Review

Pharmaceutical BIOPROCESSING

Pharm. Bioprocess.
(2014) 2(1), 75–83

Virus clearance methods applied in bioprocessing operations: an overview of selected inactivation and removal methods

Viral safety is an integral component of the process design for products derived from biological sources. While each step may contribute to viral safety, specific steps to remove and or inactivate potential viral contamination should be incorporated. The purpose of this article is to provide a brief overview to some of these specific viral clearance steps and how they may be applied in a manufacturing process. Each of these methodologies have a proven ability to remove or inactivate viruses, however selection and implementation of these methods is dependent on a variety of factors beyond the potential reduction achieved. The application of a selected method should be appropriately investigated and characterized before the implementation into the manufacturing process and subsequent viral safety assessment.

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Manufacturers of biopharmaceuticals are required to demonstrate that their manufacturing processes create products that are safe for use in humans. This approach is required for therapies, such as monoclonal antibodies, blood products, recombinant products, tissue-derived materials and some medical devices, where risk assessments identify a gap in the viral safety. One major concern for these types of products is the potential for viral contamination derived from the starting materials and/or raw materials. Manufacturers are therefore required to evaluate their process for the ability to remove and/or inactivate viral contaminants that may be present in their product.

When designing the manufacturing process, viral safety should always be a consideration. While many process steps have the capability to reduce potential viral contamination, the inclusion of specific steps to address viral safety should be considered. Preference is given to steps that can demonstrate robust viral reduction. Robust steps are those that demonstrate significant viral reduction/inactivation when performed under a wide range of operating conditions such as pH, protein concentration, ionic strength and temperature. It is the intention of this article to focus on those steps that can be considered as robust.

Virus clearance studies do not evaluate every step in a manufacturing process; only those that may be considered likely to contribute to inactivation and/or removal of virus. To segregate handling of viruses within a manufacturing environment, these types of studies are typically performed in specialized laboratories. Completion of these studies requires both expertise in virology and a detailed understanding of process steps in order to design a study that will meet regulatory requirements.

Designing a study

Virus clearance studies are executed to support products from Phase I through to product license [101]. The scope of these studies will be different for early phases compared with prelicense products. The specific design is well-documented in guidance documents including EMEA/CHMP/

FUTURE SCIENCE

10.4155/PBP.13.61 ISSN 2048-9145

Key Term

Log reduction factor: When evaluating viral reduction for a given process step, the reduction is the difference between the total virus in the spiked load sample and the total virus in the product-containing sample. The results of TCID50 assays are provided as Log_{10}/ml. Virus reduction is calculated as (ICH Topic Q5A (R1).

BWP/398498/2005 [1], ICH Q5A [102] and CPMP/BWP/268/95 [2], which all provide clear guidelines for the design of viral clearance studies.

Preliminary studies may also be conducted in advance of these regulatory studies to support optimization of critical process parameters.

Based on the European Medicines Agency guidance document for Investigatory Medicinal Products (IMP) [1], at early phase, there is no requirement to demonstrate viral partitioning. This approach has been adopted by other regulatory authorities, such as the US FDA [3], where at Phase I testing the load, load hold and product samples are generally considered to be sufficient. However, some additional testing at this stage may be beneficial to provide additional process characterization and to identify those steps with the capacity to remove viruses, which will facilitate the design of the prelicense study. For example, any step demonstrating less than 1.0 log_{10} reduction in this early study can subsequently be excluded in the later study.

The scale-down process used within the viral clearance study must be demonstrated to mimic the manufacturing process as closely as possible. When designing the study, care must be taken to ensure the critical process operations are covered. For instance, where the virus reduction filter is subjected to a pressure drop this should be included in the study with appropriate testing to characterize the impact. Pressure drops may be planned and controlled in a manufacturing environment, occurring when the filter may be de-pressurized between the end of product filtration and start of the rinse; however, pressure drops may also be unplanned, for example, where there is a malfunction in supply or equipment.

There may be occasion to complete additional validation studies to address process change, scale-up, deviations and viral contamination.

The study design must meet the regulatory requirements for the product at the appropriate development stage. There is clear guidance in the regulatory guidelines for IMP and for marketing authorization which outline the testing strategy. At early phase, where there may be subsequent changes in the process, there may be little value to include additional testing to evaluate viral partitioning. This approach is referenced in the IMP guidance document [1].

