Use of a Noninfectious Surrogate to Predict Minute Virus of Mice Removal During Nanofiltration

David Cetlin
MockV Solutions, Inc., RD 22, Baltimore Road Rockville, Maryland, 20850

Melanie Pallansch
Texcell N.A., 4991 New Design Road Frederick, Maryland, 21703

Coral Fulton
Texcell N.A., 4991 New Design Road Frederick, Maryland, 21703

Esha Vyas
Asahi Kasei America Inc, 1855, Elmdale Avenue, Glenview, Illinois, 60026

Aesha Shah
Asahi Kasei America Inc, 1855, Elmdale Avenue, Glenview, Illinois, 60026

Taka Sohka
Asahi Kasei America Inc, 1855, Elmdale Avenue, Glenview, Illinois, 60026

Arun Dhar
Animal and Comparative Biomedial Sciences, Aquaculture Pathology Lab University of Arizona, 1117 E Lowell Street, VSM Building, Room 201, Tucson, Arizona, 85721

Luke Pallansch
Texcell N.A., 4991 New Design Road Frederick, Maryland, 21703

Daniel Strauss
Asahi Kasei America Inc, 1855, Elmdale Avenue, Glenview, Illinois, 60026

DOI 10.1002/btpr.2694

Viruses can arise during the manufacture of biopharmaceuticals through contamination or endogenous expression of viral sequences. Regulatory agencies require "viral clearance" validation studies for each biopharmaceutical prior to approval. These studies aim to demonstrate the ability of the manufacturing process at removing or inactivating virus and are conducted by challenging scaled-down manufacturing steps with a "spike" of live virus. Due to the infectious nature of these live viruses, "spiking studies" are typically conducted in specialized biological safety level-2 facilities. The costs and logistics associated with these studies limit viral clearance analysis during process development and characterization. In this study, a noninfectious Minute Virus of Mice-Mock Virus Particle (MVM-MVP) was generated for use as an economical small virus spiking surrogate. An immunoglobin G containing solution was spiked with live MVM or MVM-MVP and processed through Planova nanofiltration units. Flux decay data was collected and particle reduction values were calculated from $TCID_{50}$ and Immuno-qPCR analysis. The data indicated comparable filtration performance and particle reduction between infectious MVM and noninfectious surrogate, MVM-MVP. This proof of concept study suggests the feasibility of utilizing MVPs for predictive size-based viral clearance assessments during process development and characterization as an alternative to homologous infectious virus.
Keywords: viral clearance, process development, chromotography , nanofiltration, minute virus of mice

Introduction

Viral contamination is an inherent risk during the manufacture of biopharmaceuticals.[1–4] Whether introduced endogenously from cell banks or exogenously through manufacturing, unmitigated viral contaminations have led to serious health implications including influenza, acquired immune deficiency syndrome, hepatitis, herpes, measles, and poliomyelitis.[5] International regulatory agencies therefore require biopharmaceutical companies to validate the "viral clearance" efficacy of their manufacturing processes prior to clinical trial or commercial approval.[6–8] Currently, viral clearance is assessed through small scale "spiking studies" whereby specific model mammalian

Correspondence concerning this article should be addressed to D. Cetlin at dcetlin@mockvsolutions.com.

viruses are artificially introduced ("spiked") into biopharmaceutical material and subsequently cleared (removed or inactivated) via purification techniques.[9,10] These studies require specialized biological safety level (BSL) laboratories and trained personnel resulting in costs that can rise above $100,000 depending on scope. Unfortunately, due to these high costs and logistics, most companies delay assessments and spend considerable up-front resources optimizing manufacturing processes without knowledge of their viral clearance efficacy. This increases the risk of insufficient viral clearance capacity, potentially delaying clinical trials or product launch and forcing companies to invest additional time and resources in redeveloping process steps.

Bacteriophages, endogenous CHO Type C retrovirus-like particles (RVLPs), and biologic feedstreams have been used in attempts to economically predict the outcomes of viral clearance studies.[11–13] However, due to the differences in physicochemical characteristics of bacteriophage models[9] and the complexities involved in producing purified and highly concentrated RVLP spiking stocks, the scope of utility for these surrogates have remained narrow.

Virus like particles (VLPs) are multiprotein structures that mimic the characteristics, organization and conformation of native infectious viruses but are themselves noninfectious.[14] These properties have made VLPs an interesting class of molecules for vaccine development[15] More recently, VLPs were introduced as potential BSL-1-compatible viral clearance spiking surrogates for biopharmaceutical process development studies.[16] The potential benefits of employing a VLP as a spiking agent includes the reduced costs, assay time and in-house flexibility not afforded by typical live viral clearance studies.. This feasibility study was conducted to test the concept of predicting *Minute Virus of Mice* (MVM) clearance through the use of a non-infectious MVM-VLP surrogate, referred to as an MVM-Mock Virus Particle (MVM-MVP). In a previous study,[16] MVM-MVPs were shown to be physicochemically similar to MVM, a small model virus commonly cited by international regulatory agencies to demonstrate clearance.[17] In this study, MVM and MVM-MVP were spiked into an immunoglobin G (IgG) solution and parallelly processed through Planova nanofiltration units. Flux decay data was collected, and particle reduction values were calculated after $TCID_{50}$ and Immuno-qPCR (I-qPCR) analysis.

