Development and Qualification of a Novel Virus Removal Filter for Cell Culture Applications

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Commercial bioreactors employing mammalian cell cultures to express biological or pharmaceutical products can become contaminated with adventitious viruses. The high expense of such a contamination can be reduced by passing all gases and fluids feeding the bioreactor through virus inactivation or removal steps, which act as viral barriers around the bioreactor. A novel virus barrier filter has been developed for removing viruses from serum-free cell culture media. This filter removes the 20 nm minute virus of mice by >3 log reduction value (LRV), the 28 nm bacteriophage ΦX174 by >4.5 LRV, the mycoplasma *Acholeplasma laidlawii* by ≥8.8 LRV, and the bacteria *Brevundimonas diminuta* by ≥9.2 LRV. Robust removal occurs primarily by size exclusion as demonstrated over a wide range of feedstocks and operating conditions. The filtered media are indistinguishable from unfiltered media in growth of cells to high densities, maintenance of cell viability, and productivity in expressing protein product. Insulin and transferrin show high passage through the filter. The virus barrier filter can be autoclaved. The relatively high membrane permeability enables the use of a moderate filtration area.

Introduction

Bioreactor Contamination. Reduction of bioreactor contamination by microorganisms is a key factor for success in the biotherapeutic business. It is relatively easy to control contamination caused by bacteria, fungi, and mycoplasma. Removal of microorganisms by filtration and growth inhibition of microorganisms with antibiotics are effective means of contamination control in biotechnological practice. The situation is different if the contamination is caused by adventitious viruses. Adventitious viruses may be present in cell culture raw materials or other feedstreams added to bioreactors and, unlike bacteria, fungi, and mycoplasma, are much less likely to be removed by "sterilizing-grade" filters. These viruses can infect the mammalian cell expression system. Cell infection and viral replication amplify the viral contaminant and can lead to high virus titers in the bioreactor. Unlike bioreactor contamination by bacteria, these high virus titers may not be apparent since cell culture parameters (culture density, protein titers) may remain within normal expectations (*1*). Standard infectivity assays designed to show the presence of infectious virus contaminants in bioreactor fluid (e.g., in vitro cytopathic effect or hemadsorption on indicator cell lines) may take weeks to develop. Bioreactor fluids are routinely harvested and released for downstream purification before these test results are complete. Therefore, the downstream purification train may be contaminated before preventive measures can be taken. Should this happen, the affected equipment and raw materials will need to be decontaminated or discarded. Infectivity assays are designed to be generic but may not be sensitive enough to identify the wide range and extremely low level of potential virus contaminants capable of infecting the expression system (*1*−*3*). This raises the remote possibility of undetected virus contamination of the final drug product.

The contamination scenarios described above are not hypothetical. In the past few years, many cases of adventitious virus contamination of cell culture and biotherapeutic products were reported. These incidents occurred randomly at various scales and were detected at various stages of processing the biotherapeutics, even in some final products (Table 1). Contamination sources may include protein supplements (e.g., serum, insulin, trypsin), buffer components (e.g., sugars, salts, vitamins, amino acids), cGMP failure by operators, or equipment barrier breaches.

Contamination Costs. We have constructed cost estimates for the hypothetical viral contamination of a 10,000 L production scale bioreactor (Table 2). These costs are illustrative only and do not represent costs borne by Genentech as a result of virus contamination. The cost of a production batch from seed fermentors to harvesting, associated QC testing costs, and the cost of disposal of contaminated fluid is estimated at $50 per liter. Drug sales associated with the contaminated batch are lost along with other batches that could have been produced during the time spent decontaminating the facility. This represents a considerable opportunity cost. The costs of decontaminating the facility may range from validation of sterility to disposal of components of unit operations (e.g., column resins, filters, pump seals). Regulatory oversight costs include notification of worldwide regulatory agencies and supporting their requests. The managerial costs of supervising/reporting on con-

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Table 1. Adventitious Virus Contamination of Cell Cultures or Final Products of Biotherapeutics[a]

virus	possible source	material
canine parvovirus (CPV)	unknown	live vaccine[b] (*4*)
minute virus of mice (MVM)	unknown	BHK cell culture (*5*)
minute virus of mice (MVM)	FBS	BHK cell culture (*6*)
minute virus of mice (MVM)	raw material	CHO cell culture (*7*, *8*)
minute virus of mice-cutter (MVMc)	unknown	BHK cell culture (*9*)
bluetongue virus (BTV)	unknown	live vaccine (*10*)
epizootic haemorrhagic disease virus (EHDV)	FBS	CHO cell culture (*11*)
bovine viral diarrhea virus (BVDV)	FBS	rIFN-α^b, rIFN-β^b (*12*)

[a] BHK, baby hamster kidney cells; FBS, fetal bovine serum; CHO, Chinese hamster ovary cells. [b] Final product.

Table 2. Cost Estimates for 10,000 L Bioreactor Contamination

batch setup & disposal cost	$0.5 million/batch
opportunity cost of lost sales	$5.0 million/batch
decontamination costs	$0.1−1.0 million/batch
regulatory oversight costs	$0.1−1.0 million/batch
total cost of contamination	$5.7−7.5 million/batch
contamination incidence rate	2 incidents/1000 batches
expected contamination cost	$11,000−15,000/batch or $1.10−1.50/L

tamination-related activities are not explicitly included. The indirect cost includes the time used for disinfecting the contaminated facilities and the resulting shutdown of product manufacturing. While the exact values of these costs are plant- and product-specific, $6−8 million is a fair estimate of the general magnitude of the contamination costs.

One published incidence rate (*7*, *8*) is given as two contaminations out of tens of millions of liters processed or roughly 1 incident per 500 batches at the 10,000 L scale. Another study (*13*) indicated that no contaminations occurred in 3000 batches over 12 years at the 2000 L scale. Informal discussions with fermentation personnel at a number of biotechnology firms indicate that other unreported contaminations may have occurred. Using the total estimated contamination cost per batch and the reported incidence rate, one can calculate a $1.10−1.50 per liter cost of the contaminations spread out over all of the batches. This expected cost represents what a risk-neutral firm might be willing to spend to avoid virus contamination. A risk-averse firm would be willing to spend a per liter cost premium above this rate to avoid the costs of a contamination incident.

Barriers to Virus Contamination in Cell Cultures. In the prevention of virus contaminations arising from contaminated raw materials, a number of approaches may be employed. Raw materials may be screened for the presence of viruses, although this is unlikely to provide sound protection because of the heterogeneity of raw materials and the inherent limitations of sampling. Some components, such as serum, can be irradiated to inactivate potential virus contaminants. This is impractical for large volume components or prepared medium solutions. High or low pH treatment is limited in its effectiveness for inactivating viruses, is not broad spectrum at either pH extreme, and can compromise the quality or osmolarity of the medium. Heat-stable liquid solutions can be autoclaved. Liquid solutions that are not stable to heat sterilization conditions may still be treated using some form of high-temperature, short-time (HTST) heat treatment. Such systems rely on microwave heating (Charm Biotechnology, Malden, MA), ohmic heating (Raztek Corp., Sunnyvale, CA), conventional steam heating (*14*), or UV treatment (Maxwell Technologies, San Diego, CA). These HTST systems are capital intensive and require significant automation and validation to ensure proper treatment of the medium. Another approach, particularly suitable for companies unable or unwilling to commit to the capital expense of HTST systems, would be the use of virus-retentive filters in place of, or in addition to, existing bacterial/mycoplasma-retentive filters. Such a filtration approach would be ideally suitable in providing virus barriers for heat-sensitive feedstreams (protein-containing media, ethanol-solubilized components, etc.) as well as for large-volume applications if the filter capacity were sufficient. Additionally, a filtration approach may be suitable for small to moderate volume tissue culture applications where the cost and complication of HTST systems may not be justified.

