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TECHNOLOGY/APPLICATION

A Consensus Rating Method for Small Virus-Retentive Filters. I. Method Development

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ABSTRACT: Virus filters are membrane-based devices that remove large viruses (e.g., retroviruses) and/or small viruses (e.g., parvoviruses) from products by a size exclusion mechanism. In 2002, the Parenteral Drug Association (PDA) organized the PDA Virus Filter Task Force to develop a common nomenclature and a standardized test method for classifying and identifying viral-retentive filters. One goal of the task force was to develop a test method for small virus-retentive filters. Because small virus-retentive filters present unique technical challenges, the test method development process was guided by laboratory studies to determine critical variables such as choice of bacteriophage challenge, choice of model protein, filtration operating parameters, target $\log_{10}$ reduction value, and filtration endpoint definition. Based on filtration, DLS, electrospray differential mobility analysis, and polymerase chain reaction studies, a final rating based on retention of bacteriophage PP7 was chosen by the PDA Virus Filter Task Force. The detailed final consensus filter method was published in the 2008 update of PDA Technical Report 41. Virus Filtration.

KEYWORDS: Virus retentive filtration, Virus safety, Bioprocessing standards, Biopharmaceuticals, Plasma-derived products.

Introduction

Cell cultures typically used in the production of monoclonal antibodies and therapeutic recombinant proteins produce endogenous type C retrovirus particles (1) and can potentially become infected by adventitious viruses (2, 3). Human source materials for plasma-based pharmaceuticals are also vulnerable to contamination by viral pathogens (4–6). These considerations have led to requirements by regulatory agencies for biopharmaceutical manufacturing schemes to include virus removal or inactivation steps (jointly termed *viral clearance*) (7, 8). Virus filtration has been observed to reliably clear viruses, including endogenous retrovirus, and virus filters are often incorporated into biopharmaceutical manufacturing schemes (9). Virus filters are membrane-based devices that remove large viruses (e.g., retroviruses) and/or small viruses

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(e.g., parvoviruses) from product by a size exclusion mechanism (10).

In 2002, the Parenteral Drug Association (PDA) organized the PDA Virus Filter Task Force to develop a common nomenclature and a standardized test method for classifying and identifying viral-retentive filters. At that time, each filter manufacturer rated its filters differently based on pore size or functional criteria. There is great interest within the filter and biopharmaceutical industry to develop a single rating system. A single rating system should promote industry uniformity by developing a single, reliable test to ensure a given level of viral clearance performance under consensus conditions for filters produced by all manufacturers. In 2005, the task force successfully developed a rating system for large virus-retentive filters based on retention of a medium-sized bacteriophage, PR772 (11–13).

Since then, the task force was charged to develop a general protocol for small virus-retentive filters. To an even greater extent than large virus-retentive filters, significant technical challenges are associated with small virus-retentive filters, for example, the potential for passage (14, 15). It should be noted that no currently marketed, small-pore virus filters claim to be absolute for 20-nm to 25-nm viruses and that non-absolute filters are known to show a range of particle or microbial retention $\log_{10}$ reduction values (LRVs) depending on fluid and process conditions (16). To surmount potential obstacles to a uniform test posed by these challenges and to support the task force's decision making during the method development, a series of feasibility studies were performed at the Center for Drug Evaluation and Research (CDER), US Food and Drug Administration (FDA) in collaboration with the four filter manufacturers (Pall® Life Sciences, East Hills, NY; Millipore® Corp., Billerica, MA; Sartorius-Stedim Biotech GmbH, Gottingen, Germany; and Asahi Kasei Medical Corp., Tokyo, Japan).

This report presents the results of the feasibility studies and describes how critical aspects of the small virus-retentive filter test method were set based on these studies. The filter performance demonstrated in this study does not necessarily predict performance in a biopharmaceutical manufacturing context. For such applications, protein formulation-specific testing of a candidate filter should be strongly considered.

Materials and Methods

Terminology

In this report V_{init}, V_{10}, V_{20}, V_n, etc. refer to the initial volume of filtrate after two hold-up volumes (i.e., void volume of the device as reported by the filter manufacturer), and to the volumetric throughputs after the flow rate across the filter has declined 10, 20, and n percent from the initial buffer flow rate, respectively. LRV can also be referred to as $\log_{10}$ titer reduction (LTR) (8, 17). LRV_{init}, LRV_{10}, LRV_{20}, LRV_n refer to the LRV of the model phage at V_{init}, V_{10}, V_{20}, V_n. ΔLRV_n refers to the change in instantaneous LRV_n relative to LRV_{init}.

Test Articles

Phage PP7 and its host *Pseudomonas aeruginosa* were obtained from the ATCC (Manassas, VA; accession numbers 15692-B4 and 15692). Coliphages PR772 and ΦX174 and their hosts *Eschericia coli* strains K12 J-53 and C were obtained from the Félix d'Hérelle Reference Center for Bacterial Viruses (Université Laval, Québec, Canada). Stocks were prepared by the CsCl gradient ultracentrifugation method as described (15, 18).

Bovine serum albumin (BSA) is a 66 kDa protein with an isoelectric point of 4.8. Five grades of BSA were purchased from Sigma (St. Louis, MO). BSA was dissolved at room temperature in standard phosphate-buffered saline (PBS, Biofluids, Rockville MD), pH 7.4 not more than 3 h prior to use to form "BSA test fluids A through E". The challenge fluids were spiked with PP7, 0.1 μm pre-filtered, and then used in the filtration studies. The following lots of BSA were used in these studies:

A. Sigma A7030 (crude fraction V), lot 10H0261

B. Sigma A3059 (99% heat-precipitated), lot 115K0703

C. Sigma A7638 (>99%, ethanol-precipitated), lots 014K7607 & 075k7572

D. Sigma A0281 (identified on certificate of analysis as "cold alcohol precipitated from A7638", i.e., BSA lot D was derived from BSA lot C), lot 075k7545

E. Sigma A1900 (98% monomer), lots 104K7540 & 036k7575

Additional model proteins were

- α-Lactalbumin from bovine milk, a 14 kDa protein with an isoelectric point of 4.5 to 4.8. The grade used in the study was identified in the certificate of analysis as "Type I, ≥85% (PAGE), lyophilized powder"; Sigma product number L5385; lot 063K7009.

- Albumin from human serum, a 66 kDa protein with an isoelectric point of 4.8. The grade used in the study was identified in the certificate of analysis as "≈99% (agarose gel electrophoresis), lyophilized powder"; Sigma product number A8763; lot 025K7555.

- Lysozyme from chicken egg white, a 14 kDa protein with an isoelectric point of 10.7. The grade used in the study was identified in the certificate of analysis as "lyophilized powder, ≈50,000 units/mg protein, >90% purity"; Sigma product number L7651; lot 114K7054.

- 10% intravenous immunoglobulin (IVIG), a plasma-derived product consisting largely of 150 kDa human IgGs, was donated by Talecris Biotherapeutics, Inc. (Clayton, NC).

Test Article Analysis

Enumeration of phage titers was performed as described (15, 18).

Filters

Pre-filters (0.1 μm nominal pore size) were Millex® 33 CVVL filter capsules (Millipore Corp., Bedford, MA) or SuporLife® 100 DCF™ 0.1 μm filter capsules (Pall, East Hills, NY). The five virus filters tested in this study were Asahi Kasei Medical Corp. Planova 15N and 20N (0.001 m^2 filtration surface area); Pall Pegasus™ SV4 (0.0011 m^2 filtration surface area in FTK200 disc holder), Sartorius Virosart® CPV (Minisart® membrane disk units, 0.0005 m^2 filtration surface area) and Millipore Viresolve® NFP (Optiscale™-25 disk units, 0.00035 m^2 filtration surface area). All filters are designed for direct flow. Each virus filter was tested for installation integrity using the manufacturer's recommended test method for the scaled-down devices.