Proven methods of viral inactivation

Current regulatory expectations for developing a manufacturing process involve the incorporation of at least two orthogonal steps to remove or inactivate viruses. Inactivation is commonly included in a process, as they are typically simple and economical steps to implement; however, these steps are most effective in the inactivation of enveloped viruses.

There are a variety of inactivation methodologies that may be used within processes. The selection of these steps will be dependent on many factors, including product type, ease of use, cost and point of use within the process. **Figure 1** demonstrates the typical types of inactivation steps that may be applied in viral clearance studies (data from BioReliance, Stirling, UK).

In designing viral clearance studies, robust steps such as extremes of pH (low or high) may be considered. Solvent/detergent treatment, although traditionally used within the plasma industry, is being incorporated into recombinant protein processes, such as monoclonal antibodies, where the product may not be stable to pH extremes.

pH extremes (low pH, ≤4.0 & high pH, ≥11)

Within a manufacturing process, there may be exposure to pH extremes. Exposure may be direct product contact (e.g., low pH treatment of monoclonal antibodies following affinity chromatography); or indirect contact (e.g., high pH sanitization of chromatography columns).

The evaluation of viral inactivation following exposure to pH extremes has been studied for a wide variety of manufacturing processes, including monoclonal antibodies, plasma-derived products, recombinant proteins, vaccines and products derived from human or animal tissues. These steps have the potential to demonstrate robust viral reduction (i.e., >3.5–4.0 log_{10}) [4] with minimal influence of changes in the operational parameters within the defined limits. While these steps may provide good viral inactivation, consideration should be given to the stability of the product under these conditions, for example acidic clipping may be observed for monoclonal antibodies held at low pH for an extended duration.

Since these inactivation steps can be considered as robust steps, it is critical that robustness is demonstrated during the validation phase, both in terms of product quality and viral inactivation. Viral inactivation, by exposure to low pH, may be influenced by a limited number of factors, for example exposure time, pH range, protein concentration and temperature [4]. It is therefore important that these parameters, where considered critical, are evaluated within the

study. Figure 2 (data from BioReliance, Stirling, UK) demonstrates the inactivation kinetics for *Murine leukemia virus* (MLV) and *Pseudorabies virus* (PRV) at two different temperatures. The inactivation kinetics was evaluated within the range of 15–25°C, assessing the upper and lower limits of the specific range (BioReliance).

The first point to note, irrespective of the temperature, is that the inactivation kinetics for the two model viruses is different. PRV demonstrates a rapid inactivation within the initial minute of the hold with no residual virus detected thereafter. The rapid decrease in viral titer for the final time point was due to the use of a large-volume titration, increasing the assay sensitivity. The MLV inactivation kinetics clearly demonstrates a slower biphasic profile. Within the data set provided, it is clear that the inactivation kinetics for PRV were relatively unaffected by the temperatures assessed, whereas a slower rate of inactivation was clearly demonstrated for MLV at the lower temperature. This influence of temperature was more pronounced at higher pH values, as might be expected. In addition, BioReliance data demonstrate that a significant improvement (e.g., 2.0–2.5 $\log_{10}$) can be achieved with a small decrease in the test pH (e.g., by reducing the pH by 0.10 pH unit). Whilst the studies referenced above indicate PRV has a greater inactivation at low pH this should not be taken as a given and should be assessed on a case by case basis.

In addition to temperature, the sample matrix may also influence the inactivation kinetics. Figure 3 (BioReliance data) illustrates the inactivation of MLV (duplicate spikes) in three different buffer matrices (citrate, acetate and glycine). At the lower pH (pH 3.5), all buffers demonstrated a similar inactivation profile and overall log reduction factor. However, when the pH was increased to pH 3.9, there was a noticeable difference in the inactivation kinetics and overall log reduction factor for the three buffers. In-house experience from previous studies will be useful in selecting an appropriate buffer matrix meeting the process requirements for the step while providing an optimal background for viral inactivation.