The virus barrier filter (VBF) is designed to protect cell culture bioreactors from virus contaminants that may be present in the gas and liquid streams feeding the bioreactor. A typical implementation is shown in Figure 1. This paper addresses filtration of liquid streams. A paper describing filtration of gas streams has been presented elsewhere (*15*).

Filter Performance Specification. The minimum proposed performance requirements of a virus barrier filter are listed in Table 3. These requirements apply to a variety of serum-free fermentation media. The primary requirement is consistent and reliable 3 log reduction value (LRV) of the small 18−20 nm murine parvovirus (MVM). Retention less than this is not worth implementing. Higher clearance levels are anticipated for larger viruses. The filter must not degrade the ability of the serum-free media to support cell growth and expression of protein product. This limits both the adsorptive binding of critical trace media components and the level and toxicity of any extractables and preservatives.

The virus barrier should have a capacity and permeability that allows for filtration of 800 L per 10 in. element in 2 h at 30 psi. This allows the filtration to occur rapidly, before significant bacterial contamination of the prepared medium can occur. It also allows for a reasonable physical sizing of filter housings within existing production areas. Finally, it allows for a reasonable cost consistent with the contamination cost presented earlier.

Filter validation imposes the need for a small-scale version of the filter for spiking studies and a test to ensure integrity at production scale. A single-use capsule or cartridge format with an easy-to-implement integrity test will make this an easy-to-use product on the manufacturing plant floor.

The virus barrier filter cartridge must also be sterilizable to avoid introducing bacterial contaminants. It is also valuable if the virus barrier can be shown to be retentive of mycoplasma and bacteria. In that way, a single device could serve both sterilizing and virus barrier functions, helping to contain costs.

Materials and Methods

Virus Barrier Filter. A series of commercially available and prototype membrane filters was tested. These

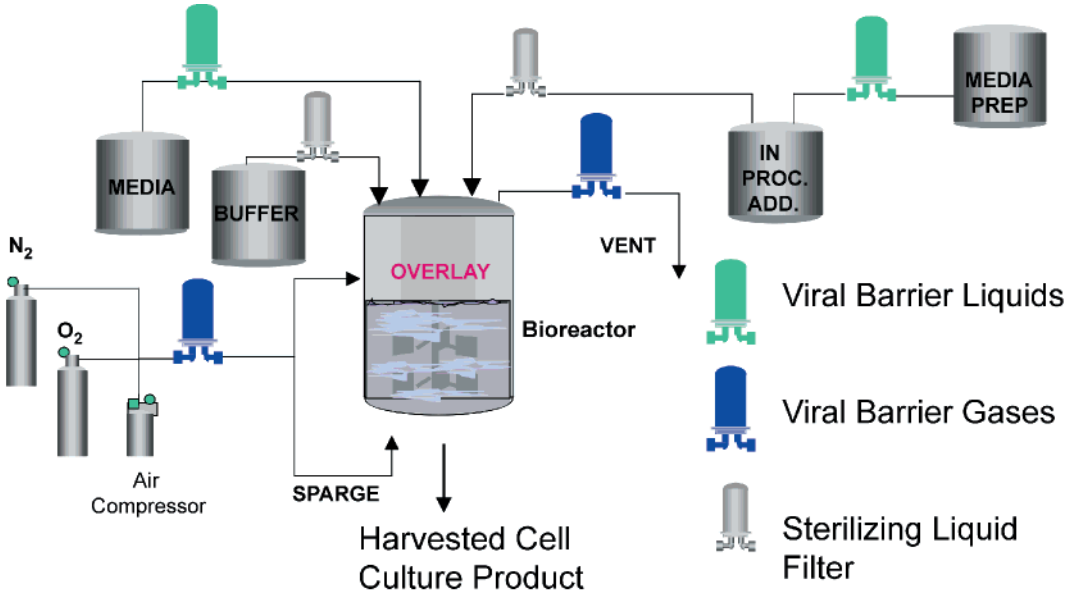

Figure 1. A typical virus barrier implementation scheme.

Table 3. Virus Barrier Specifications

≥3 LRV of MVM
no media degradation
800 L in 2 h at 30 psi per 10 in. filter
scale-down version available for spiking studies
integrity test to ensure retention
easy-to-use
sterilizable
sterilizing

filters included a variety of structures (microfilters, ultrafilters, and composite ultrafilters), base polymer compositions (PVDF, PES, regenerated cellulose), and surface modifications (hydrophilic and hydrophobic). The membrane selected for the virus barrier filter consists of a composite ultrafiltration membrane. This composite membrane does not have large voids within and below the surface, which can compromise physical strength and retention (*16*). It is composed of hydrophilic regenerated cellulose. The membrane is pleated and manufactured into 4 in. prototype cartridges. Performance testing was done using 47 mm disks or 4 in. cartridges, containing 1.5 ft^2 of membrane. Each device was run in a normal flow operating mode.

Cell Culture. Three cell culture media, Medium A, Medium B, and Medium C were used for virus challenge tests, filter capacity tests, cell culture tests, and protein retention studies. All media are serum-free and protein-free, except where indicated for protein retention experiments. They are based on the common DMEM/F12 formulation. Medium A contains animal-derived protein hydrolysate at a concentration of 0.5% (w/v). Medium B is hydrolysate-free. Medium C is a concentrated form of Medium A and is used as a nutrient supplement during the course of the cell culture production runs. Media A and B contain Pluronic F-68 (BASF, Mt. Olive, NJ) at a concentration of 1 g/L. Cell culture Medium D is similar to Medium B, serum- and hydrolysate-free, and is used for MVM dilution during infectivity assays.

The media used for cell culture experiments were filtered through the 4 in. (1295 cm^2) VBF prototype device. The devices were immersed in water and autoclaved prior to use at 121 °C for 30 min on a liquid cycle. Approximately 50 L of Media A and B were processed through each 4 in. capsule via constant pressure filtration at 5−10 psig. Approximately 10 L of Medium C was processed through a 4 in. device using a peristaltic pump. All media (control and VBF test) were passed through sterilizing-grade 0.1 μm filters prior to use.

ΦX174 Experiments. ***Materials.*** Bacteriophage mutant ΦX174 (ΦX cs 70 am −3) and *Escherichia coli* (HF4714) bacterial host strain were supplied by Dr. C. E. Dowell of Promega Corporation (Madison, WI). Host culture suspensions were inoculated 18 h prior to assay procedure. Components for Sorensen's phosphate buffer solution (pH 7.3), bottom plate agar, and overlay molten agar were obtained from Fisher Scientific (Pittsburgh, PA). Standard 16 mm × 100 mm and 16 mm × 150 mm test tubes and 15 mm × 100 mm Petri dishes were obtained from Fisher Scientific (Pittsburgh, PA). Pipets measuring from 100 to 1000 μL with standard tips were used for fluid transfer. The system included a 5 L pressure vessel (Millipore, Bedford, MA) equipped with a pressure gauge. The ΦX174 challenge suspension was prepared at 1 × 10^7 pfu/mL using either Sorensen's buffer (plus 1 g/L Pluronic F-68) or Medium B.