As the purpose of this study was to develop a rating method suitable to potentially all small-pore virus filters, and not to characterize specifically the five virus filters used in method development, the filters were blinded and are referred hereto as Filters 1–5.

Filtration operating conditions

The filtration methodology included the collection and assay of fractions taken during the course of filtration (~1.5 mL grab samples or instantaneous samples). Samples collected directly from the filter devices are referred to as "grab samples". The test filters were small-scale disk or hollow fiber devices supplied by the four filter manufacturers for use in scale-down validation studies. Devices were first pre-wetted with PBS (without protein).

Five separate challenge solutions, denoted "BSA test fluid" A through E for the five BSA types listed above, were processed through a 0.2 μm filter before being added to the pressure vessel. The system was then pressurized and the volume of filtrate measured every 1 min to 5 min for the duration of the test using precision timers and balances (buffer and protein solution density is assumed to be 1 g/mL). The initial two hold-up volumes (containing mostly buffer, not model protein:phage solution) were discarded and not counted towards throughput in liters per square meter (L/m^2). The initial flow rate of buffer was used to calculate flow rates at specific time or passage volume points. The degree of flow decay from the initial flow varied with the particular filter and protein:phage combination. Samples were collected directly from the filter devices at the start of the run (V_{init}, the initial sample after the initial two buffer hold-up volumes) and at the target volume points. Samples were collected directly from the filter devices (grab samples) at 0, 50, 100 L/m^2, etc. These samples measure the phage retention capability of the filter at the time of sample collection, as opposed to pooled filtrate samples typically collected in validation studies, which measure virus retention of the filter over the course of an entire run. LRVs were calculated as follows: Log_{10} (phage titers in the challenge solution ÷ phage titers in the filtrate grab samples).

Filters 1 through 5 were evaluated in a matrix of filtration operating conditions for the endpoint definition/target LRV assessment (Table I). The matrix was

TABLE I
Filtration Matrix

Test fluid (BSA)	A	B	C	D		E
Filter lot*a*	1	1	1	1	**2**	1
10^6 pfu/mL crude pp7		√		√*b*	√	√
10^6 pfu/mL CsCl pure pp7	√	√	√	√	√*b*	
10^8 pfu/mL crude pp7			√	√	√*b*	
10^8 pfu/mL CsCl pure pp7 + 10^4 pfu/mL PR772	√			√*b*	√	√

a BSA test fluid D was tested with two filter membrane lots for each of the five filter types. All other BSA test fluids were tested with one membrane lot.
b The filter was run to 200 L/m^2 with samples collected at V_{init}, 25, 50, 75, 100, 150, and 200 L/m^2.

designed to vary and bracket challenge conditions, phage preparation procedures (crude vs CsCl-purified), phage spike titer (10^6 to 10^8 pfu/mL), BSA quality (five sources), and co-spiking with PR772. All filters were run in constant pressure mode at the operating pressure recommended by the filter manufacturer. Except as noted, the filters were run to 100 L/m^2 or 75% flow decay. Approximately 1.5 mL grab samples were taken at V_{init} and at 25, 50, 75, and 100 L/m^2. PP7 titers were measured in filter loads and grab samples at V_{init}, 50, 100 L/m^2; grab samples at 25 and 75 L/m^2 were retained for investigation of unexpected results.

In a separate study to compare model proteins (Figure 2), samples of Filter 4 were run under constant pressure mode (30 psi, 2.1 x 10^5 Pa). Filter 4 was selected for this evaluation because in the experience of CDER, it is the most sensitive filter type to flow decay and protein quality (data not shown).

Dynamic Light Scattering (DLS) Test Articles

The phages in this study (PP7, ΦX174, PR772) were purified at CDER/FDA using CsCl gradient ultracentrifugation. Samples were received (University of Wisconsin—Madison) shortly before the initiation of the stability testing program (less than one week). For DLS experiments, phage samples were diluted to a concentration of approximately 10^{11} plaque forming units (pfu) per milliliter in PBS with added model proteins as appropriate. Once prepared, samples were refrigerated until just before DLS analysis.

DLS Procedure

DLS was performed on a Coherent (Santa Clara, CA) 488 nm argon laser and a Malvern (Worcestershire, UK) model 4700c sub-micron particle analyzer. PR772, PP7, and ΦX174 solutions were filtered through a 0.1 μm pore size polyvinylidene fluoride Millex-VV Millipore filter into a light scattering cuvette for DLS experiments. DLS measurements were made in triplicate at each of five angles: 45°, 60°, 75°, 90°, and 110°. In DLS, the intensity of scattered light is measured and converted into a normalized, first-order electric field autocorrelation function. Two different analysis methods were used to estimate virus particle size from DLS data: the cumulant method and CONTIN method. The cumulant method assumes nothing about the distribution form and simply fits a third order polynomial to the log of the normalized correlation function. The first moment of this fit (K1) is the mean, and the second moment (K2) is the variance. The average diameter (D_{avg}) and polydispersity index (PDI) are found from K1 and K2. The PDI is the normalized variance (dimensionless), given by:

$$PDI = K2 \div (K1)^2 \quad (1)$$

CONTIN analysis performs an inverse transformation on the DLS data and returns a set of size distribution solutions based on intensity or volume averages. Using CONTIN analysis, it is possible to resolve the data into different size classes (multiple peaks), whereas the cumulant method averages all of the data to calculate a single average diameter.

Electrospray Differential Mobility Analysis (ES-DMA)

The phages in this study (PP7, ΦX174) were prepared for ES-DMA analysis (19) at CDER/FDA by CsCl gradient ultracentrifugation and suspended in a 10 mmol/L ammonium acetate, pH 7.0 buffer solution.

Upon receipt at the National Institute of Standards and Technology (NIST), the solutions were further dialyzed for at least 18 h into a 2 mmol/L buffer solution using 10 kDa molecular weight cut-off Slide-A-Lyzer cartridges (Pierce, Rockford, IL) at room temperature. Some samples were further diluted 10- to 20-fold without significantly affecting the measured size. Samples were subsequently refrigerated at 4 °C until use.

The solution containing the virus was electrosprayed through a 25 μm inner diameter capillary with a sharpened tip. Potentials from approximately 1.9 kV to 2.7 kV with gas flow rates of 0.2 L/min of CO_2 and 1.0 L/min of air achieved the stable cone-jet condition necessary to obtain reliable results. The highly charged droplets emitted from the electrospray were neutralized as described (19), leaving the vast majority of the positive ions in the +1 state. The droplets of virus particles dry as they pass through the approximately 80 cm of plastic tubing connecting the exit of the electrospray to the entrance of the differential mobility analyzer.

The flow containing the dry virus particles joins a flow of nitrogen gas at 30 L/min in the annular analysis chamber (nanoDMA, TSI, Inc., Shoreview, MN), while an electrostatic potential as strong as −12 kV attracts the positively charged virus particles. Those particles for which the electrical force balances the drag force pass into a collection slit. As the electrical force is set by the potential on the central electrode and the neutralizer, only particles of a particular size pass through the collection slit into the condensation particle counter (CPC). In the CPC, the size-selected particles nucleate droplets in a saturated butanol environment, which grow large enough to be counted with 90° light scattering.

