# Impact of Virus Stock Quality on Virus Filter Validation

## A Case Study

**by Mark Cabatingan**

Manufacturers of biologicals are required to demonstrate that their downstream processes are capable of clearing adventitious or endogenous viruses that may be present in their product streams. Process engineers typically validate a viral clearance step (filtration, chromatography, heat treatment, or chemical inactivation) in scale-down mode by spiking relevant mammalian viruses into protein solutions representative of their process streams. The effectiveness of that operation for removal or inactivation of viruses with a range of physiochemical properties is then determined. In general, log reduction values (LRV) of 4 $\log_{10}$ are expected for an effective viral clearance step.

In addition to validating viral clearance, scale-down studies are also used to establish process parameters. For filtration, those parameters include operating pressure or filtrate flux, volumetric throughput per unit of filter area, and maximum extent of flux decline. A key assumption is that conditions in a scaled-down spiking study are representative of conditions in the full-scale manufacturing process. Experience in mammalian virus spiking studies with virus filters such as Viresolve NFP (normal flow parvovirus) filters suggests that the quality of virus stocks used in spiking studies can significantly affect filter performance, potentially making scale-down studies less representative

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of related manufacturing processes. Simple modifications to virus preparation methods, however, can reduce negative impacts of virus spikes on filter performance, making scale-down studies more relevant to real processes.

Model viruses are typically chosen to span the range of size and type most likely to be encountered in a bioprocess. Minute mouse virus (MMV) is a small (18–26 nm), nonenveloped, single-stranded DNA virus in the family *Parvoviridae* that has been found as an adventitious contaminant in biomanufacturing, especially with murine hybridoma and Chinese hamster ovary cell expression systems (**1**). Because of its relatively small size and the fact that it is a demonstrated adventitious contaminant, MMV is often used in virus spiking studies.

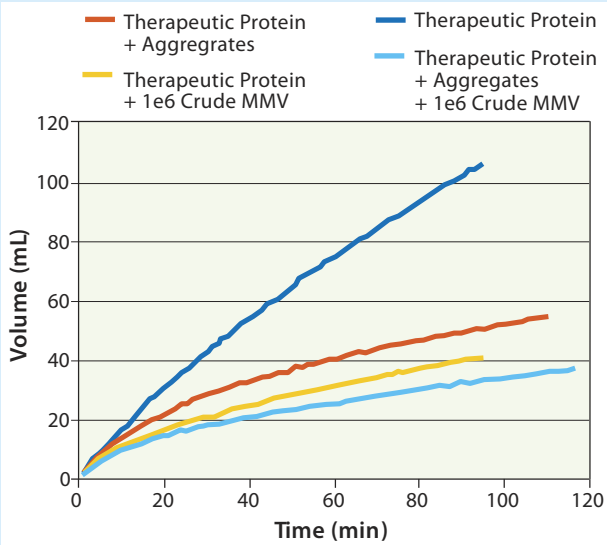
Bacteriophages are useful models for studying the mechanisms of virus retention because they are easily purified to high titers, and infectivity assays using them are relatively fast (generally one day). The bacteriophage ΦX174, a 25- to 35-nm nonenveloped DNA virus in the family *Microviridae*, can be used as a size model for small viruses (**2**).

Cell lines used to manufacture monoclonal antibodies and other therapeutic proteins typically express retrovirus-like particles (RVLPs) as an endogenous contaminant (**3, 4**). Xenotropic murine leukemia virus (X-MuLV) is an enveloped, 80- to 110-nm diameter, ss-RNA virus in the family *Retroviridae* that is often used as a model for RVLPs. This article presents data from studies using all three viruses to demonstrate the impact of contaminants in virus preparations on filter performance, using Viresolve NFP as an example.

Nanofiltration is a proven method for removing viruses from protein streams by a size-exclusion mechanism. In the case of virus filters, viral retention experiments are carried out using small-scale devices with process parameters scaled-down appropriately. For example, OptiScale 25 devices (with 3.5 $cm^2$ of membrane area) are typically used with NFP membranes.