Assay Procedure. The samples extracted from the filtration system were diluted 1:10 in concentration using 10 mL Sorensen's buffer tubes. Final dilution tubes were plated in triplicate using 0.10 mL of sample and 0.10 mL of host culture suspension and vortexed in 3 mL of phage overlay molten agar (overlay stored in block heater at 46−48 °C). This mixture of phage and host was transferred to phage agar, plated, and incubated at 37 °C overnight. Plaque counts were obtained using a Manostat colony counter (Manostat, Barrington, IL). Only the dilution tubes that yielded plaque counts of 30−300 were used for the LRV determination, as plaque counts outside of this range are not statistically significant. The mean and standard deviation were calculated to determine LRV using eq 1 with concentration units expressed in pfu/mL.

$$\text{LRV} = \text{Log}_{10}\left[\frac{\text{feed sample (upstream) concentration}}{\text{filtrate sample (downstream) concentration}}\right] \quad (1)$$

Autoclave Study. The VBF cartridges were evaluated for the ability to maintain LRV after autoclaving. These experiments were run using ΦX174 challenge solution prepared at 1 × 10^7 pfu/mL in Sorensen's buffer with

Pluronic F-68 (1 g/L). The disks and cartridges were wet with 2-propanol (70% v/v) and flushed with water prior to autoclaving. Cartridges were placed in Optiseal housings (Millipore, Bedford, MA), and disks were in stainless steel filter holders. The devices were autoclaved on a liquid cycle at 125 °C for 59 min.

Minute Virus of Mice (MVM) Experiments. ***Materials.*** Chemicals and enzymes were from Sigma (St. Louis, MO), Boehringer Mannheim (Indianapolis, IN), and Perkin-Elmer (Foster City, CA). The cell culture media were from the Genentech medium preparation facility (South San Francisco, CA) and Gibco Life Biotechnology (Gaithersburg, MD). Stocks of MVM were obtained from MA Bioreliance (Rockville, MD).

Challenge with Minute Virus of Mice (MVM). MVM was partially purified by differential centrifugation procedures and resuspended in a 10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 7.5 buffer. The purified virus was stored at −70 °C before challenge experiments. Approximately 1 or 2 L of serum-free cell culture Medium B containing 0.1% Pluronic F-68 was spiked with partially purified MVM at a ratio of 1:100. This initial load was first prefiltered with a small pore size filter (Millipore, Bedford, MA) to create a prefiltrate containing only monomeric MVM particles. This prefiltrate was then passed through the 4 in. VBF cartridge. Samples were collected from the load, prefiltrate, and VBF filtrates. These samples were assayed by the microplate 50% tissue culture infectivity dose ($TCID_{50}$) endpoint assay and a real-time quantitative polymerase chain reaction (PCR) assay.

Samples from filter challenges were assayed by microscopic examination of virus-caused cytopathic effect (CPE) on 96-well cell culture plates. Human newborn kidney cells, NB324K cells (kindly provided by Dr. Peter Tattersall, Yale University, New Haven, CT), were grown in the plate to approximately 20−30% confluence prior to virus inoculation. Samples assayed undiluted and in 1:10 serial dilutions with cell culture Medium D were inoculated into the plates in six replicates and absorbed for 90−120 min. The plates were fed with MEM (Gibco BRL, Gaithersburg, MD) supplemented with a trace element mixture, 2.8 μg/mL linoleic acid, 2 mM glutamine, 50 μg/mL recombinant human insulin (rhInsulin), and 50 μg/mL gentamycin. The inoculated plates were examined at 7 and 11 days postinfection. Virus titer was calculated according to the standard Karbar method. The removal of MVM was calculated according to eq 1 with concentrations expressed in units of $TCID_{50}$/mL.

A real time quantitative PCR assay was established specifically for detecting MVM genomic copy number. For MVM, one copy equals one virion in the absence of any free DNA fragments. The assay uses fluorogenic 5′-nuclease chemistry called Taqman PCR (MVM TM-PCR) (*17*) to determine the removal of intact virus particles by the VBF cartridge. A portion of sample from the load, prefiltrate, and VBF filtrates was analyzed. The mechanism of the assay is described in the literature (*18*, *19*). To purify single-stranded DNA of MVM, a 700 μL sample of each was incubated at 37 °C for 30 min in the presence of 2.5 μL of DNase I (10 U/μL, Boehringer Mannheim, Indianapolis, IN) and 10 mM $MgCl_2$. The reaction was terminated by adding 700 μL of phenol/chloroform/isoamyl alcohol (25:24:1) with rigorous mixing for at least 30 s. Approximately 700 μL of the aqueous phase was transferred to a fresh sterile tube after centrifugation at 14,000 rpm for 10 min at 4 °C. The centrifugation step was repeated, and 650 μL of the aqueous phase was transferred to another fresh sterile tube containing 72 μL of 3 M sodium acetate. The contents were mixed well and precipitated with 650 μL of 100% isopropyl alcohol (IPA) at −20 °C overnight. Virus DNA was pelleted by centrifugation at 14,000 rpm for 30 min (4 °C) and washed once with cold (−20 °C) 70% ethanol. After complete drying of the tube on a 50 °C heat block for approximately 30 min in a laminar flow hood, 65 μL of TE (10 mM Tris-HCl, pH 8, 0.1 mM EDTA) was added to solubilize the invisible DNA pellet.

MVM TM-PCR of each sample was performed in triplicate on a clean 96-well plate designated for thermocycling amplification on the ABI Prism 7700 Sequence detector (PEB) (Perkin-Elmer, Foster City, CA). Ten microliters of the purified sample were added to 40 μL of reaction master mix per tube. The master mix consisted of 6 mM $MgCl_2$; 250 μM of dATP, dCTP, dGTP, and 600 μM dUTP; 3 μM Primer mix of forward and reverse primers; 1 μM probe; and 2.5 units of Amplitaq Gold Taq polymerase. The probe was covalently labeled at the 5′ end with a fluorescein, FAM (Reporter dye), and at the 3′ end with a Rhodamine derivative, TAMRA (quenching dye). Taq polymerase can remove the complementarily hybridized probe via its 5′ → 3′ exo-nuclease activity. The thermocycling amplification was controlled at 50 °C for 2 min and 95 °C for 10 min followed by 50 cycles at 95 °C for 15 s and 60 °C for 1 min. The raw data were analyzed with the software installed on the Prism 7700 sequence detection system (version 1.6.3). The virus levels in the filtration load and filtrates were expressed as particles/mL. The reduction factor was calculated according to eq 1.

Bacteria and Mycoplasma Experiments. ***Materials.*** *Brevundimonas diminuta* (American Type Culture Collection ATCC #19146) was grown in saline lactose broth for 24 h at 30 °C. Peptone (Difco, Detroit, MI) was prepared by dissolving 1 g of peptone per liter of water and was sterilized by autoclaving at 121 °C for 30 min. Tests were run in accordance with standard validation testing (*20*). The membranes were sanitized by soaking in sterile ethanol (70% v/v) prior to loading aseptically into preautoclaved holders. The integrity of the filters was determined by a pressure hold test immediately before and after the retention tests, as follows. Sterile silicone tubing (Millipore, Bedford, MA) was attached downstream of the test holders. The other end of the silicone tubing was placed in a beaker of sterile water. Sterile air was applied to the upstream side of the membrane at a constant pressure of 30 psi for several minutes. The integrity of the filter was indicated by the absence of bubbles from the silicone tubing. After the pressure hold test, the filters were flushed with 200 mL of sterile water to remove the ethanol.

