



Barbara Tipton, Jeri Ann Boose, William Larsen, Joanne Beck, and Thomas O'Brien

Two types of depth filters are studied for their ability to rid protein solutions of retroviruses and parvoviruses. Such filters are relatively economical and easy to use, so as secondary virus-removing devices, they are beneficial for downstream processing of mammalian cell cultures. Results indicate that depth filters are more effective on retroviruses than parvoviruses.

Barbara Tipton is a process development associate scientist, and **Joanne Beck** is an associate director of process development, at Amgen Inc., One Amgen Center Drive, Thousand Oaks, CA 91320; **Jeri Ann Boose** is director of validation clearance at BioReliance, 9900 Blackwell Road, Rockville, MD 20850; and **Thomas O'Brien** is director of laboratory and scientific applications support services, and corresponding author **William Larsen** is a senior applications specialist, at CUNO Incorporated, 400 Research Parkway, Meriden, CT 06450, 203.238.8941, fax 203.238.8798, wlarsen@cuno.com, www.cuno.com. Together, they have 50 years of experience in biopharmaceutical processes.

Retrovirus and Parvovirus Clearance from an Affinity Column Product Using Adsorptive Depth Filtration

Bioprocess streams using mammalian cultures can contain infectious virions for three reasons: because cultures typically require rich nutrients, ideal for viruses (1); because the producing cell line might harbor endogenous retroviruses; and because prescreening efficiency cannot reach unity in detecting viruses in raw materials or cell lines because of detection limitations (2). Bioprocess designers and validators must, therefore, assess existing unit operations for inherent viral inactivation or removal capabilities, and design additional unit operations specifically for inactivation or removal purposes. Infectious virions cannot be allowed to spread throughout a production facility and into final products.

Adsorptive depth filters have been reported to effectively remove viruses during Cohn fractionation of human immunoglobulin (3). Log reduction values (LRVs) of about 2.3–5.4 using depth filters with a 0.7- μm mean flow pore diameter were observed with a panel of five viruses including porcine parvovirus (PPV) during viral clearance validation of a 20% ethanol-bearing filtration step (Fraction III supernatant). Negatively charged bioprocess contaminants such as DNA (4) and submicron membranous debris derived from mammalian cells (5) are known to be adsorptively removed from bioprocess streams by depth filters, suggesting that virus removal by ion exchange should be possible.

Study Parameters

Our study was designed to determine the virus-removing efficiencies of adsorptive depth filters in 100% aqueous systems such as those typically encountered during downstream processing of mammalian cell cultures. Because virus removal through Cohn fractionation was thought to be a result of electrostatic or ion-exchange

interactions, we hypothesized that virus removal can occur during 100% aqueous-based processing. On the assumption that the downstream virus removal from mammalian systems would be sensitive to ion-exchange interactions, we sought a bioprocess that fit the following constraints:

- pure product,
- $\text{pH}_{\text{process}} = 7$,
- $\text{pI}_{\text{product}} > \text{pH}_{\text{process}}$, and
- low ionic strength.

An established biotechnology company (Amgen, Inc. www.amgen.com) provided a protein purification procedure with a processing step that met those constraints. A depth filter was used to provide both viral reduction and clarification of the affinity column eluate before frozen storage and further downstream processing. The formal studies we describe are designed to serve as proof-of-principle tests for determining the capacity of two types of depth filters for PPV and xenotropic murine leukemia virus (XMuLV). Our study is a subset of investigations undertaken to characterize the overall process for viral clearance. In particular, the present validation study is a subset of a larger effort to characterize the virus removal capabilities of the following filtration sequence:

affinity column eluate \Rightarrow *depth filter* \Rightarrow *ion exchange column eluate* \Rightarrow *50 nm viral filter*

Parvoviruses and retroviruses. Parvovirus virions are spherical, 18–26 nm in diameter, and nonenveloped. Mammalian parvoviruses have narrow host ranges and are resistant to heat, detergents, organic solvents, and extremes of pH (less than three or greater than 10). In humans, parvoviruses are associated with rheumatoid arthritis, spontaneous abortions, fetal death, and complications in several types of anemia. Retrovirus virions are spherical, have a diameter of 80–100 nm, and are enveloped.