**MANY** factors affect filter performance in viral clearance validation studies. . . . Properties of the **VIRUS SPIKE** itself are an often-overlooked source of contaminants that can affect filter performance. Potential spike **CONTAMINANTS** include serum and host-cell proteins, lipids, and host-cell DNA.

**Figure 1:** Impact of virus spike and protein aggregates on NFP performance (OptiScale devices with Viresolve NFP membrane at 30 psi)




Although viral clearance experiments are designed to reproduce actual process conditions at very small scale, in some cases the results of such studies are not always scalable from laboratory to manufacturing. For example, introduction of virus spikes into a protein solution can cause virus-retentive filters to plug much more rapidly than in normal operations where process streams are unspiked. Therefore, adding a virus spike into a protein solution reduces flux and throughput when compared with an unspiked product stream. Because current practice is to size virus filters based on volumetric throughput achieved during a virus spiking study, manufacturers of biologicals may use more filter area than would be required in their manufacturing processes. That can lead to increased filtration costs and unnecessary complexity in a process step.

## Filter Performance

Many factors affect filter performance in viral clearance validation studies including product type and composition, buffer composition, protein concentration and purity, and the levels of protein aggregates (due to age, freeze/thaw, shipping, etc.), for example. Such factors can affect throughput, flux (pressure differential), process time, and potentially virus retention. Various methods help users understand and limit the effects of some variables on filter performance during spiking studies (**5**, **6**). However, properties of the virus spike itself are an often overlooked source of contaminants that can affect filter performance. Examples of potential contaminants in virus stocks include serum proteins, host-cell proteins, host-cell DNA, and lipids.

Separation of small viruses from therapeutic proteins such as monoclonal antibodies is challenging because of the low size differential between pathogen and product. For example, the hydrodynamic radius of an IgG molecule is about 8–10 nm, whereas a typical small virus particle is about 20 nm in diameter. In general, virus filters are expected to allow >95% of IgG molecules to pass through while achieving a >4 $\log_{10}$ removal of small viruses. As a result, a general issue with such filters is that they can be sensitive to fouling with protein aggregates or contaminating materials in virus stocks (**7**, **8**).

Figure 1 illustrates the effect that feedstream properties and virus spikes can have on NFP performance. Volume and time data for the filter under a number of conditions are compared. The protein used is highly monomeric and shows very little flux decline over time. Adding a crude MMV spike containing 1% fetal bovine serum (FBS ) in its stock decreases throughput at any given point, the result of decreasing flux over time. The same phenomenon can be seen when aggregates of a protein

**Figure 2A:** Crude MMV preparation method

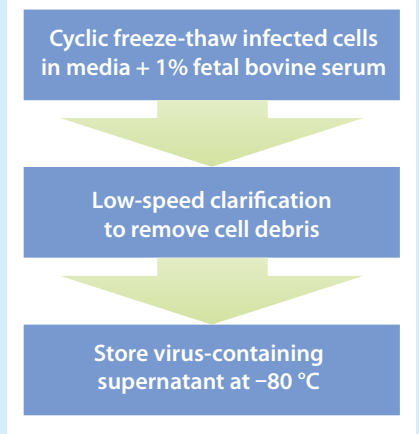



**Figure 2B:** Clean MMV preparation method

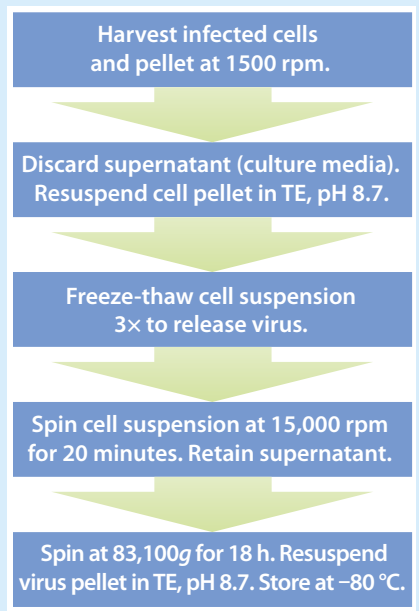


**Figure 3A:** Impact of MMV stock protein concentration on capacity (1 mg/mL IgG in FA buffer, pH 7.2, OptiScale 25 devices with Viresolve NFP membrane at 30 psi)