Conversion to size was performed as per the manufacturer's instructions (http://www.tsi.com/documents/1933792g-3080.pdf) with a Cunningham slip correction factor of

$$C_c = 1 + Kn\left[\alpha + \beta \exp\left(\frac{-\gamma}{Kn}\right)\right] \quad (2)$$

where $Kn = 2\lambda/d$, $\alpha = 1.257$, $\beta = 0.40$, $\gamma = 1.110$, and the gas mean free path at room temperature $\lambda = 66$ nm. We employed a well known charge distribution to determine the complete particle size distribution, as opposed to only the distribution of +1 charged particles measured in the system. The equation was specified by the commercial vendor,

$$f = 10^{\sum_{i=0}^{5} a_i [\log(d_p/1nm))]^i} \quad (3)$$

where d_p represents the diameter of the particle, and a_o through a_5 are −2.3484, 0.6044, 0.4800, 0.0013, −0.1553, and 0.0320, respectively. The number-average diameter (i.e. the average diameter weighted by count as opposed to mass or volume) was then calculated with the following equation:

$$\bar{d} = \frac{\sum_i d_i N_i}{\sum_i N_i} \quad (4)$$

The average and standard deviation reported for the triplicate measurement represent the average and standard deviation of $\bar{d}$.

Polymerase Chain Reaction (PCR)-based PP7 Identity Test. Two sets of unique oligo-nucleotide primers were selected to amplify ≈500 bp fragments from the 5' (nt 382–404, 5'-CCA TTC GCG TGA GGT TGA CTG TG-3'; nt 874–896, 5'-TGC TGG CAC GCG GAT TAC AGG TT-3') and 3' region (nt 1769–1791, 5'-CGC AGG TCG AAG ATC TTG TCG TC-3'; nt 2244–2266, 5'-5'-TGG TGC TAG CCG CCT ATC CTC AA-3') of the PP7 RNA genome. The oligo-nucleotides were designed from the published PP7 nucleotide sequence (Genbank accession number X80191) (20) by the FastPCR version 5.1.70 beta1 software (PrimerDigital, Helsinki, Finland). The FastPCR software dynamically optimizes best primer length based on the general nucleotide structure of the primer such as complexity, nucleotide composition at 3' and 5' ends of primers, the melting temperature of the 10 bases at the 3' and 5' ends, a self-complementarity test, and secondary (non-specific) binding. Default parameters were chosen and two sets of primers were chosen that had relatively high annealing temperatures (~60 ° C). The uniqueness of the oligonucleotide sequences was verified by a standard Basic Local Alignment Search Tool (BLAST) analysis (21) against the "entire nucleotide collection" (nr/nt) in GenBank (http://www.ncbi.nlm.nih.gov/BLAST). Beside PP7, no other sequence in nr had a similarity, with no gaps, higher than 90%. Genomic nucleic acids from PP7, ΦX174, and PR772 were purified with a QIAamp Viral RNA kit (Qiagen, Chatsworth, CA). Mouse genomic DNA was purified by standard phenol extraction/ethanol precipitation methods (22). Reverse transcriptase PCR

TABLE II
Potential Model Phages

Phage	Family	Host	Host considerations	Phage size (nm)		Isoelectric point (pI)		Suitable buffer system for storage (2–8 °C)
				ICTV[a]	DLS[b]	Available literature[c]	Chromato-focusing[d]	
MS2[e]	*Leviviridae*	*E. coli* C3000	BSL 1	26	Not Measured	3.9	Not Measured	Tris-NaCl + 2 mM $MgCl_2$, pH 8.1
ΦX174	*Microviridae*	*E. coli* C	BSL 1	26 to 32	26 to 30	6.6	6.5	Borate/ EDTA pH 9.5
PP7	*Leviviridae*	*Pseudomonas aeruginosa*	BSL 2[f]	26	30 to 33	NA	4.3 to 4.9	PBS pH 7.4

[a] International Committee on the Taxonomy of Viruses (31). Sizes in ICTV represent a consensus of virologists and are generally based on multiple electron microscopy measurements.
[b] Dynamic light scattering from (15) and this study.
[c] Electrophoretically determined (e.g., whole-particle micro-electrophoresis and isoelectric focusing) isoelectric point measurements complied by Dowd et al. (32).
[d] Isoelectric point calculated based on phage chromatofocusing (33).
[e] MS2 was ruled out early because of ease of use issues (data not shown) and because historically it had been used to test virus retention by gas filters (24), not liquid filters.
[f] Additional state health permit needed in Hawaii

(RT-PCR) for 35 cycles was performed using standard methodology (22) with an annealing step temperature of 58 °C. Separate amplification of the 500 bp amplicons was detected by electrophoresis of the PCR reaction mixtures on 1.0% agarose gels in tris-borate buffer.

Results and Discussion

In 2005, the PDA Virus Retentive Filter Task Force successfully developed and prototyped a method to classify large virus-retentive filters based on retention

TABLE III
Dynamic Light Scattering Particle Size Distribution Profile of Multiple Preparations of PP7 and ΦX174

Preparation	Time (months)	D_{avg} (nm)	PDI[a]	Intensity Peaks (nm)	Volume Peaks (nm)
PP7 (1)	0	32	0.07	33	33
	2	31	0.10	32	32
	4	31	0.08	33	32
PP7 (2)	0	33	0.10	33	33
ΦX174 (1)	0	34	0.23	37	32
ΦX174 (2)	0	48	0.32	34, 122[b]	33, 122[b]
ΦX174 (3)	0	34	0.25	33	26
ΦX174 (4)	0	40	0.23	45	31
ΦX174 (5)	0	41	0.26	44	31
ΦX174 (6)	0	40	0.16	43	33