Table 1. Characteristics of the two filters used in this study

Depth Filter Type	Anion-Exchanging Characteristic	pK _a	Charge Capacity	Nominal Pore Diameter (μm)
VR05	Weak	7.0	Low	0.3
VR07	Strong	none	High	0.3

Table 2. Parvovirus (PPV) clearance results

Sample	Units	Depth Filter (Anion-Exchange Characteristics)	
		VR05 (Weak)	VR07 (Strong)
Initial challenge solution initial count (t=0, unfiltered)	PFU/mL	5.8 × 10 ⁷	
Final challenge solution final count (t=75 min, unfiltered)	PFU/mL	5.2 × 10 ⁷ (9% loss of infectivity)	
Volume filtered	mL	202	182
Experiment duration	min	68	73
Filtrate count: Fraction 1 (size)	PFU/mL	8.1 × 10 ⁶ (0–120 mL)	2.2 × 10 ⁶ (0–120 mL)
Filtrate count: Fraction 2 (size)	PFU/mL	4.2 × 10 ⁶ (120–202 mL)	6.5 × 10 ⁵ (120–182 mL)
Filtrate LRV: Fraction 1	LRV	0.9 ± 0.5 ^a	1.4 ± 0.6 ^a
Filtrate LRV: Fraction 2	LRV	1.1 ± 0.8 ^a	2.0 ± 0.5 ^a
Count removed by filter	PFU	1.0 × 10 ¹⁰	1.0 × 10 ¹⁰
Virus recovery count (material balance)	PFU	8.1 × 10 ⁹ (68% recovery)	8.0 × 10 ⁹ (80% recovery)
^a 95% confidence interval			

Retroviruses are more labile than parvoviruses. Mammalian retroviruses are associated with leukemias, lymphomas, sarcomas, carcinomas, immunodeficiencies, and autoimmune diseases (6).

Depth filters. The depth filters we used in this study were first developed for the plasma fractionation industry to replace asbestos prefilters. They are still widely used in that industry. In the biopharmaceutical world, they are used to remove whole cells and cellular debris in large-scale mammalian cell culture operations. The filters contain diatomaceous earth (a naturally occurring silica) and cellulose. Depth filters are manufactured in a range of nominal porosities from 10 to 0.3 μm. They are typically 5-mm thick and held together with polymeric binding resins. The binding resin within them serves to covalently bind dissimilar construction materials together, which gives the resultant

media wet strength and confers a positive charge to media surfaces.

The single-use depth filters we used are intended for dead-end or single-pass operations. They have a high effective surface area per unit of cross-sectional filter area, and the void volume within the media matrix is also high. These characteristics confer to depth filters a higher debris capacity than membrane filters, which are easily plugged by debris. The filters used in this study meet FDA's regulations for construction material and USP's Biological Reactivity Tests, In Vivo, Class VI plastics (7,8). The VR series of depth filters are specifically designed for removal of adventitious viruses based on anion-exchange binding. Table 1 details the characteristics of the two filters studied.

Design of experimental challenge. There is no standard rate of use for depth filters in pharmaceutical processes. In plasma fractionation applications, depth filters are

typically used for simultaneous debris removal and viral clearance with a use rate of about 50–200 L/m² and a conservative flux (≤ 2.5 L/m²/min). For 47-mm depth filter pads (40-mm effective diameter and 12.6-cm² effective cross-sectional area), throughputs are, therefore, about 60–240 mL. Our study purpose was to determine the removal characteristics of the depth filters at a use rate of about 100 L/m² and to determine the filter's removal characteristics at about 150% of that use rate to demonstrate whether the filters had excess capacity under excess loading conditions.

Materials and Methods

These studies were performed in compliance with European Union, Japanese, United Kingdom, and U.S. FDA good laboratory practices (GLPs).

Depth filters. We used Zeta Plus VR-series (CUNO Incorporated, www.cuno.com, filters B0204-VR05 and B0204-VR07) viral reduction filter pads.

Protein binding. The isoelectric pH (or pI) of the 110-kD protein we were considering was high (> 6.0) compared with the process pH used in these studies (5.0). We used a 1% solution (w/v) of pure protein in a 20-mM acetic acid/sodium acetate buffer with a pH of 5.0. One of each of the 47-mm depth filters was soaked overnight in 20 mL of the 1% solution with continuous, gentle agitation. Initial and final samples of the 1% solution were analyzed for protein content. Negligible protein uptake (less than 5%) was observed for both filters.