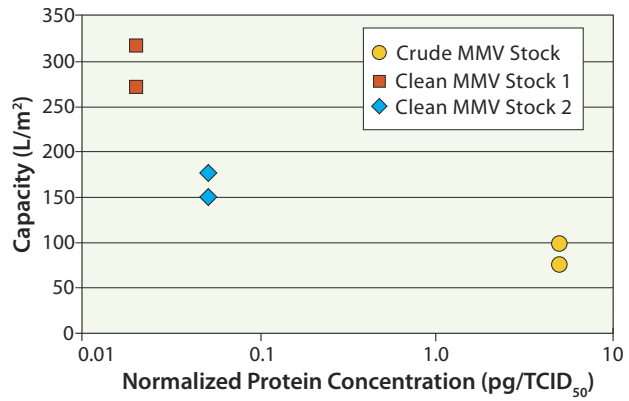



**Figure 3B:** Impact of MMV preparation method on NFP performance (1 mg/mL IgG in FA buffer, pH 7.2, OptiScale 25 devices with Viresolve NFP membrane at 30 psi)

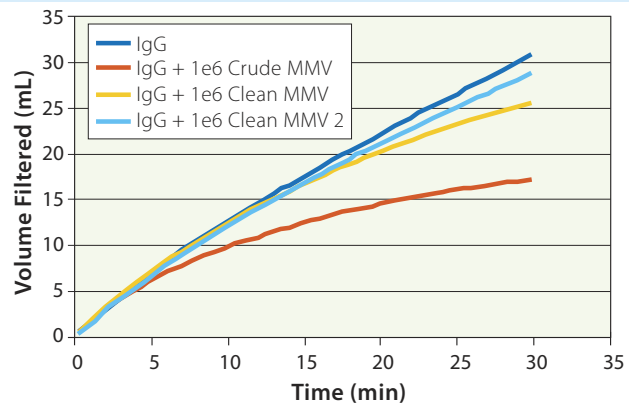



**Figure 3C:** Impact of MMV preparation method on LRV (1 mg/mL IgG in FA buffer, pH 7.2, OptiScale 25 devices with Viresolve NFP membrane at 30 psi)

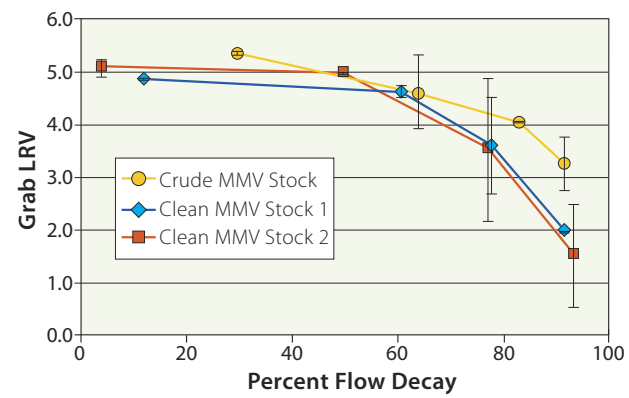



**Table 1:** Comparison of crude and clean MMV stocks (Bio-Rad DCProtein assay used for determining total protein content)

| | Crude MMV | Clean MMV |
|---|---|---|
| $Log_{10}$ Stock Titer ($TCID_{50}$/mL) | 8.4 ± 0.5 | 10.0 ± 0.5 |
| $Log_{10}$ Total Virus Per Infected Flask | 9.8 ± 0.5 | 9.2 ± 0.5 |
| Total Protein in Stock (mg/mL) | 1.7 ± 0.1 | 0.6 ± 0.4 |
| Normalized Protein Content (pg/$TCID_{50}$) | 5.35 ± 1.3 | 0.04 ± 0.02 |

(generated by heat treatment) are spiked into clean protein. When both virus and aggregates are added together, flux decline is even more dramatic. Basing filtration capacity on scale-down experiments that resemble the bottom three curves of the figure can be costly, especially if results are more likely to resemble the top curve for pure protein at the point in manufacturing when that viral clearance step is used.