[a] Polydispersity index
[b] Second peak likely represents aggregated phage

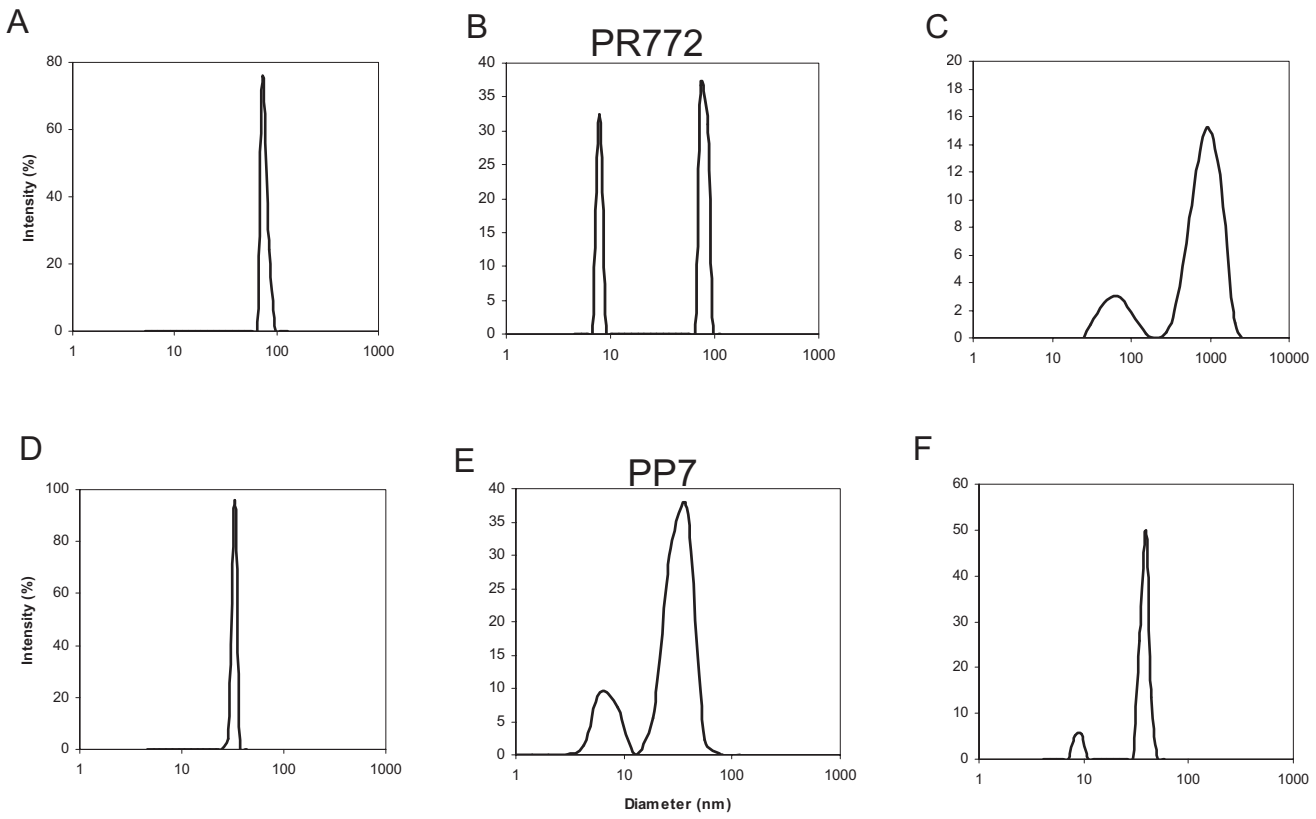

Figure 1

Dynamic light scattering (DLS) particle size distributions were calculated by CONTIN. (a) 4.5 x 10^{10} pfu/mL PR772; the intensity peak was 76 nm ± 10 nm. (b) 4.5 x 10^{9} pfu/mL PR772 co-incubated with 1 mg/mL BSA; two intensity peaks were 8 nm ± 1 nm (BSA) and 79 nm ± 11 nm (PR772). (c) 4.5 x 10^{9} pfu/mL PR772 co-incubated with 1 mg/mL lysozyme; intensity peaks were mixed aggregates of 68 nm ± 44 nm and 944 nm ± 682 nm. (d) 1.0 x 10^{11} pfu/mL PP7; the intensity peak was 33 nm ± 4 nm. (e) 1.0 x 10^{11} pfu/mL PP7 co-incubated with 1 mg/mL BSA; two intensity peaks were 7 nm ± 3 nm (BSA) and 33 nm ± 19 nm (PP7). (f) 1.1 x 10^{11} pfu/mL PP7 co-incubated with 1 mg/mL lysozyme; intensity peaks were 9 nm ± 1 nm (presumably monomer or dimer/tetramers of lysozyme) and 38 nm ± 6 nm (PP7 partially complexed with lysozyme).

of a 64 nm to 82 nm coliphage, PR772 (11–13). The next obvious challenge for the task force was to develop a method for small virus-retentive filters. Because of technical challenges associated with small virus-retentive filters—for example, the potential for fouling or passage (14, 15)—a series of development studies was performed to support the task force's decision making during the method development. The development studies addressed the following issues:

- Choice of model phage (PP7 vs ΦX174) with respect to
  - Stability
  - Ease of preparation and use
  - Size and monodispersion
  - Filtration properties
  - Availability of an identity test
- Choice of model protein with respect to
  - Absence of interactions with model phage
  - Comparability to commercial process fluids with respect to filtration behavior
- End point definition and target LRV for the final method

Early in the process, the task force decided to focus on model proteins and phages for which some experience had been gained previously on filtration properties (14, 15, 23–28). It was also decided that the model proteins should be readily available from

TABLE IV
Phage Sizes Measured by Electrospray Differential Mobility Analysis (ES-DMA)

Phage	ES-DMA measured diameter (nm)[a,c]	ES-DMA replicates (n)	ICTV[b] consensus size (nm)
PP7	23.2 ± 5.4	3	26
ΦX174	26.5 ± 6.8	3	26 to 32

[a] To reduce interference with salts, phage samples (2 to 5 x 10^{12} pfu/mL) were dialyzed 10- to 20-fold with 10 mmol/L ammonium acetate, pH 7.0 before ES-DMA
[b] International Committee on the Taxonomy of Viruses (31)
[c] Expanded uncertainty, U, assumes the distribution to be normally distributed, and a coverage factor of $k = 2$ where $U = k\,u_j$. One standard deviation, u_j, was estimated by dividing the range of the distribution by 6 as specified for type B uncertainties (http://physics.nist.gov/Pubs/guidelines/TN1297/tn1297s.pdf).

multiple commercial sources (i.e., either research and assay product vendors or manufacturers of plasma-derived products). It was agreed that the phage should be relatively well characterized, for example, the genome sequence and basic structural information should be known (20, 29–31), it and should be available from reference collections (e.g., American Type Culture Collection, ATCC). Based on these factors, potential model proteins—BSA, human serum albumin (HSA), IVIG, α-lactalbumin, and lysozyme—and small bacteriophages (Table II) were identified.

Phage Stability

To investigate the suitability of ΦX174 and PP7 for use in filter testing, long-term storage stability experiments were performed. MS2 was ruled out because no report of its use in liquid virus filtration had been published. The phage was also tested for freeze/thaw stability and sensitivity to short-term, low-pH excursions. Both phages, when purified by CsCl gradients, are stable for at least 5 to 12 months at 2 °C to 8 °C; crude preparations of PP7 in nutrient broth are not.

PP7 is sensitive to short-term, low-pH excursions (pH ≤ 4.5 for > 30 min), while ΦX174 is stable at pH ≥ 2.5. Both phages can be freeze/thawed at least 2–3 times with minimal impact on titer.

DLS of Phage and Phage:Protein Co-Mixtures

Phage Light Scattering. To confirm the size of PP7 and ΦX174, we performed DLS of two CsCl-purified PP7 preparations and six ΦX174 preparations. As can be seen, the volume peak (i.e. peak of the size distribution based on volume measurements) of both phages generally ranges from 31 nm to 33 nm (Table III), a size consistent with use of these phage for testing of small virus-retentive filters. The polydispersity index for both PP7 preparations was roughly one-half to one-quarter that of the ΦX174 preparations, suggesting that PP7 can be produced in a monodispersed form more easily and reliably than ΦX174. The intensity peaks of the ΦX174 preparations were also larger. As the intensity peaks are the most direct measurement output by light scattering instrumentation, this data also suggests that PP7 may be consistently smaller.

Uncertainties (expanded) in the caption to Figure 1 were estimated assuming a coverage factor of $k = 2$, the distribution to be lognormal, and by taking the geometric average of one-third of the difference between the mean and the upper and lower bounds of the distribution for type B uncertainties (http://physics.nist.gov/Pubs/guidelines/TN1297/tn1297s.pdf).