Materials and Methods

Expression, purification and analysis of MVM-MVP

Non-infectious MVM-MVPs were assembled from recombinant expression of the major MVM capsid protein gene (VP2) in an Sf9/baculovirus system. The cloning and production of recombinant MVM-MVP were described in a recently published paper.[16] After harvest of Sf9 cells expressing recombinant MVM protein, purification was accomplished through sucrose gradient centrifugation followed by Cesium Chloride gradient centrifugation. Fractions containing MVM-MVP (according to SDS-PAGE with Coomassie Blue staining) were pooled and dialyzed into a formulation buffer. Western Blot analysis utilizing an anti-MVM-MVP antibody (MockV Solutions) confirmed the presence of MVM-MVPs. Negative staining transmission electron microscopy (TEM) was utilized to visualize and quantify MVM-MVP preparations. Stock solution of MVM-MVP was finalized by diluting the particle concentration to 12 $\log_{10}$ TEM Counts/mL (1.0×10^{12} particles/mL) with formulation buffer. Size exclusion chromatography-multi angle light scattering (SEC-MALS) was performed using a Malvern OMNISEC to evaluate MVM-MVP polydispersity. Briefly, samples were prepared by centrifugation at 12,000 rpm for 20 min and transferring the supernatant into HPLC vials. 100 μL's of each sample was injected into the system and eluted with PBS at a flow rate of 1 mL/min. A right angle light scattering detector was used to calculate MVM-MVP molecular weight (BSA was used as calibration standard and was injected before and after samples).

Production of infectious MVM

MVM (strain Prototype [P]) was propagated at Texcell N.A. (Rockville, MD). Initially, A9 cells were grown in serum-free media and subsequently purified by ultracentrifugation and mixed-mode chromatography, per proprietary procedures. The resulting virus possessed high titers, low contamination of host-cell DNA and protein and a virion monomeric content >90% as determined by dynamic light scattering (data not shown). The titer of purified MVM preparations was approximately 8.50 $\log_{10}$ $TCID_{50}$/mL.

Planova filtration procedure

All filtration runs were performed using 0.001 m^2 Planova 35 N, 20 N and BioEX filters (Asahi Kasei Medical Co., Ltd.). Table 1 outlines the experiments conducted.

For buffer matrix runs, 10 mM NaPhosphate, 40 mM NaCl, pH 7.0 buffer was made and filtered through a 0.2 μm Nalgene Rapid-Flow PES Vacuum filter (Nalgene RapidFlow, Thermo Fisher Scientific). For IgG matrix runs, h-IgG (Equitech Bio, Cat H60–0001) was diluted to 0.1 g/L in buffer from a stock of ~10 g/L. This material was then filtered through a 0.2 μm Nalgene Rapid-Flow PES. For MVM spiked runs, a ~0.6% (v/v) addition of MVM stock was then spiked into ~160 mL's of matrix (Buffer or IgG). For MVM-MVP runs, various amounts of stock solution (1×10^{12} particles/mL) were added to achieve the different target total spiking amounts as shown in Table 1. For example, a 0.06% (v/v) spike of MVM-MVP stock was added to deliver 11 $\log_{10}$ particles/mL in 160 mL's of matrix.

Prior to processing, load samples were taken, and the remaining volume was filtered through a 0.22 μm Nalgene Rapid Flow PES filter. Previrus Filtered samples were taken and the load solution was processed through a Planova 35 N, 20 N or BioEX filter using air at constant pressure (14 psi for 35 N and 20 N, 45 psi for BioEX) until a 150 L/m^2 throughput was achieved. For each run, once the target load throughput was reached, the pressure was released, buffer was added to the system and 15 L/m^2 was processed. Load, Previrus Filter, Filtrate and Fractions (fractions from select runs only) were taken and aliquoted for $TCID_{50}$ or I-qPCR analysis. A schematic representation of the procedure is shown in Figure 1.

As shown in Table 1, buffer matrix runs (no IgG) were conducted with 20 N and BioEX filters to determine the effect of IgG on filtration performance and particle removal. No-particle-challenge IgG runs (no MVM or MVM-MVP spike) were performed on each filtration type to generate baseline throughput curves.

Preliminary MVM-MVP experiments were conducted with 20 N filters to determine the optimal MVM-MVP spiking challenge. Target amounts of 8.0, 9.7, and 11.0 $\log_{10}$ particles,

Table 1. Summary of Filtration Experiments

Filter	Matrix System	Particle Type	Target Challenges	No. of Experiments
35 N	IgG	None	NA	1
		MVM	8.5 $\log_{10}$ $TCID_{50}$	1
		MVM-MVP	11.0 $\log_{10}$ particles	1
20 N	Buffer	MVM	8.5 $\log_{10}$ $TCID_{50}$	1
		MVM-MVP	11.0 $\log_{10}$ particles	1
	IgG	None	NA	1
		MVM	8.5 $\log_{10}$ $TCID_{50}$	2
		MVM-MVP	8.0 $\log_{10}$ particles	1
			9.7 $\log_{10}$ particles	1
			11.0 $\log_{10}$ particles	4
BioEX	Buffer	MVM	8.5 $\log_{10}$ $TCID_{50}$	1
		MVM-MVP	11.0 $\log_{10}$ particles	1
	IgG	None	NA	1
		MVM	8.5 $\log_{10}$ $TCID_{50}$	2
		MVM-MVP	11.0 $\log_{10}$ particles	2

were spiked into IgG. In addition, an extreme MVM-MVP load experiment was conducted to assess the potential of MVM-MVP breakthrough. For this experiment, 12.0 $\log_{10}$ particles was spiked into IgG matrix and loaded onto a BioEX filter. Fractions (~50 mL each) were collected throughout filtration and analyzed individually and as a combined pool via I-qPCR.