It has been shown with a number of viral filters that virus breakthrough can occur as a function of volume filtered (**2, 9, 10**). For NFP filters, studies using the bacteriophage ΦX174 have shown that LRV decreases more as a function of flux decline and that the mechanism of fouling affects the degree of decline (**2**). In these studies, cake fouling (simulated with 90-nm beads) produced no net loss of LRV, but protein fouling resulted in significant loss at high flow decay. Plugging with 21-nm particles, on the other hand, produced LRV loss curves between those seen in cake and protein fouling.

A potential issue with virus spiking studies is that components of virus preparation can foul membranes in a way that may be atypical of fouling mechanisms encountered in manufacturing. With virus breakthrough a general phenomenon for virus removal filters, one could argue that fouling by contaminants in virus spikes should be minimized so that conditions in filter validation most closely represent those in the manufacturing process.

## Impact of MMV Stock Preparation

A lack of standard methods for preparation and analysis of virus stocks used in filter validation studies can lead to quantitative and qualitative differences in contaminants from laboratory to laboratory, or even from stock to stock within a single lab. Some contaminants that may be present in virus stocks are serum proteins carried over by a growth medium, host-cell proteins and DNA, cell wall fragments, and lipids. Virus-removal filters are typically implemented at a point in downstream purification processes where such contaminants are absent. If the contaminants are not representative of the manufacturing process and have the potential to foul virus filters and affect observed retention capabilities, it is advisable to use cleaner virus stocks in filter validation studies.

To demonstrate the effects of virus stock quality on filter performance, we compared two MMV propagation methods (Figure 2). In the crude method (Figure 2A), cells undergo minimal processing: lysing by freeze–thaw cycles followed by low-speed centrifugation to remove large cellular debris. Thus, many supernatant components are contained in the virus stock. The "clean" MMV stock method was adapted from literature (**11**) and diverges from the crude method at its very first step (Figure 2B). Cells are spun down at harvest, and the resulting supernatant is immediately discarded. The cells are then resuspended in protein-free TE buffer (Tris + ethylenediaminetetraacetic acid, EDTA) for cell lysis. The sample is subjected to ultracentrifugation two more times, and the resulting virus pellet resuspended in TE to yield a nearly protein-free solution.

The method for producing clean MMV stocks outlined in Figure 2B consistently gives 1.5 $\log_{10}$ higher titers and lower total protein concentrations (Table 1) than the method in 2A. Furthermore, when total protein concentration is normalized to infectivity, it is clear that for any given spike titer, about 100-fold less protein is added to the challenge solution with a clean MMV preparation. As can be seen in Figure 3A, higher stock protein concentrations correlate with lower capacities using spiked IgG solutions. Even with clean MMV stocks, some variation in total protein concentration correlates with varying capacity. Thus, measuring protein content may be useful in screening virus stocks to be used for filter validation studies.

The benefit of using cleaner MMV stocks in virus-spiking studies can be clearly seen in Figure 3B. In the presence of a crude MMV spike, the volume of IgG filtered over time is significantly lower than for unspiked IgG. Spiking with clean MMV stocks, on the other hand, significantly improves filter performance such that the curves are similar to those for unspiked protein. In a validation study, that would lead to more accurate sizing of the virus filter. The crude MMV curve in Figure 3B indicates significantly more flow decay than with protein alone or with clean MMV stocks. As seen in Figure 3c, the "grab" (instantaneous) LRV with clean MMV spikes is about 0.5–1.0 $\log_{10}$ lower than with the crude MMV stock, particularly at high levels of flow decay. Those data are consistent with the idea that crude virus stocks contain contaminants that can foul virus filters by mechanisms different from those seen when using unspiked protein.