The retention test was performed by passing 100 mL of the *B. diminuta* challenge suspension through each filter at 30 psi. The filtrate was passed through 0.45 μm assay filters (Millipore, Bedford, MA) and transferred to tryptic soy agar (TSA) plates (Binax, Waterville, ME) and subsequently incubated at 30 °C. The plates were examined at 2 and 7 days for colonies.

Acholeplasma laidlawii (American Type Culture Collection ATCC #23206) was used for the mycoplasma experiments. Forty-eight hours prior to the challenge test, 50 mL of fortified commercial broth, FCB (Difco, Detroit, MI), was inoculated with *A. laidlawii* stock. This seed culture was incubated at 37 °C, 7% CO_2 for 18−24 h. After incubation, 100 mL of modified Hayflick broth (*21*) was inoculated with 10 mL of the seed culture. The Hayflick culture was incubated at 37 °C, 7% CO_2, for 18−

24 h. The challenge inoculum was prepared by diluting the Hayflick culture into sterile mycoplasma buffer.

The empty stainless steel filter holders and reservoirs were autoclaved at 121 °C for 60 min. Swinnex filter holders (Millipore, Bedford, MA) were loaded with 0.22 μm GSWP filters (Millipore, Bedford, MA) and autoclaved at 121 °C for 20 min. After cooling overnight, the test apparatus was aseptically assembled in a laminar flow hood (Baker Company, Sanford, ME).

The VBF filters were wet with isopropyl alcohol (70% v/v) prior to being loaded into the filter holders. Two hundred milliliters of sterile reagent grade water was used to flush the alcohol for each filter. Pressure hold tests, at 30 psi, were done before and after the challenge tests as a means of detecting gross leaks or defects. A sterility check of the test system was conducted prior to the challenge tests, as follows. One hundred milliliters of sterile mycoplasma buffer (8.64 g/L Na_2HPO_4 and 3.98 g/L NaH_2PO_4) was passed through each test filter at 15 psig. The filtrates were passed through GSWP filters, which were then removed from the Swinnex filter holders in a laminar flow hood and plated onto Fortified Commercial Agar, FCA, (Difco, Detroit, MI). The plates were incubated at 37 °C, 7% CO_2 for 6−7 days. One hundred milliliters of the diluted Hayflick challenge culture was passed through each filter at 30 psig. The filtrates were assayed on GSWP filters as described above. Following the challenge filtration, each filtration system was flushed with 100 mL of sterile mycoplasma buffer, which was subsequently assayed on GSWP filters as described above.

After incubation, each assay filter was stained with Dienes Stain (Remel, Lenexa, KS) and examined for the presence of mycoplasma colonies by viewing under 40× magnification. The results are reported in terms of LRV, as defined by eq 1.

Cell Culture Experiments. The cell culture experiments were performed using standard suspension cell culture practices with a Chinese hamster ovary (CHO; K1-derived) cell line expressing a humanized monoclonal antibody. The cells were passaged twice in both control and VBF-filtered test medium (these passages denoted as "inoculum train"). The cells were then inoculated into production cultures. The inoculum cultures were performed in duplicate 20 L stainless steel bioreactors operated at 15 L working volume. The production cultures were performed in duplicate 2 L glass bioreactors operated at a working volume of 1.5 L.

Inoculum cultures (Medium A) were controlled at pH 7.15 via CO_2 sparging and/or 1 M Na_2CO_3 addition. Dissolved oxygen was controlled at 30% of air saturation via sparging of air and/or oxygen. Temperature was maintained at 37 °C. Cultures ran for approximately 3 days before transfer to the next inoculum train passage or to production cultures.

Production cultures (Medium A initial batch) were controlled at pH 7.15 via CO_2 sparging and/or 1 M Na_2CO_3 addition. Dissolved oxygen was controlled at 60% of air saturation via sparging of air and/or oxygen. Temperature control began at 37 °C and shifted to 33 °C approximately 2 days into the 12 day production culture. Medium C was added as a nutrient supplement to the production culture at 125 mL/L approximately 3 days post-inoculation. Additional glucose was added as necessary to prevent glucose exhaustion.

All cultures were monitored daily for viable cell number and percent viability by trypan blue exclusion and for packed cell volume (expressed as a percentage of culture volume). Production culture supernatant samples were analyzed for antibody concentration via a Protein-A HPLC method.

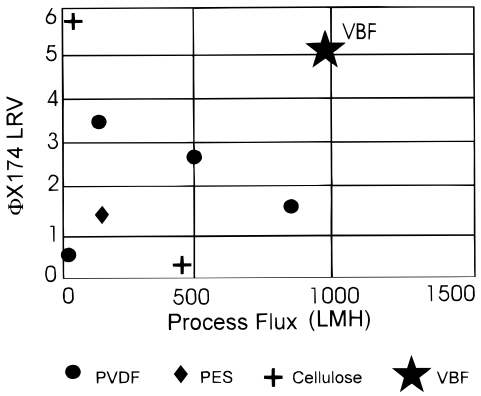

Figure 2. Selecting the membrane for the VBF.

Protein Retention Experiments. For protein retention studies, rhInsulin, (MW = 6 kDa) or bovine apo-transferrin (MW = 76−81 kDa) were added to cell culture medium at approximately 10 mg/L. The media solutions were then subjected to standard VBF filtration test conditions, as described in the filter sizing section. Filtrations were performed twice for rhInsulin and three times for bovine apo-transferrin. Filtrate samples were collected at various time points and from the pooled filtrate. The insulin was assayed by an in-house ELISA method (coefficient of variation approximately 20%), and the apo-transferrin was assayed by the modified Biuret (Sigma, St. Louis, MO) assay (coefficient of variation approximately 10%).

Filter Sizing Experiments. Virus barrier filter disks were wet by immersion in ethanol (70% v/v) and transferred with forceps onto a layer of polypropylene nonwoven support material into a 47 mm holder. The 47 mm holder was sealed and attached to a pressure vessel containing the feed material. The pressure was adjusted, and the system was tested to ensure that it was integral. The time was recorded with a stopwatch as soon as the inlet valve was opened. The filtrate was collected in a graduated cylinder or tared collection vessel.

Results and Discussion

Membrane Screening. A series of membranes with different compositions and retention ratings was selected and compared with the performance specifications listed in Table 3. For any given membrane family, an increase in nominal pore size is accompanied by a drop in virus retention and a rise in permeability. Small-scale tests of these membranes with ΦX174 in buffer resulted in the performance map shown in Figure 2. The membrane selected as the virus barrier filter met the performance targets and demonstrated superior performance relative to all other types of membranes tested.

ΦX174 Retention Experiments. The sensitivity of the ΦX174 clearance by the VBF to changes in processing parameters was evaluated to determine critical variables, worst case conditions, and robustness. As shown in Figure 3, the presence of Pluronic F-68 appears to lower ΦX174 clearance and was included in all further testing. Nevertheless, the VBF consistently showed log reduction values greater than the target of three, independent of the presence of Pluronic F-68 (Figure 3). The ΦX174 clearance was not significantly influenced by membrane lot-to-lot variation (Figure 4) for each type of cell culture media. In all cases, the LRV was greater than the target of three.