Quantification of MVM-MVP by I-qPCR analysis

An I-qPCR assay was developed and utilized to quantify MVM-MVP in samples generated during the study. First, samples were applied to the wells of a microwell strip coated with Guinea Pig anti-MVM-MVP polyclonal antibody (MockV Solutions). After incubating at room temperature, wells were washed, and a biotinylated antiMVM-MVP polyclonal antibody was added. The strips were incubated and washed again. Next, Neutravidin (Thermo Fisher) was added to the wells and the strips were incubated and washed. A biotinylated oligonucleotide was then added to the wells and the strips were incubated and washed a final time. Recovery buffer was then added to each well. Each strip was then placed on a heat block and incubated at 95°C for 5 min. After a quick centrifugation to remove condensate, fluid was then transferred from each strip well to an empty PCR plate. Quantitative PCR (qPCR) was performed according to set cycling parameters in a 25 µL reaction containing 1× Universal Master Mix (ThermoFisher), nuclease free water (ThermoFisher), 0.3 µM of each of forward and reverse primers, 0.2 uM of the probe and 5 µL of sample (transferred from the heated microwell strips to the empty PCR plate). Results from unknown samples were compared to a standard curve of cycle threshold (Ct) values generated through a dilution series of known MVM-MVP concentrations.

To determine the limit of quantification (LOQ) of the I-qPCR assay, buffer blanks were run alongside every standard curve during the course of the study. The standard deviation from their average was determined. Using a signal to noise ratio of 10:1, the following formula was applied to calculate LOQ:

$$\mathrm{LOQ} = \mathrm{AVG} + (10\sigma)$$

where AVG is the average MVM-MVP concentration of the buffer blank samples (back-calculated from buffer blank Ct values) and σ was the calculated standard deviation of buffer blank samples run during the course of the study.

MVM $TCID_{50}$ analysis

The titer of infectious MVM was determined by $TCID_{50}$ assay at Texcell N.A. Samples were serially diluted (5-fold) using high glucose DMEM medium containing 1% FBS. Monolayers of 324 K cells in 24-well plates were infected using at least eight 0.25 mL replicates of the appropriate dilution of sample. Plates were then incubated at 37°C with 5% CO2 and individual wells were observed for CPE on days 10–12 after infection. Virus titers in $TCID_{50}$/mL were calculated by the Karber method using the equation:

$$\log \mathrm{TCID}_{50}/\mathrm{mL} = d + [f\,(s - 0.5)] + 0.6 \quad (5)$$

where d is the log 10 (starting dilution), f is the log (dilution factor) and s is the sum of the proportion of CPE positive wells at each dilution. The assay possessed acceptable accuracy and precision over a dilution range of 9.0 $\log_{10}$ with a lower LOQ of ~1.0 $TCID_{50}$/mL when using large-volume testing (i.e., 5.4 mL per assay).

Log reduction values analysis

MVM and MVM-MVP log reduction values (LRV) were calculated as shown in the equation below:

$$\mathrm{LRV} = \log_{10}\left\{\frac{(Cl \times Vl)}{(Cp \times Vp)}\right\}$$

where C is the MVM or MVM-MVP particle concentration (via $TCID_{50}$ for MVM or I-qPCR for MVM-MVP), V is the volume and l or p denote of the previrus-filtered solution and filtrate, respectively.

Results

MVM-MVP production and load challenge study

According to TEM and SEC-MALS analysis, MVM-MVP stock solution was comprised of intact, spherical particles that exhibited a single major peak at a retention time of ~6.5 min; corresponding to a molecular weight of roughly 4,000 kDa (expected mass of 3,840 kDa[18]). No traces of lower molecular weight species or VP-2 monomer were present (Figure 2).

Prior to comparing MVM vs. MVM-MVP performance, a range of MVM-MVP spiking amounts was assessed to determine optimum challenge. Target load challenges of 8.0, 9.7,

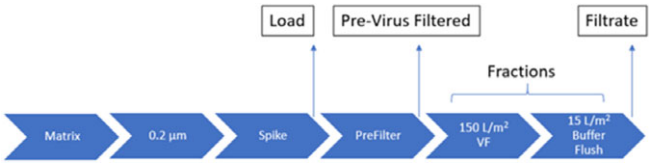


Figure 1. Schematic Diagram of Filtration Procedure. Matrix was first sterile filtered and then spiked before prefiltration and viral filtration (VF) processing with a buffer chase. Samples collected are indicated above.

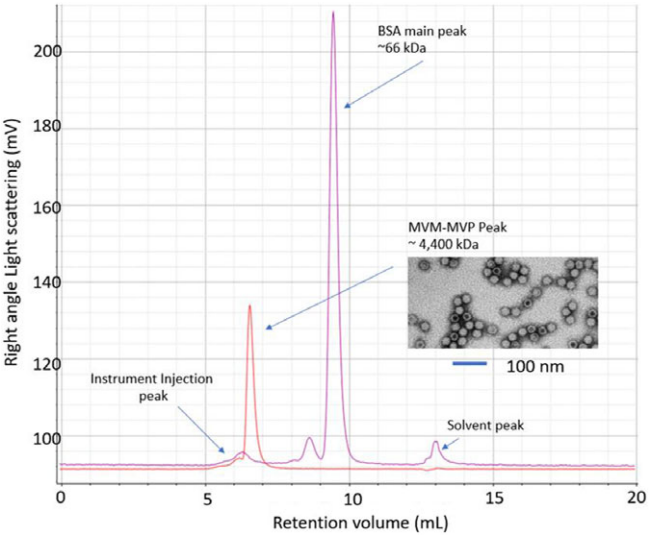


Figure 2. SEC-MALS chromatograph overlay of MVM-MVP stock solution diluted 1:50 in PBS (with an accompanying TEM image) and BSA standard at 2 mg/mL. Instrument injection and solvent peaks can be seen at ~5.5 and 13.0 mL's retention volume, respectively.

and 11.0 $\log_{10}$ particles in IgG matrix were prepared and processed through 20 N filters. Filtration performance as a function of normalized flux vs. throughput for each run is shown in Figure 3. At a throughput of 150 L/m^2, normalized flux decay was calculated and compared by subtracting the difference between Initial Flux and Instantaneous Flux from 100.

$$\text{Normalized Flux} = 100\% \times (\text{Instantaneous Flux})/(\text{Initial Flux}).$$

IgG matrix processed through 20 N with no MVM-MVP spike is shown as a reference.
Based on these data, 20 N performance was comparable over the range of MVM-MVP challenges tested and similar to IgG matrix baseline.