## Impact of X-MuLV Propagation Methods

Experiments similar to those above were conducted with X-MuLV. Crude X-MuLV stocks were prepared as outlined in Figure 4A. Briefly, cells were infected, then passaged four times in DMEM (Dulbecco's modified Eagle medium) with 2.5% FBS. Culture supernatant was then harvested and clarified by low-speed centrifugation, and the clarified supernatant was stored at –80 °C. As shown in Figure 4B, the procedure for making clean X-MuLV stocks was adapted from previously described methods (**12**), including an extra clarification step (0.45-µm filtration), virus pelleting by extended low-speed centrifugation, and storage in buffer with either no protein or a small amount as a stabilizer (0.01% BSA).

As with MMV above, crude X-MuLV stocks have higher protein content that correlates to lower capacity of spiked buffer (Figure 5A). Furthermore, crude X-MuLV spikes can significantly diminish the filterability of an IgG solution through NFP. Spiking with clean X-MuLV gives volume–time curves that are very similar to those for the protein alone. Both crude and clean X-MuLV were fully retained in these experiments (data not shown). Given the higher stock titers and lower impact on filter capacity, however, clean X-MuLV stocks have the potential to yield higher claimable LRV in validation studies. That is in addition to the obvious economic benefit of more accurate filter sizing.

## Discussion

The quality of the stock virus used in viral filter validation studies exerts a significant impact on filter performance. Crude virus preparations carry over proteins from their growth media as well as cellular debris from their hosts. Membrane fouling with crude virus stocks, probably attributable to several mechanisms, tends to reduce flow rate and total processed volume. In addition, flow decay leads to breakthrough, especially for small viruses.

The purpose of scale-down experiments is to mimic actual process conditions as closely as possible, and spiking with crude virus preparations fails to achieve this goal. Bioprocessors frequently overestimate their necessary process filtration membrane area based on premature filter plugging during such spiking studies. Moreover, the multiple mechanisms of filter plugging due to impurities in crude virus stocks may confound attempts to understand filter performance under conditions of actual use.

**Figure 4A:** Crude X-MuLV preparation method (typical stock titers 1e5–1e6 $TCID_{50}$/mL)

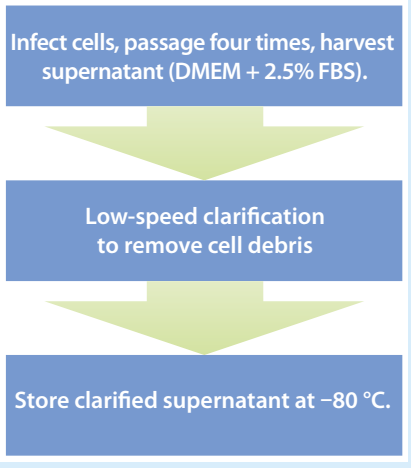
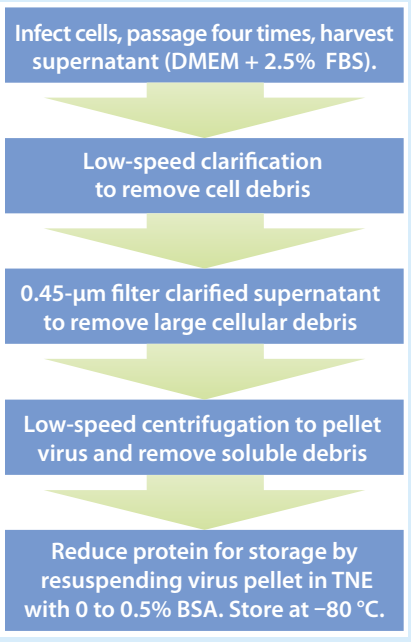