The use of high pH solutions (0.01 to 1 M sodium hydroxide) has been demonstrated to be highly effective in the inactivation of a wide variety of viruses including nonenveloped viruses characterized with a high resistance to physiochemical inactivation, for example, *Murine minute virus* (MMV) and *Reovirus type 3* (REO). Sodium hydroxide solutions are generally used as column sanitization agents, where resin stability permits, and can be demonstrated to achieve >4 $\log_{10}$ inactivation for viruses such as MLV, PRV, MMV and REO (BioReliance data). The application

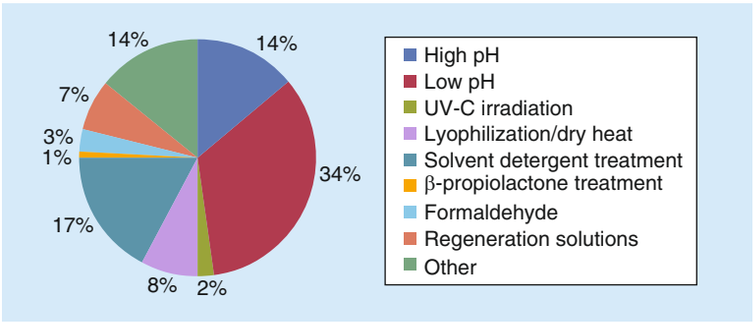

Figure 1. Inactivation step analysis.
Data from BioReliance, Stirling, UK.

of high pH sanitization solutions to a chromatography column (where applicable) will reduce the potential for 'carryover' of residual virus from the previous use. High pH may also be included as a direct product contact step within the manufacturing process (e.g., extraction of animal derived tissues) and can be successfully demonstrated to provide good viral inactivation with examples of >4 $\log_{10}$ for viruses, such as por-

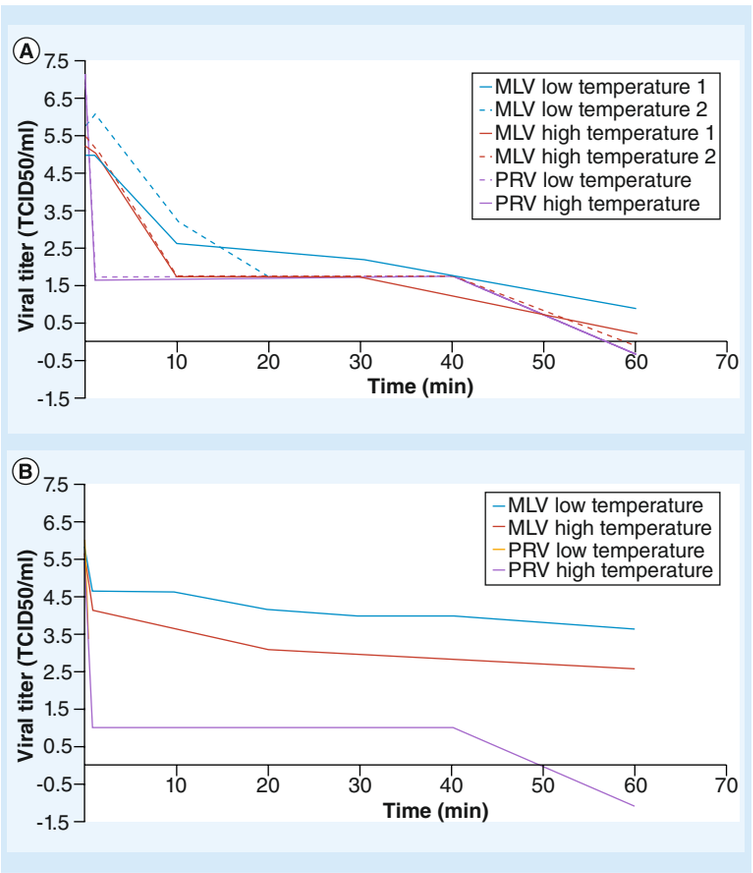

Figure 2. Inactivation kinetics for *Murine leukemia virus* and *Pseudorabies virus*. Temperature effects on inactivation kinetics at **(A)** pH 3.75 and **(B)** pH 3.85.
MLV: *Murine leukemia virus* ; PRV: *Pseudorabies virus*.
Data from BioReliance, Stirling, UK.