I-qPCR performance and LOQ

An I-qPCR assay was developed and utilized to quantify MVM-MVP in solution (Figure 4A). During the course of the study, four separate standard curves were generated. Average Ct values with standard error from each MVM-MVP concentration were plotted against MVM-MVP concentration (blue data points, Figure 4B). In addition to the standard curves, Ct values derived from four separate sets of buffer blank data were back-calculated to MVM-MVP concentration via standard curves and plotted on Figure 4b (orange "X" data points). These values were averaged together (7.1×10^4 particles/mL, Figure 4C) and plotted on Figure 4B (orange diamond data point). The standard deviation of these averages (3.3×10^4 particles/mL), was multiplied by 10 to determine the assays LOQ (4.1×10^5 particles/mL). Thus, the I-qPCR assay enabled analysis of MVM-MVP concentrations over a ~3.5 $\log_{10}$ range, spanning from ~1.0×10^9 to ~ 4.0×10^5 particles/mL.

MVM vs. MVM-MVP spiking experiments

P20 and BioEX filters were challenged with Buffer or IgG matrix containing a target of 8.5 $\log_{10}$ $TCID_{50}$ MVM or 11.0 $\log_{10}$ particles. As a negative control, 35 N filters were challenged with MVM and MVM-MVP-spiked IgG matrix. Filtration performance as a function of flux vs. throughput is shown in Figure 5A–D. IgG matrix processed through each filter with no particle spike are shown as a reference in each panel.

For 20 N and BioEX IgG runs, flux decay was calculated and compared at 150 L/m^2 throughput (Table 2).

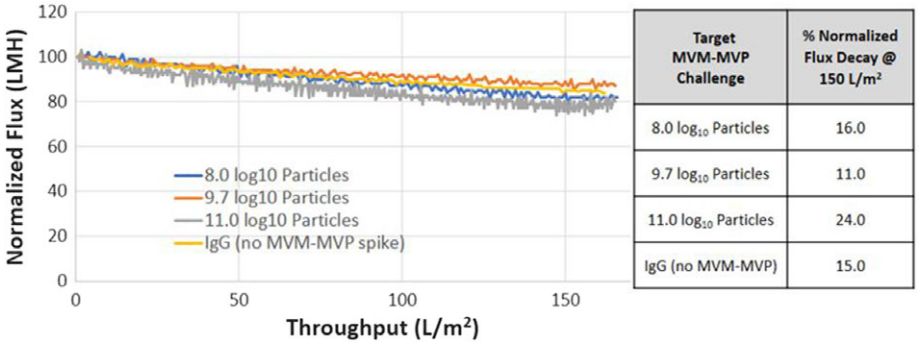
For each experiment, actual MVM or MVM-MVP challenge and subsequent clearance was determined. "Filtered Load" MVM titers of post-prefilter samples were measured by $TCID_{50}$ while MVM-MVP concentrations were determined through I-qPCR analysis. According to the "Filtered Load" results, actual MVM challenges were between 8.0 and 8.4 $\log_{10}$ $TCID_{50}$ MVM while MVM-MVP challenges ranged between 10.6 and 11.7 $\log_{10}$ particles according to I-qPCR. Postnanofilter "Pool" samples were analyzed and LRVs were determined. Tables 3–5 show 20 N, BioEX and 35 N results for IgG spiking experiments (buffer matrix results not shown). The LOQ Values for each assay ($\leq 1.4 \times 10^1$ and $\leq 4.1 \times 10^5$ for $TCID_{50}$ and I-qPCR, respectively) were used when determining LRV and included in the Tables where appropriate.

Extreme MVM-MVP challenge

A BioEX filter was challenged with a target spike of 12.0 $\log_{10}$ Particles in IgG matrix. Filtration performance as a function of flux vs. throughput was collected and shown in Figure 6A. Flux decay at a throughput of 150 L/m^2 was measured to be 46%. Intermediate filtrate fractions collected during processing were analyzed and overall clearance was determined through I-qPCR analysis (Figure 6B). Actual values are reported instead of the LOQ; however, for the combined Pool sample, the LOQ was used to determine LRV.

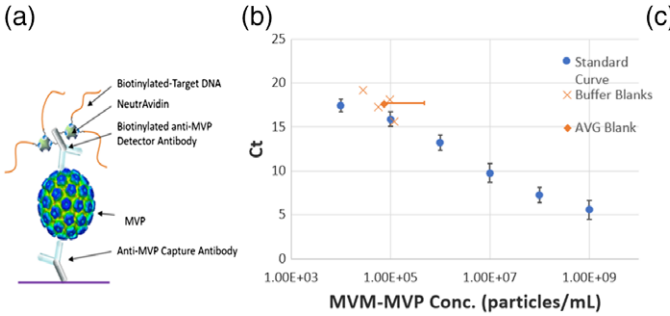
Discussion

We sought here to test the feasibility of predicting MVM clearance and nanofiltration performance with a non-infectious MVM-MVP particle. First, nanofiltration performance was assessed by challenging Planova BioEX, 20 N and 35 N filters with 8.5 $\log_{10}$ $TCID_{50}$ MVM or 11.0 $\log_{10}$ MVM-MVP spiked loads (in IgG or buffer systems). The results revealed that MVM and MVM-MVP throughput and flux decay profiles were comparable among each filter type and load system. Next, we analyzed and compared the removal of each particle. $TCID_{50}$ analysis of MVM 20 N and BioEX pools showed complete clearance of MVM (LOQ achieved), resulting in LRV determinations of >4.7 (Tables 3 and 4). Similarly, I-