Sample preparation. A heterogeneous mixture of soluble protein in clarified mammalian culture broth was applied to a preequilibrated affinity column under conditions optimized for the capture of protein product. The column was washed with buffer to remove proteins not specifically bound by the column. Protein product was then eluted from the column using a step-function pH shift. This yielded a fraction containing essentially pure product that was then adjusted to pH 5.0 and diluted with 20-mM acetic acid/sodium acetate buffer, at pH 5.0, to a concentration of 1.0 mg of protein/mL. The fraction was then stored at –70 °C before the depth filter challenge testing.

PPV cultivation and purification. An American Type Culture Collection (www.atcc.org)

Table 3. Retrovirus (XMuLV) clearance results

Sample	Units	Depth Filter (Anion-Exchange Characteristics)	
		VR05 (Weak)	VR07 (Strong)
Initial challenge solution initial count (t=0, unfiltered)	TCID ₅₀ /mL	3.9 × 10 ⁵	
Final challenge solution final count (t=75 min, unfiltered)	TCID ₅₀ /mL	6.6 × 10 ⁵ (0% loss of infectivity)	
Volume filtered	mL	199	170
Experiment duration	min	67	60
Filtrate count: Fraction 1 (size)	TCID ₅₀ /mL	< 6.8 ^a (0–120 mL)	< 6.8 ^a (0–120 mL)
Filtrate count: Fraction 2 (size)	TCID ₅₀ /mL	< 6.8 ^a (120–199 mL)	< 6.8 ^a (120–170 mL)
Filtrate LRV: Fraction 1	LRV	>4.8 ± 0.5 ^b	>4.8 ± 0.5 ^b
Filtrate LRV: Fraction 2	LRV	>4.8 ± 0.5 ^b	>4.8 ± 0.5 ^b
Count removed by filter	TCID ₅₀	7.8 × 10 ⁷	6.6 × 10 ⁷
Virus recovery count (material balance)	TCID ₅₀	1.7 × 10 ⁶ (2% recovery)	1.7 × 10 ⁵ (0.3% recovery)

^aNo virus was detected. This theoretical titer was based on a Poisson distribution.
^b95% confidence interval

PPV strain (NADL-2-VR-742) was used to infect porcine testicle cells (PT-1). The culture was infected three days before being subjected to multiple freeze–thaw cycles. Thawed culture was then clarified by centrifugation, and virions in the supernatant were concentrated using ultracentrifugation. The resulting viral pellets were resuspended in 10-mM Tris at pH 7.5 with 100-mM NaCl and 1-mM EDTA. This PPV stock solution was then aliquotted and stored at –60 °C or less until use.

XMuLV cultivation and purification. An Advanced Biotechnologies (www.abionline.com) XMuLV strain (10-131-001) was used to infect multiple cultures of mink lung cells (MLC). Harvests of chronically infected MLC cultures were pooled, then clarified and concentrated by ultracentrifugation. The resulting viral pellets were resuspended in 10-mM Tris at pH 7.5, 100-mM NaCl, 1-mM EDTA, and 0.5% bovine serum albumin (BSA). The XMuLV stock solution was then aliquotted and stored at –60 °C or less until use.

PPV enumeration. The sample PPV titer was based on the measurement of plaque-forming activity in pig kidney cells (PK-13), using serial tenfold dilutions of sample.

Each sample dilution was used to inoculate three replicate dishes (0.5 mL per dish), and assessed for the number of plaque-forming units (PFUs). We determined the PPV stock solution titer by averaging the triplicate results from serial threefold dilutions. The PPV stock solution used in the studies contained 6.8 × 10⁸ PFU/mL.

XMuLV enumeration. The XMuLV sample titer was based on the 50% tissue culture infectious dose (TCID₅₀) endpoint in feline cells (PG-4, S+L–). Tenfold, serially diluted samples were inoculated into eight replicate microplate wells (50 µL per well) and incubated. The solution titer was determined by averaging the results of three independent TCID₅₀ determinations using threefold dilutions. The XMuLV stock solution used in these studies contained 7.8 × 10⁷ TCID₅₀/mL.

Spiked sample preparation. Approximately 410 mL of challenge solution was prepared by combining 400 mL of room temperature, column-purified product with 10 mL of PPV or XMuLV stock solution. A low spike ratio (2.4%) was selected to ensure that the chemical characteristics of the challenge solution remained unaltered and to control costs. This spike ratio is large enough to

demonstrate the potential for large LRVs (values in excess of 4.0 are considered highly effective).

After mixing, two aliquots of the resulting challenge solution were removed for either PPV or XMuLV enumeration. One was enumerated immediately (t=0 min); the other was set aside and enumerated at the end of the challenge experiment (t=75 min). The difference in titer between the initial and final samples measures virus stability in the challenge solution.