Phage:Protein Interactions. To determine which model proteins in our candidate panel interact with phage, DLS studies of protein:phage co-mixtures were performed. If the phage and model proteins physically interact, for example, by electrostatic interaction, the main DLS phage peak calculated by the CONTIN program is predicted to shift towards higher sizes. In the absence of interactions, separate phage and protein peaks should resolve at the sizes of the individual components. In Figure I, it is evident that neither PR772:BSA or PP7:BSA co-mixtures interact in a manner detectable by our DLS analysis. In contrast, lysozyme appears to interact with both phages. The nature of this interaction could be electrostatic interactions between the negatively charged phage and the positively charged lysozyme. In contrast, BSA is a negatively charged protein, providing an explanation for the lack of interaction with the two phages.

Electrospray Differential Mobility Analysis (ES-DMA). Recently, ES-DMA has shown utility for measuring the diameters of high-resistance, non-enveloped viruses such as MS2, adenovirus, cowpea mosaic virus, and rice yellow mottle virus (34–36). Recovery of infectious virus from the instrument is possible if stress on the virus is minimized by maintaining the electrospray droplet size larger than the virus size (35). Because viruses are not infectious unless they are intact, this finding argues that ES-DMA measures the actual size of intact virus, not fragments. Reproducible recovery was obtained for small, high-resistance viruses like MS2, but not for large, fragile viruses like T-even phages (35). Thus, small, high-resistance phages like PP7 and ΦX174 (31) can be predicted to be accurately measured by ES-DMA as well.

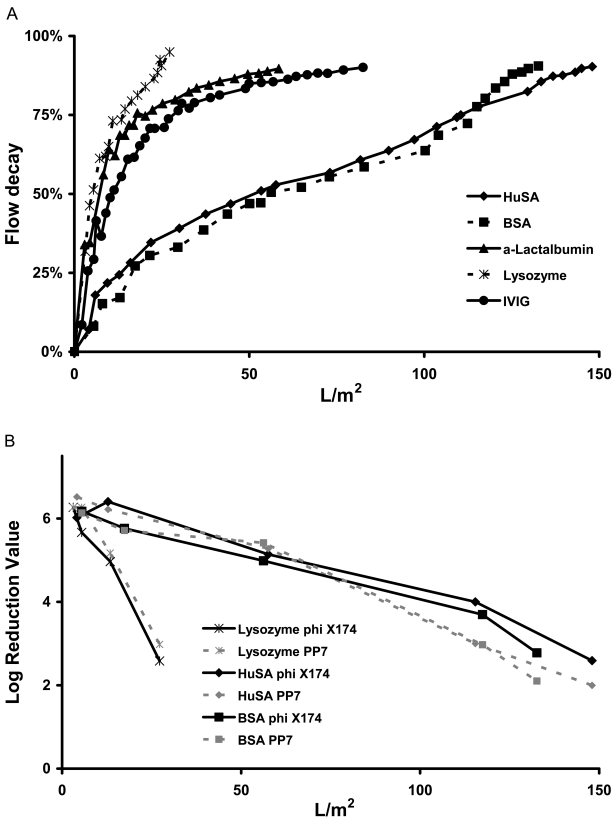

Figure 2

Flux decay and instantaneous LRV vs volumetric throughput for the five potential model proteins. All five model proteins were dissolved at 2.5 mg/mL in PBS, except IVIG which was diluted in purified water, and co-spiked with 10^7 pfu/mL PP7 and ΦX174. Samples of Filter 4 were run under a constant, 30 psi pressure (2.1 x 10^5 Pa). (a) Flux relative to initial flux monitored every 1 to 5 min. (b) Instantaneous LRV measured at V_{init}, V_{25}, V_{50}, V_{75}, and V_{90}.

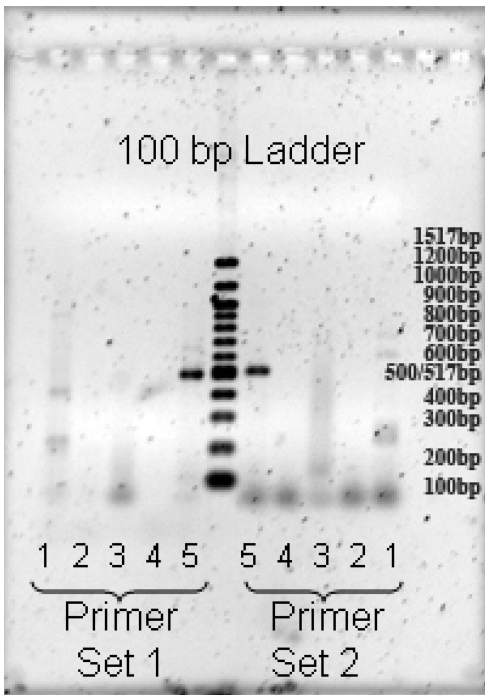

Figure 3

RT-PCR based identity test for PP7. One microliter of RT-PCR products run on an agarose gel at 100 volts/m for 30 min (22). Primer set 1 amplifies an amplicon from the 5' region of the PP7 genome (nt 382–896), and primer set 2 amplifies a 3' region amplicon (nt 1769–2266). Lane 1 contains the reverse transcriptase/amplification reaction for ΦX174; Lane 2, PR772; Lane 3, mouse DNA; Lane 4, water; Lane 5, PP7.

To verify our DLS measured sizes of PP7 and ΦX174, high-titer, CsCl-purified preparations of both phages were evaluated by ES-DMA. Measurements were performed in triplicate on samples that had been extensively dialyzed against 10 mmol/L ammonium acetate, pH 7.0 to eliminate salt interference. The ES-DMA-measured sizes of PP7 and ΦX174 were 4 nm to 7 nm smaller than as measured by DLS (Table IV). This observation can be attributed to technical differences between ES-DMA and DLS. DLS measures the hydrodynamic diameter of particles in solution. This measurement is influenced by factors such as hydration of the particle, as well as motion in solution (37). Thus, a DLS measurement is usually larger than the actual hard physical size of a particle. In contrast, ES-DMA may somewhat underestimate the size of a phage par-