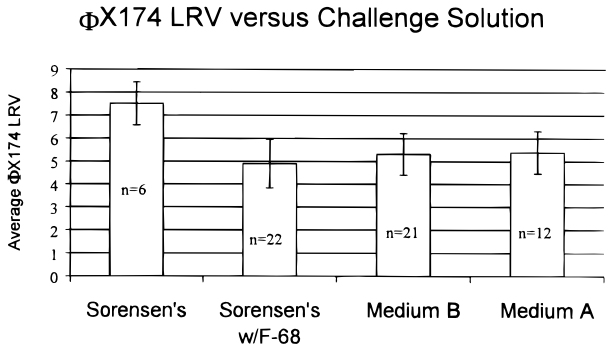

Figure 3. LRV insensitivity to solution type with Pluronic F-68 present (n = number of disks tested; bars = ± one standard deviation from mean). The 47 mm VBF device was tested at 10 psi using 100 mL of 1×10^7 pfu/mL challenge solution.

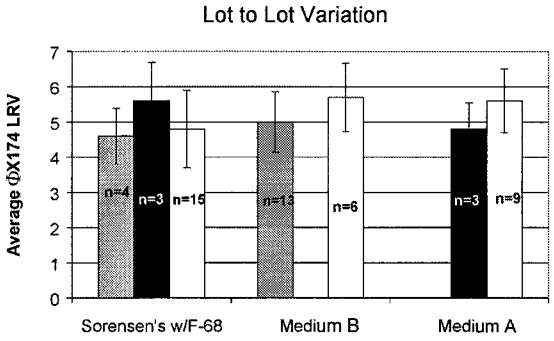

Figure 4. LRV insensitivity to membrane lot (n = number of disks tested; bars = ± one standard deviation from mean). The 47 mm VBF device was tested at 10 psi using 100 mL of 1×10^7 pfu/mL challenge solution. Lot 1 is shown as shaded □, lot 2 is shown as ■, lot 3 is shown as unshaded □.

To evaluate virus clearance for batch processing, the ΦX174 concentration in the filtrate was sampled at various points from 1 to 100 mL/cm^2 throughout the process. Two media types were tested yielding a LRV for ΦX174 of greater than three (Figure 5). The ΦX174 LRV increased with process volumes so that small volumes represent a worst case challenge. This behavior is consistent with the Viresolve composite membrane (Millipore, Bedford, MA) run in TFF mode but differs from that reported for a cellulosic hollow fiber membrane (*22*). As shown in Figure 6, challenge pressures of 10 and 30 psi made little difference in the ΦX174 clearance. At both pressure levels, the LRV was greater than 4.5 and a two-sided t test indicates equivalency ($p = 0.5$). To evaluate the ability to manufacture the VBF membrane into a pleated cartridge, two membrane lots and two media were used to compare ΦX174 LRV obtained using flat stock membrane disks with LRV obtained using pleated cartridges. The LRV data from disks and cartridges were compared using a two-sided sample t test and found to be equivalent (Figure 7) (lot 1 $p = 0.92$ and lot 2 $p = 0.57$). This qualifies the 47 mm disk as a scale-down model for virus spiking.

As listed in Table 3, virus barrier filters must be sterilizable to avoid contamination of cell culture media. Heat sterilization of cartridges was investigated using an autoclave. The data in Figure 8 show that, using Sorensen's medium with Pluronic F-68, the average virus clearance of cartridges without autoclaving was statistically equivalent to the average of those that had been autoclaved ($p = 0.78$). Two additional membrane lots (data not shown) confirmed these results ($p = 0.92$, $p = 0.57$).

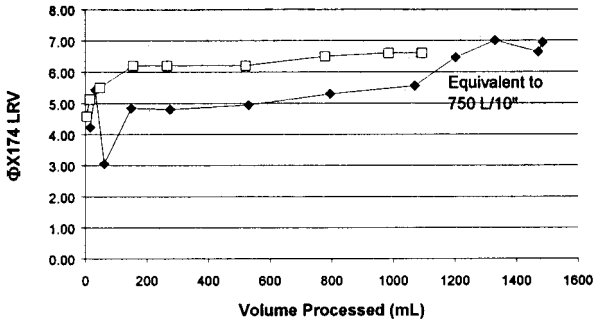

Figure 5. No drop in LRV with volume processed. The 47 mm VBF device was tested at 10 psi using 100 mL of 1×10^7 pfu/mL challenge suspension. Medium B is shown as ◆, and Sorensen's is shown as □.

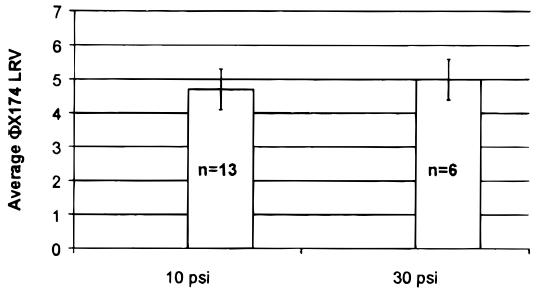

Figure 6. LRV is independent of pressure (n = number of disks tested; bars = ± one standard deviation from mean), 100 mL of 1×10^7 pfu/mL ΦX174 challenge solution in Medium B using 47 mm VBF device.

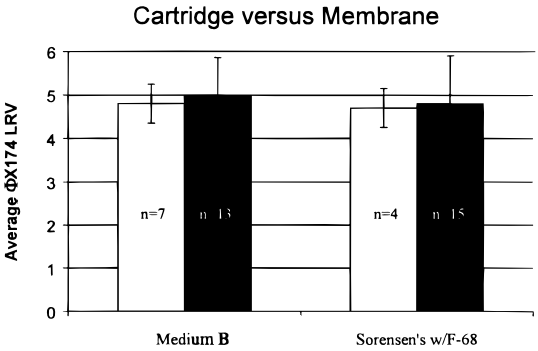

Figure 7. VBF in membrane disks (47 mm shown as □) or Optiseal cartridges (shown as ■) produced similar LRVs using different media using 100 mL of ΦX174 challenge solution at 1×10^7 pfu/mL for the 47 mm disk and 1500 mL of challenge suspension at 1×10^7 pfu/mL for the Optiseal cartridge at 10 psi (n = number of disks tested; bars = ± one standard deviation from mean).