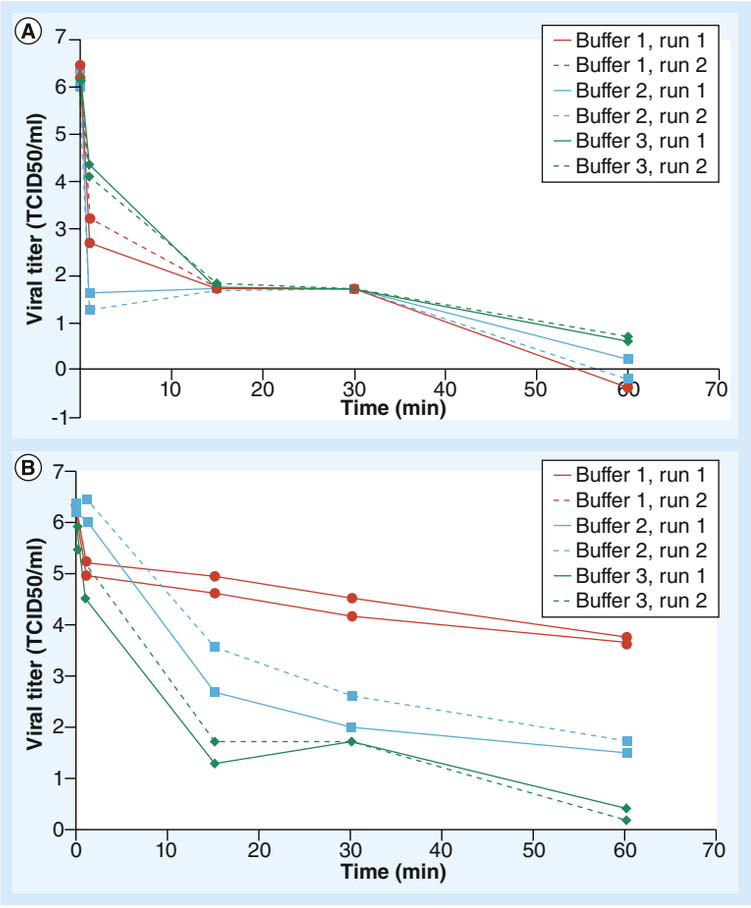

Figure 3. The influence of murine leukemia retrovirus inactivation kinetics in three different buffer matrices: glycine, acetate and citrate. (A) pH 3.5 and **(B)** pH 3.9.

cine parvovirus, MMV, bovine adenovirus and polio (BioReliance data).

» Solvent detergent treatment

Solvent detergent treatment is a very well-characterized method that can be applied for the inactivation of enveloped viruses. The methodology was developed in the early 1980s [5] with application in the reduction of pathogens in plasma-derived products detailed by Hellstern and Solheim [6]. Although initially developed for the plasma industry [7], this methodology has been applied to other products with equal success. Detergent solubilization is used frequently in the manufacture of viral vaccines. For example, influenza and solvent detergent treatment have been used in a number of recombinant protein manufacturing processes. The detergent disrupts the lipid structure of the viral envelope, and this process is enhanced by the presence of the solvent. The disruption of the lipid envelope renders the virus incapable of infection. Typically, Polysorbate 80 or Triton™ X-100 are used at a concentration of approximately 1% combined with tri-*n*-butyl phosphate at 0.3%. Under these conditions, viral inactivation in excess of 4 $\log_{10}$ (BioReliance data) can be achieved for a variety of enveloped viruses after just 1 h exposure time.

Key Terms

Parvovirus: Parvoviruses are known contaminants of Chinese hamster ovary cell fermenters, and are also potential contaminants of rodent-derived biopharmaceuticals. Recent studies have shown that parvoviruses are very common in bovine serum and may be present at high titer. Parvoviruses are very stable in the environment and resistant to inactivation by gamma-irradiation and other physical treatments. They pose a severe challenge to nanofiltration systems because of their small size (~18–24 nm).

Risk mitigation: Methodical and structured evaluation on the extent of exposure to a risk and/or the likelihood of its occurrence.

Risk mitigation & other methods of viral inactivation

» Risk mitigation

Risk mitigation against the introduction of viruses into the manufacturing process is key; constructing a robust risk assessment is necessary to evaluate the potential for viral contamination. Consideration must be paid to the raw materials used in cell culture; the removal or replacement of naturally occurring materials (e.g., animal derived) will significantly reduce the risk of adventitious viral contamination.