**Figure 4B:** Clean X-MuLV preparation method (typical stock titers ~1e7 $TCID_{50}$/mL)



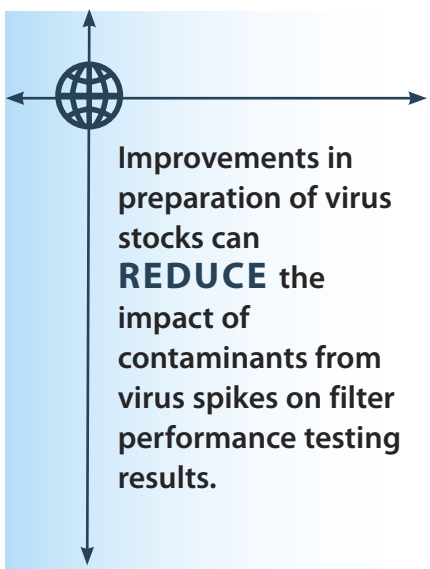



**Figure 5A:** Impact of virus stock protein concentration on capacity ($10^5$ $TCID_{50}$/mL challenge in FA buffer, pH 7.2, OptiScale 25 devices with Viresolve NFP membrane at 30 psi; Bio-Rad DC protein assay; control capacity >10,000 L/$m^2$)

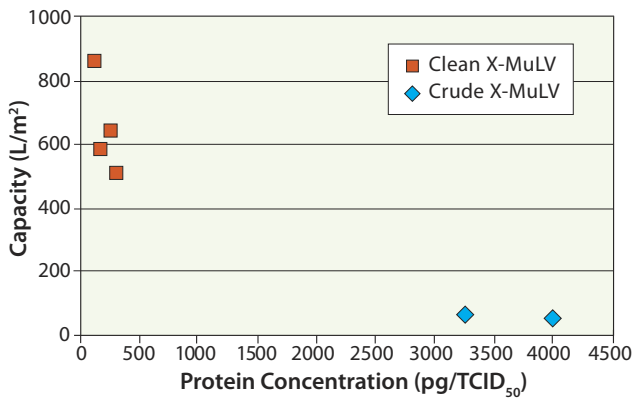
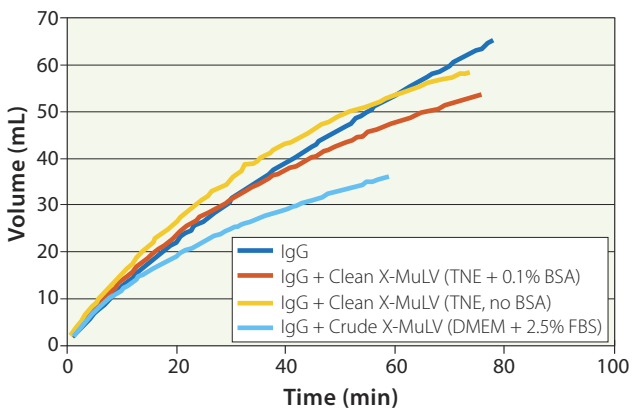



**Figure 5B:** Impact of X-MuLV preparation method on NFP performance (1 mg/mL IgG in FA buffer, pH 7.2, OptiScale devices with Viresolve NFP membrane at 30 psi)




Clearly, improvements in preparation of virus stocks can reduce the impact of contaminants from virus spikes on filter performance. By minimizing extraneous proteins and cellular debris, the clean preparation method results in virus spikes that better represent real-world process conditions.

Our company is continuing to develop methods to purify virus stocks used in filter validation studies. More work must be done to improve lot-to-lot consistency of virus stocks and further reduce the presence of proteins and other contaminants. Given the observed lot-to-lot variation even with clean virus stocks, it would be useful to investigate methods for measuring levels of other contaminants such as DNA and lipids that might adversely affect filter performance. Additionally, tools such as dynamic light scattering analysis to measure the size and monodispersity of virus particles should be used to verify the suitability of virus stocks for filter validation studies (**13**). It also may be beneficial to have industry standards either on virus purification methods or on the purity requirements for virus preparations. In the absence of such standards, it may be necessary to consider alternative spiking methods to deal with the impact of virus preparation contaminants on filter performance (**14**).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