Target MVM-MVP Challenge	% Normalized Flux Decay @ 150 L/m²
8.0 $\log_{10}$ Particles	16.0
9.7 $\log_{10}$ Particles	11.0
11.0 $\log_{10}$ Particles	24.0
IgG (no MVM-MVP)	15.0

Figure 3. 20 N filtration performance among 8.0 (blue) 9.7 (orange) and 11.0 (gray) log10 particle challenges in IgG. For comparison, non-MVM-MVP spiked IgG was processed through a 20 N (yellow).



Run #	Buffer Blank Avg. MVM-MVP Conc. (particles/mL)
1	5.3×10^4
2	9.2×10^4
3	1.1×10^5
4	2.6×10^4
AVG	7.1×10^4
STD Dev.	3.3×10^4
LOQ	4.1×10^5

Figure 4. Schematic overview of I-qPCR assay (a) Graph of MVM-MVP dilution series (b). I-qPCR Threshold cycle (Ct) values at each concentration tested were averaged from four independent curves (blue points). Error bars were calculated from the standard deviation at each concentration divided by the square root of four (for each of the four data points). Ct results from four Buffer Blank readings, generated by background signal, were plotted graphically (orange X) at corresponding calculated MVM-MVP concentrations. The average Buffer Blank concentration (orange diamond) and standard deviation were used to determine the assays LOQ (orange horizontal error bar) and are shown quantitatively (c).

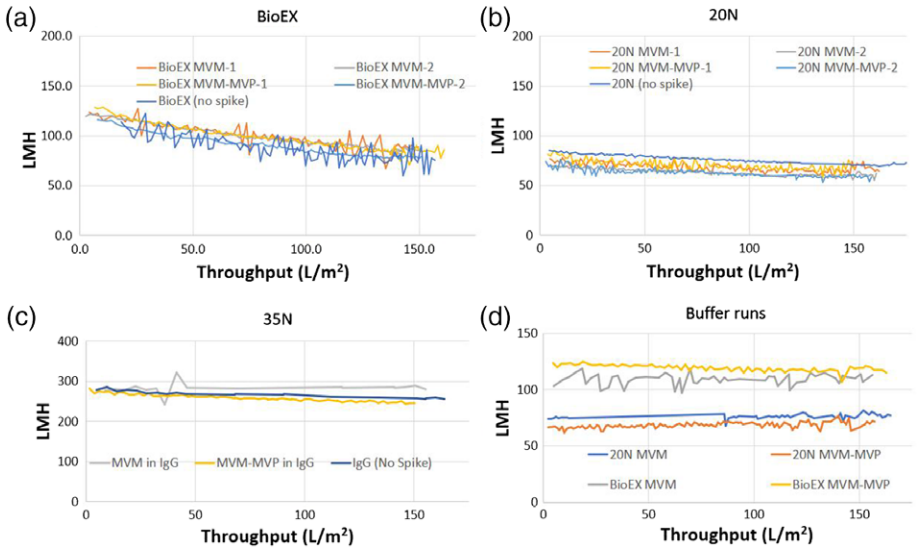


Figure 5. BioEX (a), 20 N (b) and 35 N (c) flux vs. throughput curves are shown for IgG spiked with either MVM (orange and gray) or MVM-MVP (yellow and light blue). As a control, nonspiked IgG was also plotted (dark blue). 20 N and BioEX flux vs. throughput curves for buffer spiked with MVM and MVM-MVP are also shown (d).

qPCR analysis of 20 N and BioEX pools demonstrated complete MVM-MVP clearance (LOQ achieved); albeit, the ability to detect breakthrough of particles was limited by the relatively low sensitivity of the I-qPCR assay. Despite this limitation, the expected absence of detectable MVM-MVP in all processed pools was achieved and enabled us to determine LRV's of ≥2.8 and ≥ 3.5 for 20 N and BioEX respectively (Tables 3 and 4). As expected, complete flow through of either particle was accomplished through 35 N processing (Table 5).

The concentration of the MVM-MVP stock solution used as spiking reagent throughout this study was determined through TEM while spiked load and process sample MVM-MVP

quantity was determined through I-qPCR. Both of these techniques quantify total particles. In comparison, $TCID_{50}$ assessments of MVM quantify only the subset of particles present that are infectious. Although previously reported MVM ratios ranging from 700–2500 particle/pfu have been cited,[19,20] the exact ratio of total:infectious MVM particles in sample preparations used during this study was unknown. Based on these previous reports, it is likely that an 8.5 $\log_{10}$ $TCID_{50}$ MVM challenge delivers at least an additional order of magnitude of non-infectious particles. These noninfectious particles would theoretically produce the same impact on filtration and flux decay as infectious particles.

To determine the optimal MVM-MVP spike challenge, an initial set of experiments was undertaken. The target MVM-MVP challenge amounts of 8.0, 9.7, and 11.0 $\log_{10}$ particles were achieved by spiking IgG matrix with a 12.0 $\log_{10}$ particles/mL MVM-MVP stock solution (as determined by TEM). These challenges equate to 11.0, 12.7, and 14.0 $\log_{10}$ particles/m^2 of filter, respectively. Filtration performance and normalized flux decay analysis (Figure 3) showed little difference among these spike challenges, and therefore a target 11.0 $\log_{10}$ particles challenge (6.7×10^8 particles/mL in 150 mL total) was selected for further comparative experiments. Despite this being a high challenge for nanofiltration (14.0 $\log_{10}$ particles/m^2) we were encouraged by the low flux decay profile and sought to utilize the fullest range of the I-qPCR assay.