Detailed characterization of raw materials using the most up-to-date technologies for screening will identify potential contamination. An appropriate sourcing and raw material testing regime will minimize the risk of viral contamination. However, testing and careful sourcing of raw materials will not fully remove the risk of viral contamination, since testing methods have a limit of detection and are influenced by sampling limitations. Therefore, incorporation of robust inactivation techniques for the treatment of raw materials can provide additional contribution to the viral safety of the product.

» Other methods of viral inactivation

Treatments such as gamma irradiation, high-temperature short-time treatment (HTST) and UV radiation in the C range (UV-C) treatment are rapidly becoming more common steps within manufacturing processes. These steps have proven viral inactivation capabilities but may have technical challenges in developing suitable scale-down models where custom equipment has been fabricated in preference to using commercially available systems.

Gamma irradiation steps can be used as a risk mitigation strategy for raw materials, such as bovine serum albumin [8]. It may also be used as a post-purification

treatment, for example sterilization of medical devices [9], irradiation at 50 kGy has demonstrated a log reduction of >3.5 $\log_{10}$ for parvoviruses.

HTST of culture media has also been used as an effective method to prevent viral contamination in bioreactors [103]. In recent times, established companies, such as Genzyme (MA, USA) and Amgen (Cambridge, UK), have reported bioreactor contaminations that have resulted in plant closure to facilitate full decontamination. While these reported contaminations were contained and no affected product was released to the market, these events are potentially high impact to both the manufacturing organizations and patients. Robust investigations have taken place and preventative measures have been implemented as a result.

HTST treatments can be assessed by the use of bench-scale systems, which use microreactors to treat liquids at high temperature for short periods of time for assessment of viral inactivation.

Murphy *et al.* reported that HTST is an effective treatment for MMV inactivation when the contamination is at a low level in the bioreactor, however may be less effective where high levels of contamination is present [10]. Log reduction values of ≥3.0 $\log_{10}$ were reported when MMV spiked at 5% into medium was treated at 100°C for 60 s [10]. Murphy *et al.* have also reported that HTST is effective in combination with other risk-mitigation strategies such as control of raw materials, training, environmental monitoring and in-process testing.

UV-C treatment is a technology that has been used in the food, plasma and biotechnology industries. UV-C treatment can be assessed by the use of a sophisticated bench-scale system, which uses a helical reactor with a maximum wavelength of 254 nm. This system delivers a uniform treatment to the materials and can be controlled for consistent exposure at the dosage required. Materials challenged with parvovirus and UV-C treated have demonstrated >6.0 $\log_{10}$ reduction.

There have been papers published which demonstrate the effectiveness of UV-C in viral inactivation in recent years. In 2009, Bae *et al.* [11] reported that the non-enveloped viruses (HAV, PPV, BPV, MMV and REO) evaluated in their study were undetectable at 3000 J/m^2.

Many of these techniques are applied in the treatment of raw materials, and although viral inactivation is important, it is also critical to demonstrate that the treatment of the raw material does not affect the efficiency of the production system. This can be further supported by Schleh *et al.* [12] who have evaluated by high temperature short time treatment and UV-C treatment for their effectiveness in the inactivation of adventitous contamination of cell culture.

» Virus reduction filtration

Membrane filtration is a fundamental part of many purification processes. It is used within processes for recombinant proteins, but also within the vaccine industry to purify virus particles and viral vectors. In more recent years, filtration technology has been developed as a method to exclude viruses from process feed streams [13]. The early filters on the market focused on the removal of larger viruses, in excess of 50 nm in size. These filters were subsequently optimized to address the greater challenge of removing small robust viruses, and have been demonstrated to be capable of a 4 $\log_{10}$ reduction,(BioReliance) something that is difficult to achieve by other methods. The mode of operation for filtration may be tangential flow filtration or, more commonly, 'dead end' filtration (Figure 4). The mode of operation and therefore the filter selected may be application specific [14,15].

This technology has been widely implemented in recombinant protein processes where manufacturers have focused on the removal of serum from their upstream process. For more complex feedstreams, where the presence of serum may be required for product stability, nanofiltration may have a limited application due to filter clogging.

The viral reduction filtration landscape has changed over the years as new-generation filters have been developed and released. Figure 5 demonstrates the changes in filter popularity (based on validation studies conducted at BioReliance) between 2003–2008 and 2008–2012. Changes in popularity of a given filter type may relate to ease of operation and capacity to remove small viruses. Table 1 provides an overview of the most popular virus filters on the market.