MVM Retention Experiments. Prefiltration of the MVM-spiked cell culture medium at 10 psi removed 1.8−2.8 logs of virus aggregates, depending on the specific preparation. The prefiltrates containing monomeric MVM were immediately filtered through the VBF cartridge. The filtrates of each test were divided into three major fractions. The first (F1) and the last (F3) fractions were 10 mL each, and the second (F2) fraction was the pool (>950 mL). Under filtration pressures of 5 and 10 psi, flow rates were 1.12 and 1.5 L/min, respectively, for virus-spiked media using the 4 in. Optiseal cartridge. A low infectivity, $\leq 10^{0.83}$ $TCID_{50}$/mL, was detected in the undiluted filtrate fractions, demonstrating a significant removal of MVM from the filtration pool by the VBF cartridge. This corresponded to a reduction of $10^{3.2}$-fold of the nonaggregated particles determined by both infectivity and virus genomic DNA copy numbers (Figure 9). No difference in virus clearance was observed in the

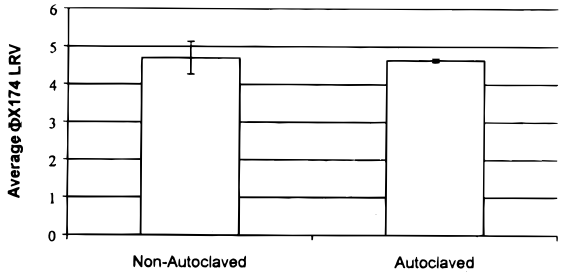

Figure 8. VBF cartridge clearance unchanged by autoclaving using a 4 in. VBF cartridge (bars = ± one standard deviation from mean) at 10 psi, using a ΦX174 challenge solution of 1×10^7 pfu/mL. Autoclaved for 1 h at 126 °C while submerged in water.

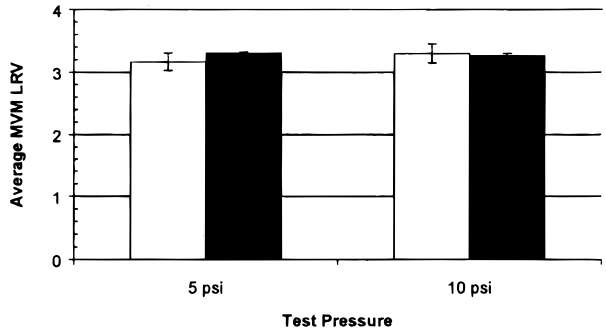

Figure 9. Removal of monomeric MVM particles from cell culture Medium B at different filtration pressure conditions. The $TCID_{50}$/mL is shown as □, and the MVM TM-PCR is shown as ■.

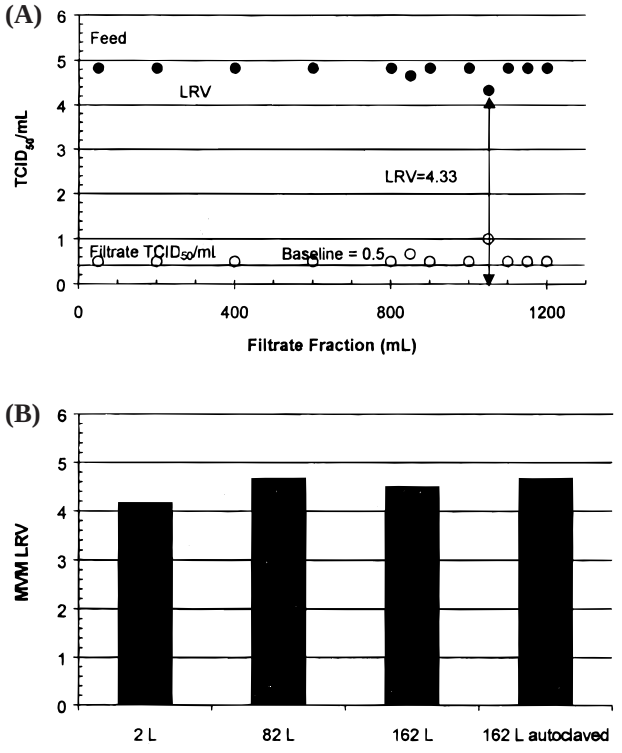

Figure 10. Throughput of VBF in removing MVM from cell culture Medium B as detected by NB324K $TCID_{50}$ assay. (A) 47 mm disk. (B) 4 in. cartridge.

filtration at 5 and 10 psi, although the flux increased with increasing pressure. These results demonstrate that the VBF removed significant levels of monomeric MVM from serum-free cell culture medium, regardless of the operational pressure or flowrate. Since removal of monomeric MVM was demonstrated, no prefiltration was used in subsequent testing.

Both 47 mm disks and 4 in. Optiseal cartridges were evaluated for MVM removal over a range of processed volume. For the disk test, 1200 mL of cell culture medium spiked with MVM (1:100) was prefiltered with a 0.1 μm Durapore VVLP membrane filter (Millipore, Bedford, MA) to remove nonviral debris that could plug the filter. This prefiltered suspension was then passed through a VBF 47 mm disk housed in a custom-designed double O-ring holder (Millipore, Bedford, MA). The flow rate was maintained at 7.7 mL/min under 10 psi pressure. As shown in panel A of Figure 10, no infectious virus was detected in either of the 800 mL filtrate fractions. Compared to MVM in the load, 5.33 logs in the cell culture medium, a significant reduction, $\geq 10^{4.83}$-fold, of infectious virus was achieved by the VBF membrane. With increasing filtration volume, a weak cytopathic effect, highest (1 log) infectivity of MVM was detected only in the undiluted filtrate fractions at 1100 mL. No MVM was detected in the other filtrates. At maximum throughput of 1200 mL or 800 L/m^2, the VBF membrane disk removed MVM by $10^{4.33}$-fold.

To confirm that the scaled-up filter device also removes MVM, 4 in. Optiseal cartridges were challenged after various treatments. Cartridges were challenged with either (1) 2 L of MVM-spiked load; (2) MVM spike after filtration with 80 L of cell culture Medium B; (3) MVM spike after filtration with 160 L of cell culture Medium B; or (4) MVM spike after filtration of 160 L of Medium B using a cartridge autoclaved for 60 min on a liquid cycle (121 °C). The virus load was 5.0−5.5 log $TCID_{50}$/mL, and the flow rate ranged from 500 to 1143 mL/min at a constant pressure of 10 psi. As shown in panel B of Figure 10, filtration with the 2 L challenge resulted in $10^{4.2}$-fold reduction of MVM infectivity, indicating that the fabricated cartridge gave consistent virus retention. Further studies showed that filtration yielded $10^{4.7}$- and $10^{4.5}$-fold reduction postfiltration with 80 and 160 L of cell culture media. Therefore, the volume of medium that can be filtered with high MVM removal capacity by the VBF cartridge is at least 160 L/4 in. cartridge, equivalent to 880 L/m^2. Furthermore, no decrease of virus reduction was observed during the filtration with the autoclaved cartridge (Figure 10B).

Mycoplasma and Bacteria Retention Experiments. In addition to the studies focusing on the small MVM and ΦX174, the VBF was challenged with mycoplasma and bacteria. A 100 mL challenge at 5.9×10^6 cfu/mL of *A. laidlawii* mycoplasma demonstrated complete removal for a reported LRV of ≥8.8. A 100 mL challenge at 1.5×10^7 cfu/mL of *B. diminuta* also demonstrated complete removal for a reported LRV of ≥9.2.

Retention Mechanism. Figure 11 shows the retention of viruses and other microorganisms by the VBF as a function of size. Both MVM and ΦX174 are icosahedral in shape. They were sized by electron microscopy as having diameters of 20 and 28 nm, respectively. *A. laidlawii* is spherical, deformable, and roughly 300 nm in diameter by electron microscopy. *B. diminuta* is cylindrical, 400 nm in diameter by 800 nm in length by electron microscopy. The data in Figure 11 show that size plays a role in the magnitude of the reduction obtained, consistent with a size-exclusion mechanism.