Analysis of the flux decay curves illustrated in Figure 5 indicate that IgG, even when present at a low concentration (0.1 g/L), was the main factor influencing filtration flux decay. As a major intent of this study was to compare the behavior of MVM vs. MVM-MVP particles across Planova filters, this finding re-enforced our strategy of spiking into low concentrations of IgG material. By minimizing the effects of IgG induced fouling, we were able to better isolate the flux decay contributions caused by MVM and MVM-MVP introduction, if any. Future studies will be performed with in-process mAb material at a range of representative process conditions to ensure the robustness of MVM-MVP removal during protein based gradual pore plugging.

Table 2. % Flux Decay for IgG spiked 20 N and BioEX Experiments

Experiment	BioEX % Flux Decay @ 150 L/m^2	20 N % Flux Decay @ 150 L/m^2
MVM-1	28%	14%
MVM-2	29%	13%
MVM-MVP-1	34%	18%
MVM-MVP-2	33%	18%
IgG (no spike)	36%	15%

Table 3. MVM and MVM-MVP spiked IgG 20 N clearance results

	Target 8.5 $\log_{10}$ $TCID_{50}$ MVM (n = 2)				Target 11.0 $\log_{10}$ particles MVM-MVP (n = 2)			
IgG Matrix Only	Filtered Load	Pool	Filtered Load	Pool	Filtered Load	Pool	Filtered Load	Pool
$TCID_{50}$ Results ($TCID_{50}$/mL)	9.8×10^5	$\leq 1.4 \times 10^1$ [LOQ]	9.8×10^5	$\leq 1.4 \times 10^1$ [LOQ]	NA	NA	NA	NA
I-qPCR Results (particles/mL)	NA	NA	NA	NA	6.8×10^8	$\leq 4.1 \times 10^5$ [LOQ]	2.5×10^8	$\leq 4.1 \times 10^5$ [LOQ]
Volume (mL)	154	164	154	162	151	153	150	158
$TCID_{50}$ or particles	1.5×10^8	$\leq 2.3 \times 10^3$	1.5×10^8	$\leq 2.2 \times 10^3$	1.0×10^{11}	$\leq 6.3 \times 10^7$	3.7×10^{10}	$\leq 6.5 \times 10^7$
$\log_{10}$ ($TCID_{50}$ or particles)	8.2	≤ 3.4	8.2	≤ 3.4	11.0	≤ 7.8	10.6	≤ 7.8
LRV	≥ 4.8		≥ 4.8		≥ 3.2		≥ 2.8	

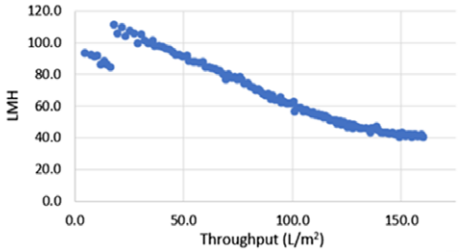
Table 4. MVM and MVM-MVP-Spiked IgG BioEx Clearance Results

	Target 8.5 $\log_{10}$ $TCID_{50}$ MVM (n = 2)				Target 11.0 $\log_{10}$ particles MVM-MVP (n = 2)			
IgG Matrix only	Load	Pool	Load	Pool	Load	Pool	Load	Pool
$TCID_{50}$ Results ($TCID_{50}$/mL)	6.8×10^5	$\leq 1.4 \times 10^1$ [LOQ]	9.8×10^5	$\leq 1.4 \times 10^1$ [LOQ]	NA	NA	NA	NA
I-qPCR Results (particles/mL)	NA	NA	NA	NA	1.4×10^9	$\leq 4.1 \times 10^5$ [LOQ]	1.8×10^9	$\leq 4.1 \times 10^5$ [LOQ]
Volume (mL)	144	150	144	157	148	162	148	159
$TCID_{50}$ or particles	9.8×10^7	$\leq 2.1 \times 10^3$	1.4×10^8	$\leq 2.2 \times 10^3$	2.1×10^{11}	$\leq 6.6 \times 10^7$	2.7×10^{11}	$\leq 6.5 \times 10^7$
$\log_{10}$ ($TCID_{50}$ or particles)	7.99	≤ 3.31	8.15	≤ 3.33	11.3	≤ 7.8	11.4	≤ 7.8
LRV	≥ 4.7		≥ 4.8		≥ 3.5		≥ 3.6	

Table 5. MVM and MVM-MVP spiked IgG 35 N clearance results

	Target 8.5 $\log_{10}$ $TCID_{50}$ MVM		Target 11.0 $\log_{10}$ particles MVM-MVP	
IgG Matrix only	Load	Pool	Load	Pool
$TCID_{50}$ Results ($TCID_{50}$/mL)	1.2×10^6	9.8×10^5	NA	NA
I-qPCR Results (particles/mL)	NA	NA	3.5×10^9	1.4×10^9
Volume (mL)	157	158	150	150
$TCID_{50}$ or particles	1.9×10^8	1.6×10^8	5.2×10^{11}	2.1×10^{11}
$\log_{10}$ ($TCID_{50}$ or particles)	8.3	8.2	11.7	11.3
LRV	0.1		0.4	

(a)



(b)

Sample	Concentration (particles/mL)
Load (151 mL's)	7.6×10^9
Fraction 1 (1-75 mL's)	2.0×10^5
Fraction 2 (75-150 mL's)	2.6×10^5
Fraction 3 (9 mL Chase)	1.6×10^5
Combined Pool (Representing 159 mL's Total)	1.9×10^5
LRV*	**≥ 4.3**

* LOQ (4.1×10^5 particles/mL) used for Pool concentration

Figure 6. Flux vs. Throughput curve BioEX filter challenged with 12.0 $\log_{10}$ particles spiked into IgG (a). I-qPCR generated MVM-MVP concentration results of the spiked load, fractions, and combined pool (b). A LRV of ≥4.3 was calculated.

