^9 pfu/ml from 1.1 L of cell lysate, approximately one-third of the estimated production scale (four 10-tier cell factories). Silver stain SDS-PAGE gels for the purified virus have bands with apparent molecular weights consistent with the five poliovirus coat proteins, with three bands (VP1, VP2, and VP3) cross-reactive with a Sabin type 1 polyclonal antibody. The purified formulated virus was infective to Vero cells with host cell genomic DNA levels less than the limit of detection (5 ng/ml) for the qPCR quantitation assay. The virus can be sterile filtered with no significant loss in recovery of viral infectivity. The purification method described provides a high-throughput and scalable chromatographic method for an attenuated chimeric poliovirus to meet the qualitative and quantitative requirements of preclinical and clinical testing.

REFERENCES

1. Gromier M, Alexander L, Wimmer E. Internal ribosomal entry site substitution eliminates neurovirulence in intergeneric poliovirus recombinants. *PNAS* 1996;9:2370–75.
2. Gromier M, Bossert B, Arita M et al. Dual stem loops within poliovirus internal ribosomal entry site control neurovirulence. *J Virol* 1999;73:958–64.
3. Gromier M, Lachmann S, Rosenfeld MR et al. Intergeneric poliovirus recombinants for the treatment of malignant glioma. *PNAS* 2000;97:6803–08.
4. McGrath M, Witte O, Pincus T et al. Retrovirus purification: method that conserves envelope glycoprotein and maximizes infectivity. *J Virol* 1978;25:923–27.
5. Rueckert RR. Piconaviruses and their replication. In: Fields BN, Knipe DM, Chanock RM, Melnick JL, Roizman B, Shope RE, editors. *Virology*, New York: Raven; 1985. p 705–38.
6. Hagen AJ, Oliver CN, Stirin RD. Optimization of poly(ethylene glycol) precipitation of hepatitis A virus used to prepare VAQTA, a highly purified inactivated vaccine. *Biotechnol Prog* 1996;12:406–12.
7. Johansson BL, Belew M, Eriksson S et al. Preparation and characterization of prototypes for multi-modal separation media aimed for capture of negatively charged biomolecules at high salt conditions. *J Chromatogr A* 2003;1016:21–33.
8. Milne RSB, Connolly SA, Krummenacher C et al. Porcine HveC, a member of the highly conserved HveC/Nectin 1 family, is a functional alphaherpesvirus receptor. *Virology* 2001;281:315–28.
9. See Genbank accession AF308635.
10. Wu P, Xiao W, Conlon T et al. Mutational analysis of the adeno-associated virus type 2 (AAV) capsid gene and construction of AAV2 vectors with altered tropism. *J Virol* 2000;74:8635–47.
11. Hamann A, Weigers KJ, Drzeniek R. Isoelectric focusing and 2D-analysis of poliovirus proteins. *Virology* 1977;78:359–62.

This project has been funded in part with federal funds from the National Cancer Institute, National Institutes of Health, under Contract No. N01-C0-12400. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. government.