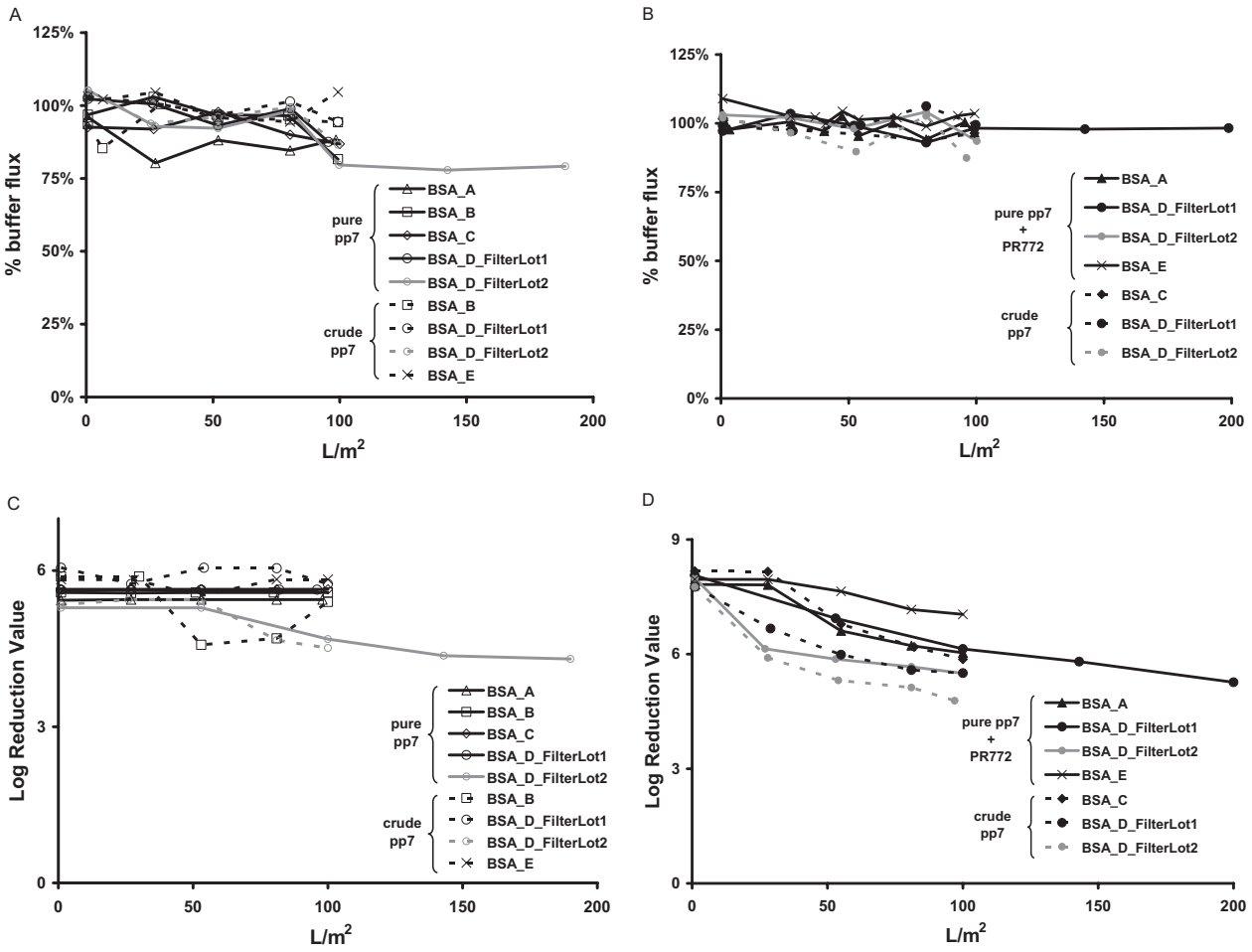

Figure 4

Results from matrix study for Filter 1. Matrix design is described in Table I. (A) Flux vs volumetric throughput for experiments spiked at ~10^6 pfu/mL PP7. (B) Flux vs volumetric throughput for experiments spiked at ~10^8 pfu/mL PP7. (C) Instantaneous LRV vs volumetric throughput for experiments spiked at ~10^6 pfu/mL PP7. (D) Instantaneous LRV vs volumetric throughput for experiments spiked at ~10^8 pfu/mL PP7.

ticle, as the capsid may compress during the electrospray process (35).

It is significant to note that, as measured by ES-DMA, PP7 is 3 nm smaller than ΦX174. It should be noted that this may reflect actual differences in the size of the phages or differences in the compressibility of the capsid, or a combination of both. The ES-DMA traces of the ΦX174 preparations contained more free capsid protein than traces from PP7 (data not shown). These observations further support the hypothesis that PP7 may be slightly smaller. In addition, the ES-DMA data, like the DLS data, argue that higher quality preparations of PP7 can be more routinely produced.

Comparison of Filtration Properties of Model Proteins

A second important property for our candidate model proteins is "filterability", that is, not rapidly fouling filters when filtered at concentrations and in buffer systems realistic of commercial processing. To compare the model proteins for filterability, 2.5 mg/mL of each model protein in PBS was co-spiked with 10^7 pfu/mL PP7 and ΦX174 and run through two types of filters: samples of Filter 4 (Figure 2) and Filter 3 (data not shown) for 300 L/m^2 or until V_{90}, whichever came first. For both filters, HSA and BSA proved to be the most filterable; difference in fouling pattern was most evident for Filter 4. In the same studies, the LRV_{init} and at V_{25}, V_{50}, V_{75}, and V_{90} of ΦX174 and PP7 were

largely equivalent. This data argues that either phage is a suitable model for testing small virus-retentive filters. Thus, the task force's final decision between PP7 and ΦX174 was based largely on convenience (i.e., PP7 is easier to prepare and can be co-spiked with PR772; S. Lute & K. Brorson, personal observations).

PP7 Identity Test

PP7 is a *Leviviridae* phage originally isolated from sewage (20, 31, 38). To provide a resource for others performing similar testing outside the scope of this study, we developed an RT-PCR based method to identify PP7 and distinguish it from other bacteriophage. Identity testing is required by United States regulation for incoming component testing in pharmaceutical manufacturing (21 CFR 211.84); implementation of this test should be considered when commissioning the filter test in a biopharmaceutical environment. Two primer sets were chosen to amplify two separate 500 bp amplicons from the 5' and 3' regions of the 3588 base pair PP7 RNA genome. The primers were selected based on complexity, nucleotide composition at 3' and 5' ends of primers, the melting temperature of the 10 bases at the 3' and 5' ends, a self-complementarity test, secondary (non-specific) binding, and melting temperatures (T_m; ≈60 °C).

To test the utility and specificity of the primers, 1 ng of genomic nucleic acid from ΦX174, PR772, mouse, and PP7 were amplified by RT-PCR for 35 cycles at an annealing temperature of 58 °C (2 °C below the T_m of the primers). As can be seen (Figure 3), both primer sets amplify 500 bp amplicons from PP7, but not from the other genomes. As a further confirmation of primer specificity, a BLAST search was performed against the entire nucleotide collection in GenBank (termed "nr/nt" by the National Center for Biotechnology Information). Aside from PP7, no other sequence in nr/nt had a similarity, with no gaps, higher than 90%.

Assessment of PP7 Removal by Five Filter Types

To determine a common endpoint definition and target LRV, a filtration matrix was designed varying phage preparation procedures (crude vs CsCl-purified), phage spike titer (10^6 or 10^8 pfu/mL), BSA quality (five sources), and ± co-spiking with 10^3 to 10^4 pfu/mL PR772 (Table I). Co-spiking with PR772, a 64 nm to 82nm bacteriophage, was tested to determine if

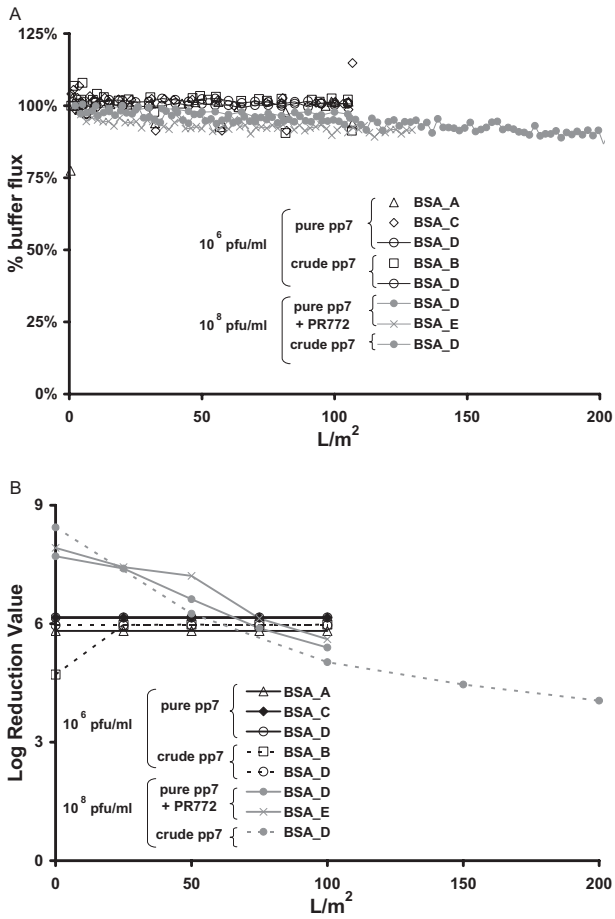

Figure 5

Results from matrix study for Filter 2. The original matrix design, described in Table I, was modified due to time and logistical constraints. (A) Flux vs volumetric throughput. (B) LRV vs volumetric throughput.

it was feasible to use PR772 as an internal installation integrity control for the filters (i.e., a test of gross defects that would allow a large bacteriophage to pass through a small virus retentive filter). BSA concentration (1 mg/mL) and buffer system (PBS) were held constant. The five filters from the four manufacturers were run to 100 L/m^2 or 75% flow decay, except as noted in the matrix (Table I). PP7 titers were measured in filter loads and grab samples at V_{init}, and at 50, and 100 L/m^2.