To observe the impacts of MVM-MVP over-spiking, we challenged a 0.001m^2 BioEX filter with a 12.0 $\log_{10}$ particles (in IgG matrix). Assuming a ratio of 100:1 total particles to infectious MVM particles, this spike would equate to a MVM challenge of 10.0 $\log_{10}$ $TCID_{50}$ (14.0 $\log_{10}$ $TCID_{50}/m^2$). According to previously conducted studies, this high challenge (2 magnitudes higher than the filter supplier recommendation of 8.0 $\log_{10}$ $TCID_{50}$) could result in a noticeable decrease in filtration performance as measured by flux decay and a potential breakthrough of virus, resulting in lower LRV's.[21] Here, at a 12.0 $\log_{10}$ particles challenge, we did observe a significant increase of flux decay in relation to throughput when compared with an 11.0 $\log_{10}$ particles challenges; however, we were not able to detect any measurable MVM-MVP breakthrough. Instead, due to the higher spiking challenge and the achievement of I-qPCR LOQ's for all fractions/pool sample analyzed, we were able to calculate a higher LRV for the 12.0 $\log_{10}$ particles challenge (≥4.3) than for any of the 11.0 $\log_{10}$ particles challenges (≥2.8–3.6). Despite this finding, spiking greater than the manufacturer's recommended challenge can lead to a risk of higher breakthrough and other undesirable artifacts. Therefore, for more reliable LRV results, we recommend an MVM-MVP spike of ≤11.0 $\log_{10}$ particles (≤14.0 $\log_{10}$ particles/m^2). Achieving higher LRV's will be anticipated after on-going improvements to I-qPCR sensitivity are made.

The utility of any viral clearance spiking agent relies on the sensitivity and dynamic range in which the agent can be accurately measured. MVM studies typically employ infectivity assays (e.g., $TCID_{50}$) which can measure the titer of infectious particles over an 8 $\log_{10}$ range to as little as <1.0 $\log_{10}$ $TCID_{50}$/mL.[22] qPCR techniques have also been established which indiscriminately measure infectious and noninfectious MVM over a 6 $\log_{10}$ dynamic range to as little as 2 MVM copies.[23] Due to the noninfectious nature of MVM-MVP's and their lack of internal nucleic acid, neither of these techniques can be implemented. To circumvent this issue, we initially developed an enzyme-linked immunosorbent assay which enabled quantification of MVM-MVP to ~1×10^7 particles/mL.[16] Although simple and effective, the narrow dynamic range limited potential LRV calculation and therefore we developed and utilized an I-qPCR assay. This assay employs an antiMVM-MVP polyclonal antibody and an externally recruited double stranded oligonucleotide sequence for TaqMan qPCR analysis. MVM-MVP concentration determinations during the course of this study were analyzed by back-calculating Threshold Cycle (Ct) values to standard curves generated from a 10-fold dilution series of known MVM-MVP concentrations. A lower LOQ was set using a 10:1 signal to noise threshold. This value represents the background signal inherent to the assay. Although MVM-MVP signals generated from nanofiltration pool samples were consistently below this limit, the relatively high value (4.1×10^5 particles/mL) constrained the mathematical ability to calculate comparable LRV's to MVM via $TCID_{50}$. Overall, assay performance was adequate for proof of concept assessments and further improvements are being undertaken to extend the dynamic range of the assay.

Overall, the results reported here indicate that MVM-MVPs behave like MVM during nanofiltration and therefore have the potential to be used as surrogates for this application. The theory of utilizing MVM-MVPs as a noninfectious surrogate for MVM during downstream process development studies is supported by previously published physicochemical comparison data.[16] MVM-MVPs are generated via *in vitro* expression of MVMs major capsid protein (VP-2) and demonstrate comparable size, surface charge and surface hydrophobicity characteristics to the live virus. Although the nanofiltration results reported here pertain only to size-based modes of separation, it is possible that charge and hydrophobic properties exhibited by MVM-MVPs would lend their use to chromatographic modes of separation as well. This will be the subject of further study. If proven, MVM-MVPs could enable downstream bioprocess scientists to economically and conveniently predict MVM clearance during process development and characterization. Design of Experiment studies could be conducted with MVM-MVP clearance as an output. This would empower scientists to explore and determine the impacts of process parameters on parvovirus clearance without the need for expensive and logistically challenging MVM studies. The concept of Quality by Design could be seamlessly applied to viral clearance supporting process optimization and leading to efficient process validation.

Conclusion

We sought to demonstrate if a noninfectious particle (MVM-MVP), assembled to mimic the physicochemical properties of MVM, could be utilized as an MVM surrogate during small scale nanofiltration experiments. To accomplish this, we developed an I-qPCR assay for quantifying MVM-MVP and compared removal and filtration performance via Planova nanofiltration to MVM. According to flux decay profiles, MVM-MVP challenged loads behaved similarly to MVM while processed through Planova 20 N, BioEX, and 35 N nanofilters. In terms of removal, Planova 20 N and BioEX filters provided complete clearance of MVM while MVM-MVP signals evinced from all pool samples were below the LOQ of the I-qPCR assay. Given the limitations imposed by the assay,

these results provide enough support to the hypothesis that MVM-MVP could indeed serve as an accurate surrogate to MVM. The use of MVM-MVPs as a surrogate to MVM during biopharmaceutical purification process development overcomes the limitations of working with the infectious virus and opens new opportunities to screen, develop and optimize novel approaches in biologic purification.