Challenge experiment. We tested depth filter pads at room temperature (24 °C) at the 47-mm diameter scale (40-mm effective diameter, 12.6-cm² effective cross-sectional area). Solutions to be filtered were processed at constant flux using a peristaltic pump (Cole-Parmer Instrument Company, www.masterflex.com, Masterflex pump #7524-10, pump head #7518-10, and tygon tubing #6409-14). A depth filter pad, autoclaved at 121 °C for 30 minutes, was mounted in an autoclaved 47-mm stainless steel filter housing (Creative Scientific Equipment, inline filter holder #47606-001). The filter was flushed with 65 mL of 10-mM acetic acid/sodium acetate buffer, at pH 5.0, at a flow rate of 6–12 mL/min to purge trace filter extractables from the system (CUNO recommendation).

The pump suction line was then removed from the buffer reservoir, and air was pumped through the filter housing briefly to displace residual buffer left in the housing on the upstream side of the filter. Well-mixed challenge solution containing either PPV or XMuLV was divided into two equal portions (approximately 200 mL each). At a constant flow rate of 2.5–3.0 mL/min (with a flux of 0.20–0.24 mL/cm²/min), one 200-mL aliquot was pumped through the VR05 filter, and a second 200-mL aliquot was pumped through the VR07 filter.

We defined initialization (t=0) of the challenge experiment as the moment when the challenge solution filtrate began to emerge continuously from the filter housing outlet. Challenge solution filtrate was collected in two fractions (0–120 mL and 120 mL to the end). Once collected, each fraction was mixed and sampled for virus enumeration. Pressure drop across the depth filter and filtrate optical density (A600, buffer blank) were monitored during

the course of these challenge experiments as indicators of normal processing behavior (low-pressure drop values and clear filtrates throughout each experiment were expected). The duration of these experiments ranged from 60 to 73 minutes.

Virus recovery. After filtering all of the challenge solution, air was pumped through the filter housing to displace residual solution left in the housing on the upstream side of the filter. The depth filter pad was immediately removed from the filter housing and immersed in 20 mL of 500-mM NaCl (a salt strength sufficient to elute virions, but not so strong that it interferes with virus assays). Viruses on the immersed pad were extracted for one hour using gentle agitation throughout. The extract was then sampled for virus enumeration.

Results and Discussion

Results are discussed for pressure drop, optical density, and parvovirus and retrovirus clearance.

Pressure drop and optical density. Pressure drop across the VR05 filters did not vary during the course of the filter challenge experiments. Pressure drop values of 2.0–2.4 psi were observed throughout both experiments. Pressure drops across the VR07 filters rose modestly during the last ten minutes of each experiment to final values of 7.0 and 6.0 psi in the parvovirus and retrovirus challenge experiments, respectively.

Filtrate optical density (A600) was well controlled by both types of filters: It never rose above a value of 0.011 absorbance units during any of the four challenge experiments. The optical densities of the unfiltered parvovirus and retrovirus-spiked samples (initial challenge solutions) were 0.030 and 0.035, respectively. Reasonable pressure drops and clear filtrates are indicative of properly functioning filters.

PPV clearance. Table 2 shows the results of the PPV tests with each filter. The PPV stock solution used in these studies contained an average of $10^{9.33 \pm 0.31}$ or 2.1×10^9 PFUs/mL. When subjected to a 41-fold dilution using column-purified product, the result was a count of $10^{7.8 \pm 0.4}$ or an average of 5.8×10^7 PFUs/mL. That diluted solution (the initial challenge solution) was used to challenge the depth filters. The PFUs measured for the

initial challenge solution were within the 95% confidence interval for the calculated value of the initial challenge solution (PPV stock solution concentration \times 0.024).

Each challenge experiment lasted about 75 minutes. The concentration of PPV in the final challenge solution (unfiltered initial challenge solution held for 75 minutes at room temperature) was $10^{7.7 \pm 0.3}$ or 5.2×10^7 PFUs/mL, which was within the 95% confidence interval for results from the initial challenge solution. Therefore, column-purified product had no inactivating effect upon PPV infectivity throughout the course of the experiment.

We collected filtrate from each depth filter in two fractions. Each fraction was assayed for viruses immediately after collection. Based on the effective filter area, filtrate fraction 1 (0–120 mL) covered the fraction interval 0–95 L/m² (0–8.9 L/ft²), and filtrate fraction 2 (120–190 mL) covered the fraction interval 95–150 L/m² (8.9–14 L/ft²).