As expected (15), the instantaneous LRV vs volumetric throughput pattern varied among the filter types (Figures 4–8). In addition, the different grades of BSA had variable effects on filtration by the five filter

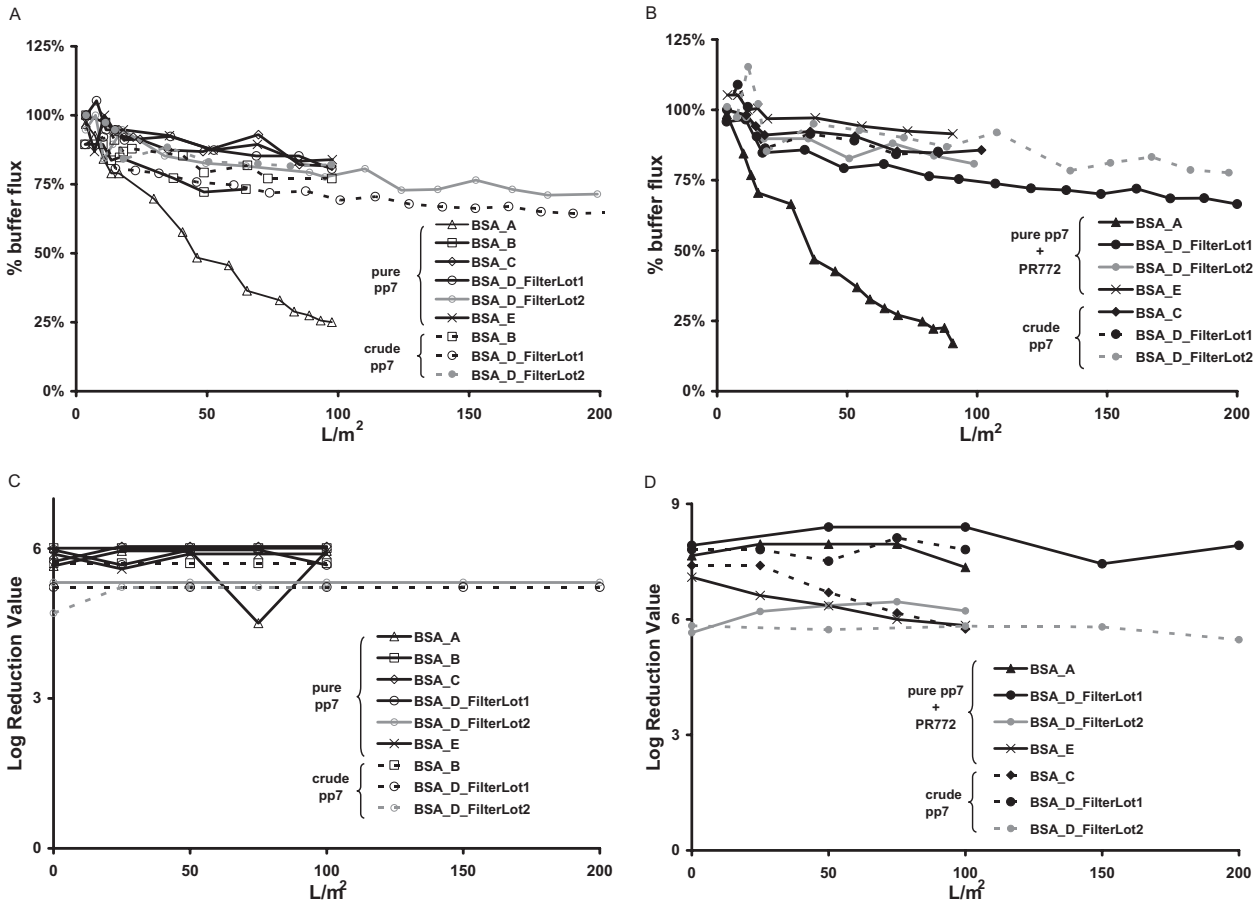

Figure 6

Results from matrix study for Filter 3. Matrix design is described in Table I. (A) Flux vs volumetric throughput for experiments spiked at ~10^6 pfu/mL PP7. (B) Flux vs volumetric throughput for experiments spiked at ~10^8 pfu/mL PP7. (C) Instantaneous LRV vs volumetric throughput for experiments spiked at ~10^6 pfu/mL PP7. (D) Instantaneous LRV vs volumetric throughput for experiments spiked at ~10^8 pfu/mL PP7.

types. However, the following observations were made from the data:

- Two feasible endpoint definitions and ratings were identified as achievable with current assay methods and filter technology.
  - —100 L/m^2 or <50% flow decay achieves an LRV of 3 $\log_{10}$
  - —50 L/m^2 or <25% flow decay achieves an LRV of 4 $\log_{10}$
  - —Based on a vote by the committee, the second was preferred.
- Upper and lower limits of 10^6 to 10^8 pfu/mL for phage are acceptable. An upper limit of 10^7 pfu/mL would result in a lower level of passage in some filters; a spiking titer of $>10^7$ is not necessary to achieve a target LRV of 4 $\log_{10}$.
- Some variability in LRV was noted on a lot-to-lot basis. To account for variability, three lots at three filters per lot can be tested.
- Co-spiking with 10^4 pfu/mL of PR772 is feasible and does not interfere with filter performance.
- The method of the phage spike preparation (e.g., crude vs CsCl gradient-purified) did not affect filterability.
- There was a variable impact of BSA quality on filterability:
  - —Filters 1, 2, and 5 exhibited only modest fouling with all grades of BSA.
  - —Filter 3 was fouled by BSA type A most rapidly.
  - —Filter 4 was fouled by BSA type D most rapidly.
  - —Because there was no consistent or logical quality trend (i.e., the crudest preparations did not foul all filters most rapidly), a BSA spec-