There is a wide selection of virus reduction filters available on the market, with Asahi, Millipore, PALL and Sartorius as the key stakeholders. When selecting a viral reduction filter, many manufacturers will select a filter

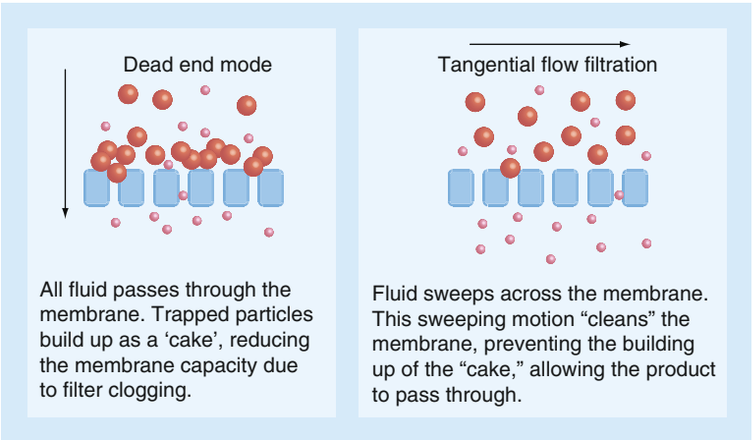

Figure 4. Mode of operation for filtration.

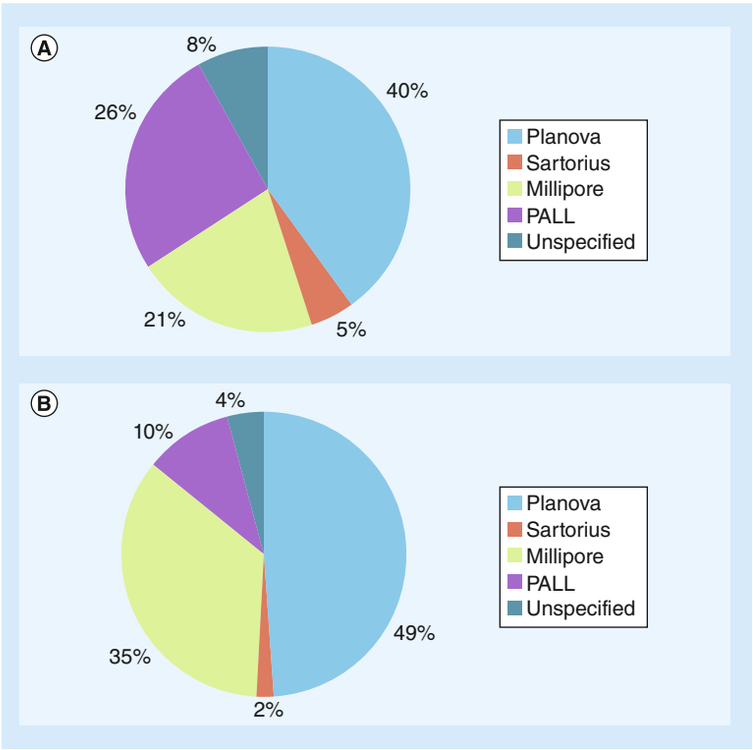

Figure 5. Breakdown of the types of viral reduction filters used over a 10-year duration. (A) 2003–2008 and **(B)** 2009–2012.

already in use within existing processes. For many applications, this may be acceptable, but care should be taken to select the most appropriate filter for a given product. The manufacturer should make use of the vendor expertise in the selection and optimization of their filtration process before moving into good manufacturing practice production. To get the maximum throughput and the best process economy, the viral reduction filter should be placed as close to the end of the process as possible. This will ensure that the feed stream is of a high purity, and it simplifies the segregation of the 'viral positive' and 'viral negative' processing within the facility.

There are a number of factors to be considered that may influence the performance of the viral reduction filter that should be considered during the development of the steps and subsequent assessment of viral reduction. These include: the quality of the feed stream, for example level of residual process impurities as well as product-related impurities, such as aggregates; the compatibility of feed stream with the filter media; the pre-filtration/preparation of the load sample; the quality of the virus spike; and the operational parameters such as filter capacity, pressure drop or cross flow and permissible flux decay [16,17].