Literature Cited

1. Anderson KP, Low MA, Lie YS, Keller GA, Dinowitz M. Endogenous origin of defective retrovirus like particles from a recombinant Chinese hamster ovary cell line. *Virology*. 1991;181:305–311.
2. Brorson K, de Wit C, Hamilton E, Mustafa M, Swann PG, Kiss R, Taticek R, Polastri G, Stein KE, Xu Y. Impact of cell culture process changes on endogenous retrovirus expression. *Biotechnol Bioeng*. 2002;80:257–267.
3. Lieber MM, Benveniste RE, Livingston DM, Todaro GJ. Mammalian cells in culture frequently release type C viruses. *Science*. 1973;182:56–59.
4. Garnick RL. Experience with viral contamination in cell culture. *Dev Biol Stand*. 1996;88:49–56.
5. Rathore AS, Sofer G, editors. *Process validation in manufacturing of biopharmaceuticals*. Boca Raton, Florida: CRC Press; 2012.
6. CBER (Center for Biologics Evaluation and Research, Federal Drug Administration). Points to consider in the manufacture and testing of monoclonal antibody products for human use. Available at: http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/OtherRecommendationsforManufacturers/UCM153182.pdf; 1997.
7. EMEA (European Medicines Agency). Guideline on virus safety evaluation of biotechnological investigational medicinal products. Available at: http://www.emea.europa.eu/pdfs/human/bwp/39849805enfin.pdf; 2008.
8. ICH (International Conference on Harmonization). Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin Q5A. Available at: http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm073454.pdf; 1999.
9. Aranha H, Forbes S. Viral clearance strategies for biopharmaceutical safety. *Pharm Technol*. 2001;25(4):22–22.
10. Shukla AA, Etzel MR, Gadam S, editors. *Process Scale Bioseparations for the Biopharmaceutical Industry*. Boca Raton, Florida: CRC Press; 2006.
11. Miesegaes GR, Lute S, Strauss DM, Read EK, Venkiteshwaran A, Kreuzman A, Shah R, Shamlou P, Chen D, Brorson K. Monoclonal antibody capture and viral clearance by cation exchange chromatography. *Biotechnol Bioeng*. 2012;109(8):2048–2058.
12. Strauss DM, Cano T, Cai N, Delucchi H, Plancarte M, Coleman D, Blank GS, Chen Q, Yang B. Strategies for developing design spaces for viral clearance by anion exchange chromatography during monoclonal antibody production. *Biotechnol Prog*. 2010;26(3):750–755.
13. Rayfield WJ, Roush DJ, Chmielowski RA, Tugcu N, Barakat S, Cheung JK. Prediction of viral filtration performance of monoclonal antibodies based on biophysical properties of feed. *Biotechnol Prog*. 2015;31(3):765–774.
14. Pillay S, Meyers A, Williamson AL, Rybicki EP. Optimization of chimeric HIV-1 virus-like particle production in a baculovirus-insect cell expression system. *Biotechnol Prog*. 2009;25(4):1153–1160.
15. Buonaguro FM, Buonaguro L. *Virus-Like Particles in Vaccine Development*. London, England: Future Medicine Ltd.; 2014.
16. Johnson S, Brorson KA, Frey DD, Dhar AK, Cetlin DA. Characterization of Non-Infectious Virus-Like Particle Surrogates for Viral Clearance Applications. *Appl Biochem Biotechnol*. 2017;183(1):318–331.
17. Romanowski P, Schleh M, Rajurs V, Chinniah S, Dehghani H. Variables affecting titer and long-term stability of virus stocks: xenotropic murine leukemia and mouse minute viruses. *Bioproc Int*. 2008;6(2):44.
18. Hernando E, Llamas-Saiz AL, Foces-Foces C, McKenna R, Portman I, Agbandje-McKenna M, Almendral JM. Biochemical and physical characterization of parvovirus minute virus of mice virus-like particles. *Virology*. 2000;267(2):299–309.
19. Lang SI, Boelz S, Stroh-Dege AY, Rommelaere J, Dinsart C, Cornelis JJ. The infectivity and lytic activity of minute virus of mice wild-type and derived vector particles are strikingly different. *J Virol*. 2005;79(1):289–298.
20. Garcin PO, Nabi IR, Pante N. Galectin-3 plays a role in minute virus of mice infection. *Virology*. 2015;481:63–72.
21. Lute S, Bailey M, Combs J, Sukumar M, Brorson K. Phage passage after extended processing in small-virus-retentive filters. *Biotechnol Appl Biochem*. 2007;47(3):141–151.
22. Strauss D, Goldstein J, Hongo-Hirasaki T, Yokoyama Y, Hirotomi N, Miyabayashi T, Vacante D. Characterizing the impact of pressure on virus filtration processes and establishing design spaces to ensure effective parvovirus removal. *Biotechnol Prog*. 2017;33:1294–1302.
23. Zhao X, Bailey MR, Emery WR, Lambooy PK, Chen D. Evaluation of viral removal by nanofiltration using real-time quantitative polymerase chain reaction. *Biotechnol Appl Biochem*. 2007;47(2):97–104.

Manuscript received Mar. 13, 2018, revision received Jun. 8, 2018, accepted Jul. 18, 2018.