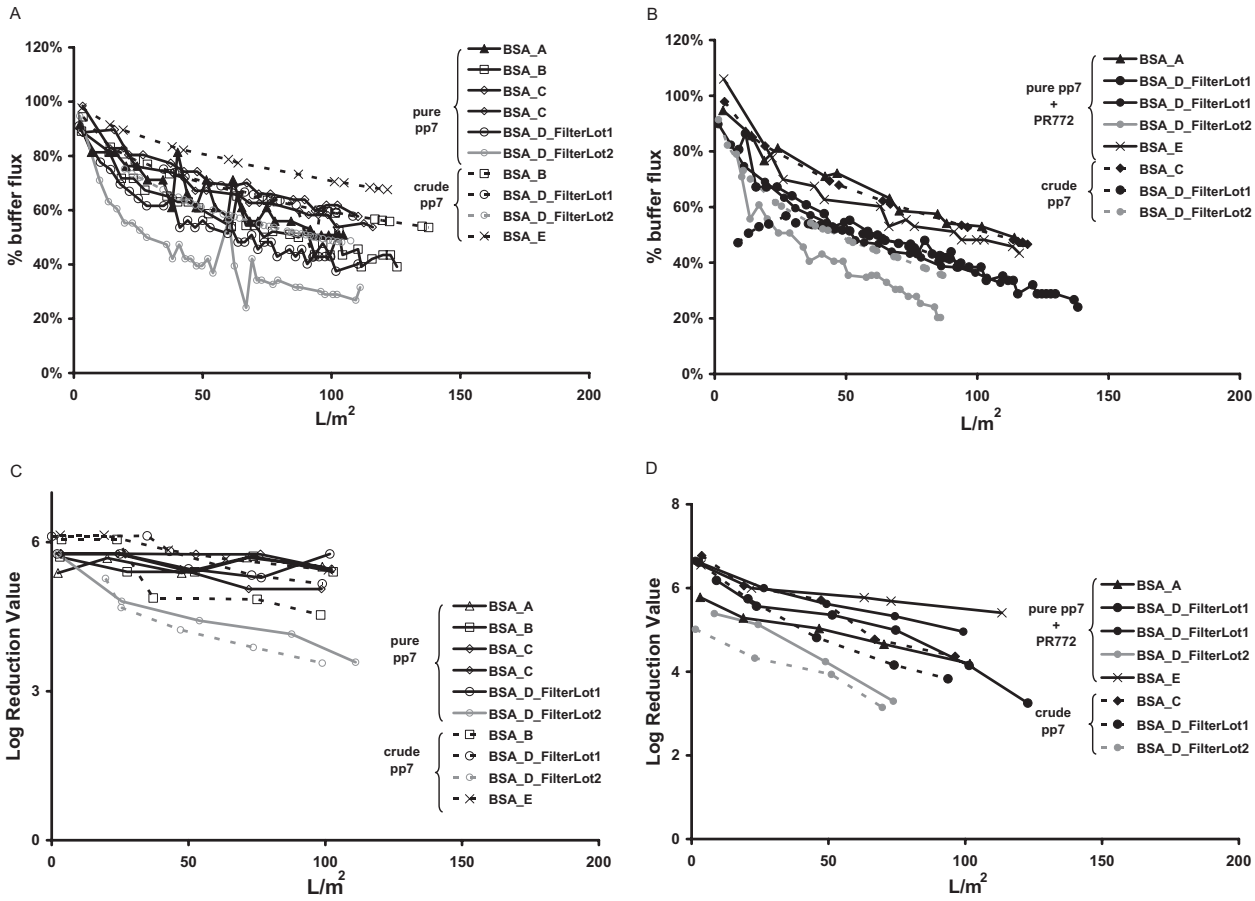

Figure 7

Results from matrix study for Filter 4. Matrix design is described in Table I. (A) Flux vs volumetric throughput for experiments spiked at ~10^6 pfu/mL PP7. (B) Flux vs volumetric throughput for experiments spiked at ~10^8 pfu/mL PP7. (C) Instantaneous LRV vs volumetric throughput for experiments spiked at ~10^6 pfu/mL PP7. (D) Instantaneous LRV vs volumetric throughput for experiments spiked at ~10^8 pfu/mL PP7.

ification can be set based on a combination of filterability and biochemical attributes such as purity and minimal aggregation.

All of the above considerations were implemented in the final consensus test method agreed upon by the PDA Virus Filter Task Force. The final consensus filter method is published in the 2008 update of the PDA Virus Filtration Technical Report (17). It was evaluated in a third party lab (CDER/FDA) in collaboration with four filter manufacturers (Pall® Life Sciences, East Hills, NY; Millipore®, Billerica, MA; Sartorius, Gottingen Germany; and Asahi Kasei, Tokyo, Japan) (39). The method and acceptance criteria based on the studies in this report and defined in the 2008 update of the PDA Virus Filtration Technical Report (17) reflect the capabilities of the current small virus-retentive filters.

Conclusion

Based on experimental data with filters from four filter manufacturers, the PDA Virus Filter Task Force selected 10^6 to 10^7 pfu/mL *Pseudomonas* phage PP7 and 1 mg/mL BSA as an acceptable model system for testing of small virus-retentive filters. Co-spiking with 10^3 to 10^4 pfu/mL PR772 was found to be an acceptable integrity test method that did not influence the flow properties of any of the filters tested. Each filter type was found to remove in excess of 4 $\log_{10}$ PP7 in a filtration run of 50 L/m^2 and 3 $\log_{10}$ in a run of 100 L/m^2. All of the above considerations were implemented in the final consensus test method agreed upon by the PDA Virus Filter Task Force. The final consensus filter method is published in the 2008 update of PDA Technical Report 41: Virus Filtration.

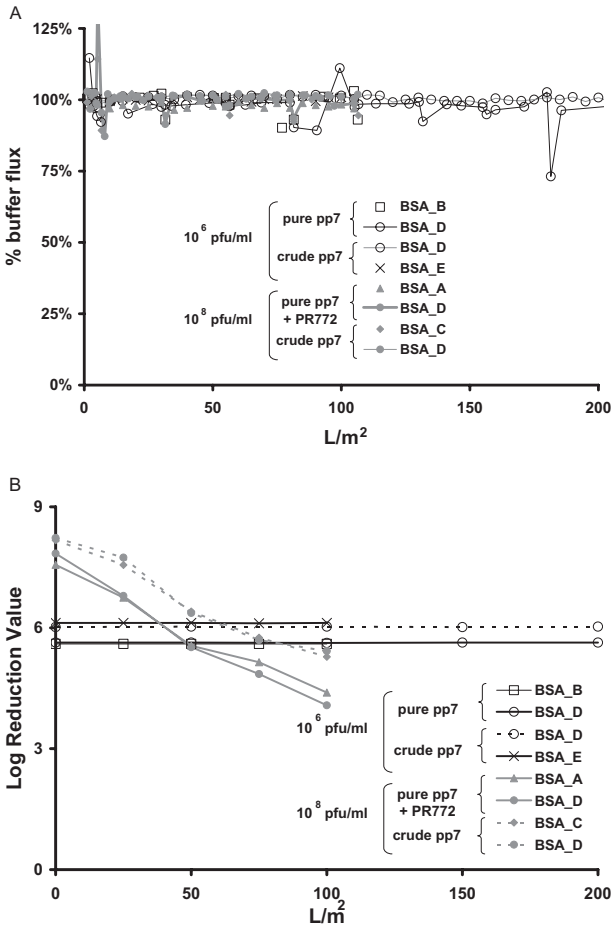

Figure 8

Results from matrix study for Filter 5. The original matrix design, described in Table I, was modified due to time and logistical constraints. (A) Flux vs volumetric throughput. (B) Flux vs volumetric throughput.

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Disclaimers

Views expressed in this article reflect those of the authors and do not constitute official positions of the FDA or the US Government. Inclusion or exclusion of individual filters in this study does not constitute an endorsement of individual filter filters or manufacturers by the FDA or the US Government.

PDA does not endorse individual filters or products from particular manufacturers.

Certain equipment, instruments, or materials are identified in this paper in order to adequately specify the experimental details. Such identification does not imply recommendation by NIST nor does it imply the materials to be necessarily the best available for the purpose.

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