Cell Culture Experiments. The results of the multiple-passage cell culture experiments are shown in Figures 12−14. It is seen that comparable growth and viability are achieved over two inoculum train passages and the production culture using medium processed

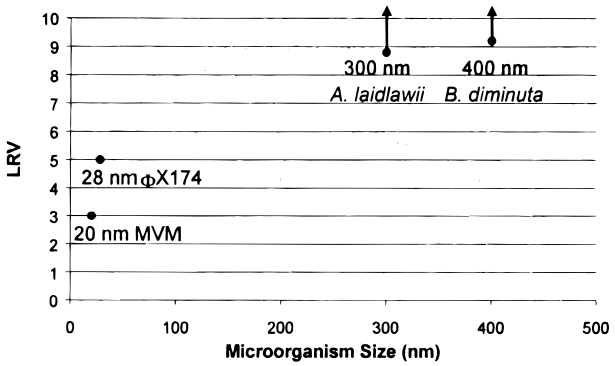

Figure 11. Virus barrier filter displayed excellent LRV for a broad range of organisms.

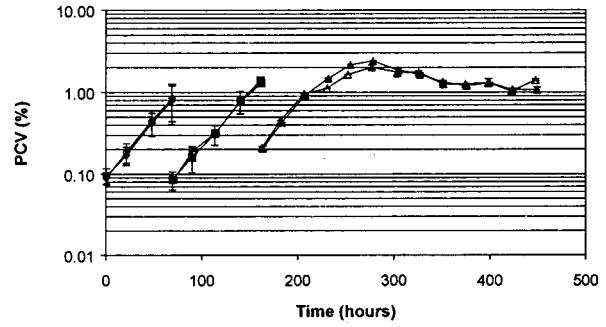

Figure 12. Growth comparison on packed cell volume (PCV) basis for inoculum and production stages. Three stages are shown, with unfiltered stage 1 shown as ●, unfiltered stage 2 shown as ■, unfiltered stage 3 shown as ▲, VBF filtered stage 1 shown as ○, VBF filtered stage 2 shown as □, and VBF filtered stage 3 shown as △.

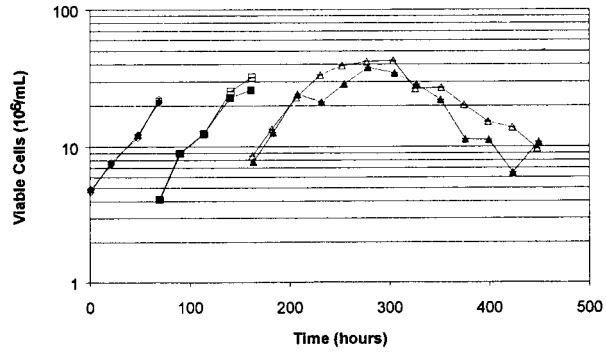

Figure 13. Growth comparison on viable cell number basis for inoculum and production stages. Three stages are shown, with unfiltered stage 1 shown as ●, unfiltered stage 2 shown as ■, unfiltered stage 3 shown as ▲, VBF filtered stage 1 shown as ○, VBF filtered stage 2 shown as □, and VBF filtered stage 3 shown as △.

through the VBF. Other measures of metabolic activity (glucose consumption, lactate production) were also comparable (data not shown) for all cultures. Antibody concentration at the end of the production culture was 101 ± 3% of control for the VBF medium (Figure 15). It was concluded that the use of the VBF device does not impact cell culture performance.

Protein Passage Experiments. The results of the protein retention studies are shown in Figure 16. Despite the variability in the data, it is seen that no significant retention occurs for either rhInsulin or bovine apotransferrin.

Filter Sizing Experiments. As stated earlier, the virus barrier must have a capacity and permeability such that the filtration process occurs rapidly with reasonable

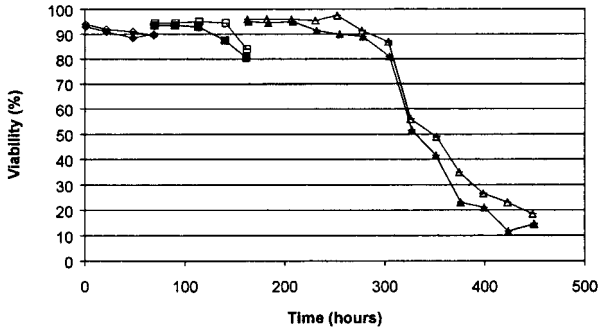

Figure 14. Culture viability comparison for inoculum and production stages. Three stages are shown, with unfiltered stage 1 shown as ●, unfiltered stage 2 shown as ■, unfiltered stage 3 shown as ▲, VBF filtered stage 1 shown as ○, VBF filtered stage 2 shown as □, and VBF filtered stage 3 shown as △.

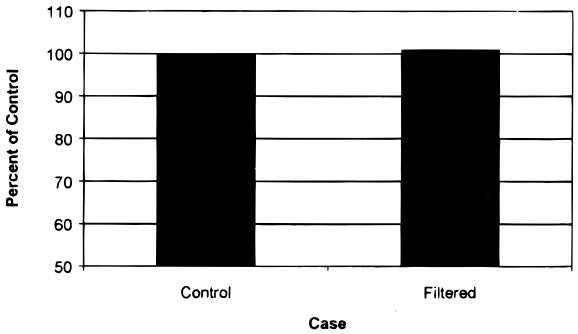

Figure 15. Comparison of antibody production by filtered media and unfiltered (control) media.

physical sizing at a reasonable cost per liter of medium. The capacity and flow characteristics of the VBF membrane were characterized by measuring the volume filtered as a function of time under constant pressure conditions. The data from these experiments were analyzed using four separate filter-blocking models (*23*): cake (eq 2), standard or gradual blocking (eq 3), complete blocking, and intermediate blocking. The cake and standard blocking models, as described below, fit the data best with R^2 values of 0.999 and 0.998, respectively.

Cake filtration: build up of particles on the surface of the filter rather than in the pores

$$t/V = K_c/2 \cdot V + 1/q_0 \qquad (2)$$

Standard blocking: particles collect on the sides of the filter pore and gradually occlude it

$$t/V = K_s/2 \cdot t + 1/q_0 \qquad (3)$$

Figure 17 is an example of the experimental capacity data for unfiltered fresh Medium A, run at 28 psid on a 13.8 cm^2 VBF membrane filter disk. When using these filter-blocking models to extrapolate filter performance, the slope of the fitted line must be stable. These experiments suggest that the typical 15−20 min test was too short for the slope to stabilize. Sizing based on these short-time results would be larger than needed. A 60 min test yielded a better slope and prediction of the required sizing.