The depth filter with the weak anion-exchanging characteristics (VR05) failed to clear an appreciable amount of parvovirus from the initial challenge solution. LRVs for filtrate fraction 1 were 0.9 ± 0.5 and 1.1 ± 0.8 for filtrate fraction 2. The large 95% confidence intervals in these two cases were caused by contamination of one set of replicate counting plates during the enumeration of each fraction.

The depth filter with strong anion-exchanging characteristics (VR07) cleared a modest amount of parvovirus from the initial challenge solution. The LRV for filtrate fraction 1 was 1.4 ± 0.6 and 2.0 ± 0.5 for fraction 2. The LRV for filtrate fraction 2 was not less than the LRV for filtrate fraction 1.

The recovery of parvovirus from each used depth filter netted about 8×10^9 PFUs. Approximately 74% of the captured viral load was recovered from each filter by the virus recovery experiments.

Retrovirus clearance. Table 3 details the results of the retrovirus clearance study. The XMuLV stock solution used in these studies contained an average of $10^{7.68 \pm 0.24}$ or 4.8×10^7 TCID₅₀/mL. When diluted 41-fold using column-purified product, the resulting titer was $10^{5.6 \pm 0.4}$ or 3.9×10^5 TCID₅₀/mL. This diluted solution (the initial challenge solution) was used to

challenge the depth filters. The measured titer of the initial challenge solution fell within the 95% confidence interval of the calculated value for the initial challenge solution (XMuLV stock solution concentration \times 0.024).

The duration of each XMuLV challenge experiment was about 75 minutes. The XMuLV concentration in final challenge solution (unfiltered initial challenge solution held for 75 minutes at room temperature) was $10^{5.8 \pm 0.4}$ or 6.6×10^5 TCID₅₀/mL, which is within the 95% confidence interval of the results from the initial challenge solution. Therefore, column-purified product had no inactivating effect on XMuLV infectivity during the course of the experiment.

Filtrate from each filter was collected in two fractions. Each fraction was assayed for viruses immediately after collection. In effective filter area terms, filtrate fraction 1 (0–120 mL) covered the fraction interval 0–95 L/m² (0–8.9 L/ft²), and filtrate fraction 2 (120–185 mL) covered the fraction interval 95–150 L/m² (8.9–14 L/ft²).

Both types of depth filters cleared retrovirus from the initial challenge solution to a significant extent. The ability to quantify exactly the level of clearance was limited by the spike-to-sample volume ratio (2.4%) and by the concentration of the XMuLV stock solution. LRVs of at least 4.3 were observed in all four fractions (the weak anion-exchanging VR05 filtrate fractions 1 and 2 and the strong anion-exchanging VR07 filtrate fractions 1 and 2). The LRV for filtrate fraction 2 was not less than the LRV for filtrate fraction 1.

The recovery of retrovirus from the depth filters netted 1.7×10^6 TCID₅₀ for filters with weak anion-exchanging characteristics and 1.7×10^5 TCID₅₀ for filters with strong anion-exchanging characteristics. On a percent recovery basis, however, these numbers were not especially large. Approximately 2% of the captured viral load was recovered from the weak anion-exchanging filters during the course of the virus recovery experiment. The recovery results for the filter with strong anion-exchanging characteristics was 0.3% of the total retrovirus count applied to the filter during the course of the experiment. A pH

shift (to about 8.0) in combination with the ionic strength shift that we used might improve retrovirus elution in future studies.

Conclusions

Using the depth filter with strong anion-exchanging characteristics, the average LRV for the PPV was 1.4–2.0 during the course of the challenge experiments. Based on those LRV results, the depth filter cannot be considered as effective parvovirus-removing step (LRV \geq 2.0 is preferred).

For depth filters with strong and weak anion-exchanging characteristics, retrovirus LRVs in excess of 4.8 were observed during the course of the challenge experiments. Based on those LRV results, both depth filters can be considered effective retrovirus-removing filters.

Large-Scale Processing Implications

Most modern biological processes that require viral clearance use nanofilters. Bioprocess designers should also consider using depth filters with anion-exchanging

characteristics as virus-retaining prefilters when protecting tangential flow filters, chromatography columns, absolute-rated 0.1 or 0.2 μ m final filters, or virus-retentive nanofilters. Viral clearance using depth filtration may be dependent on the feedstream being processed, so depth filters should be evaluated for the specific process or product conditions being used.

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