On the basis of this type of analysis, the amount of fluid processed at 28 psid during the specified 2 h time limit can be predicted. Figure 18 illustrates the predicted curves for the four types of plugging models. All of the models predict approximately 950 L/m^2 will be filtered

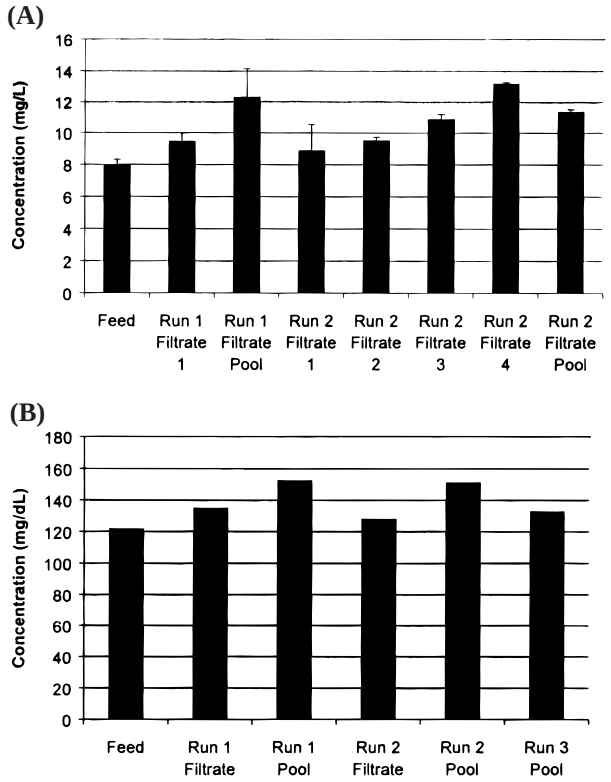

Figure 16. Protein retention in VBF filtered cell culture medium. (A) rhInsulin concentration of feed sample, filtrate samples after 50 mL filtration increments and sample from pooled filtrate through 47 mm VBF at 20 psig. Bars represent range of two assays taken from each sample. (B) Transferrin concentration of feed sample, filtrate samples after 50 mL filtration increments and sample from pooled filtrate through 47 mm VBF at 20 psi.

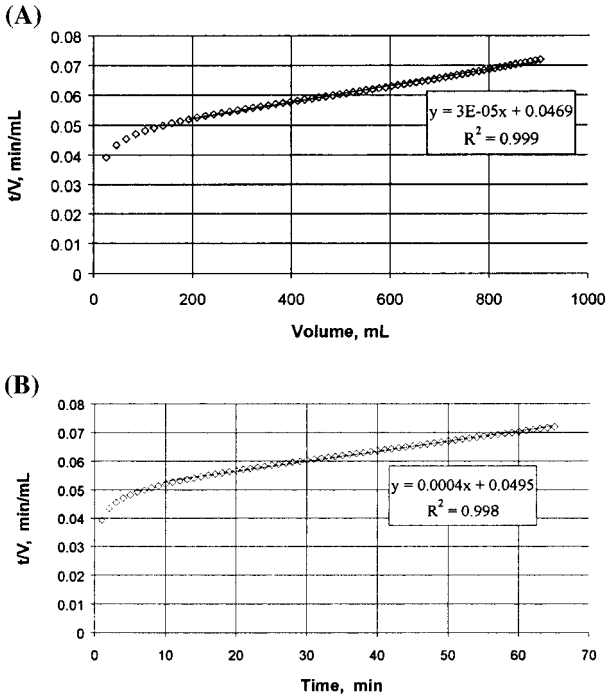

Figure 17. Filter blocking methods used to estimate VBF capacity. (A) Cake filtration model. (B) Standard blocking model.

within the 2 h. Therefore, to process the required 800 L, a 10 in. device will require a reasonable 0.85 m^2 of surface area.

It was also found that the components and quality of the feed solution greatly influence the filter sizing. A less-refined protein hydrolysate, substituted in Medium A, reduced capacity by as much as 50-fold. Feed pressure also influenced filter sizing. The predicted sizing did not have a direct linear relationship with pressures from 10 to 30 psi (Figure 19). This indicates that the higher the flux of the filter, the higher the plugging rate. Feed pressure can be increased to reduce filter area required until the capacity of the filter becomes the limiting factor.

Tests were also performed on Medium B resulting in similar sizing and behavior (data not shown). Prefiltration of the feed solution was not examined in this study but may play a significant role for some feed solutions.

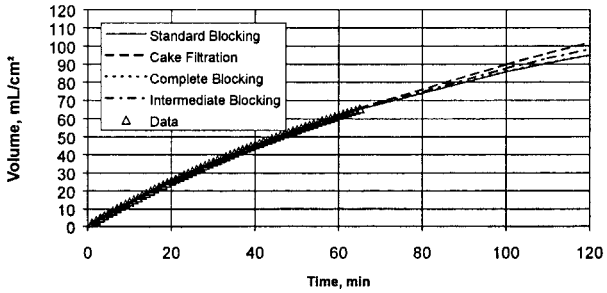

Figure 18. Extrapolated data from four filter blocking models showing that approximately 95 mL/cm^2 would be filtered within 2 h.

Conclusions

A VBF concept was defined to meet the need for prevention of bioreactor contamination. A prototype filter product was then developed and qualified to meet the performance specifications listed in Table 3. Robust retention of >3 LRV of viruses was demonstrated over a range of feedstocks, membrane lots, operating conditions (autoclaving, pressure, volume/area loading), and virus types. Retention occurs primarily by a robust size-exclusion mechanism.

Filtered cell culture media were equivalent to unfiltered media in their ability to support cell growth and expression of a recombinant protein product. Insulin and apo-transferrin protein supplements were not significantly retained by the VBF.

Filter sizing studies demonstrated the capability of the virus barrier filter to process 800 L of fresh serum- and protein-free cell culture media in 2 h with a 10 in. cartridge at 30 psi. Filter capacity is sensitive to media components, and care must be taken in sourcing and prefiltering these components. This filter sizing enables rapid, economical operation.

Scale-down 47 mm disks are shown to give equivalent virus retention to pleated cartridges. They are hence qualified for validation clearance studies.

A preliminary diffusion-based integrity test has been developed for the virus barrier filter. This integrity test shows good sensitivity in predicting ΦX174 LRV in disks and cartridges (data not shown) and is consistent with standard manufacturing operations. A complete discussion of this test and its performance will require additional cartridge fabrication and testing.

The VBF is an easy-to-use device for preventing bioreactor contamination. The cartridge format, moderate sizing, and easy-to-use integrity test permits rapid installation and testing. Single use operation avoids reuse/cleaning validation. Preuse autoclaving conveniently maintains system sterility.

The capability of the VBF to completely retain bacteria and mycoplasma was demonstrated. This enables the

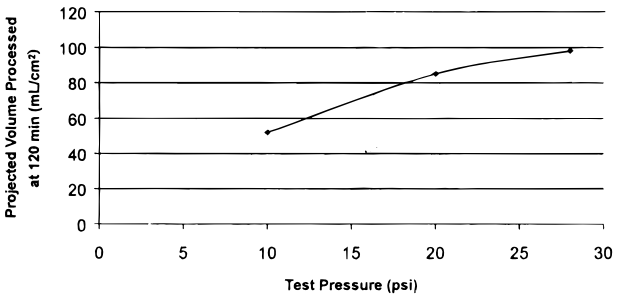

Figure 19. Increasing pressure increases volume processed for 2 h run.

VBF to potentially replace 0.1 or 0.2 μm sterilizing filters. Additional work correlating the integrity test with retention of these microorganisms is required to permit a sterilizing claim and enable this filter replacement.

This project was a technical collaboration between Millipore Corp. and Genentech, Inc., in defining, developing, and testing this new technology. The collaborative approach between customer and supplier is an effective one in reducing development cycle times.

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Notation

K_c	cake filtration constant (min/mL^2)
K_s	standard blocking constant (1/mL)
q_0	initial flowrate through filter (mL/min)
R^2	correlation coefficient
t	elapsed filtration time (min)
V	cumulative filtrate volume (mL)

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