Virus-reduction filters are designed as single use; therefore, it is important to operate the filters at a high capacity to minimize the filter area required to

Table 1. Overview of the key viral reduction filters on the market.

Product	Retention	Mode of operation	Material	Integrity test
Millipore				
Viresolve 180™	>6 log Retro	TFF	PVDF	CorrTest™
Viresolve 70™	>4 log Parvo	TFF	PVDF	CorrTest
NFR	>6 log Retro	'Dead end'	PES	Diffusion-based test
NFP	>4 log Parvo	'Dead end'	PVDF	Diffusion-based test
Viresolve Pro and Pro+	>4 log Parvo	'Dead end'	PES	Diffusion-based test
Sartorius				
Virosart® CPV	>4 log Parvo	'Dead end'	PES	Diffusion-based test
Virosart HC	>4 log Parvo	'Dead end'	PES	Diffusion-based test
PALL				
DV50	>6 log PR772	'Dead end'	PVDF	Diffusion-based test
DV20	>3 log PP7 >6 log PR772	'Dead end'	PVDF	Diffusion-based test
Pegasus® LV6	>6 log PR772	'Dead end'	PVDF	Diffusion-based test
Pegasus SV4	>4 log PP7	'Dead end'	PVDF	Diffusion-based test
Asahi Kasei†				
Planova® 35N	>6 log SV40	TFF or 'Dead end'	Regenerated cellulose	Leakage + gold particle
Planova 20N	>4 log PPV	TFF or 'Dead end'	Regenerated cellulose	Leakage + gold particle
Planova 15N	>4 log PPV	TFF or 'Dead end'	Regenerated cellulose	Leakage + gold particle
Planova BioEX	>4 log PPV	TFF or 'Dead end'	Regenerated cellulose	Leakage

PES: Polyether sulfone; PP7: 25 nm in size; PR772: 53–82 nm in size; PPV/Parvo: 18–24 nm in size; PVDF: Polyvinylidene fluoride; Retro: 80–110 nm; SV40: 40–50 nm in size; TTF: Tangential flow filtration.
†Dead end mode is the recommended mode of operation, although these filters can and have been used in the TFF mode.

process a batch. The feed stream composition in terms of product-related impurities (aggregate) and process impurities (e.g., residual host cell proteins and DNA) may all influence the filter throughput.

An appropriate pre-filtration strategy needs to be developed to 'protect' the viral reduction filter from clogging. This may be a simple size exclusion filter such as a 0.22 or 0.10 µm filter, or removal of contaminants from the absorptive properties of the filter matrix. In designing the study, it is important to consider whether the pre-filtration will be included 'in line' or disconnected during the spiking study.

Once the filtration capacity has been fixed, it is important that this capacity can be validated during the spiking study. The quality of the virus spike is therefore a critical factor in the validation goal. There have been many case studies presented by the Parenteral Drug Association [17] demonstrating how the viral spike quality can influence product throughput, flux decay and the log reduction factor. For viral reduction filtration, it is critical that purified virus spikes are used when evaluating this step [17].

The operational parameters preset for the filtration process may also influence the log reduction factor obtained where there is the potential for viral breakthrough, for example, small viruses such as MMV and PPV. Operation pressure, buffer pH and ionic strength are all factors that have the potential to influence viral breakthrough.

Vendors market all viral reduction filters with specific viral retention claims. For the small pore filters (20 nm in size), it is anticipated that viruses in excess of 20 nm will be retained with no viral breakthrough achieving in the order of 5.0–6.0 $\log_{10}$ reduction. For small viruses, such as parvovirus, it is anticipated that while some breakthrough may occur, typically a 4 $\log_{10}$ reduction can be achieved. Figure 6 summarizes the performance of a selection of commercially available filters with respect to retention of MLV (80–110 nm) and MMV (18–24 nm). For MLV, no infectious virus was detected in the filtrate sample, with the majority of the runs reporting log reduction factors in excess of 4 $\log_{10}$. Where samples were cytotoxic and required dilution prior to assay, a reduced log reduction factor was reported (2.0–3.99 $\log_{10}$).

The retention of parvovirus by these small-pore filters demonstrated an increased observation of lower reduction factors due to viral breakthrough. This was more apparent for NFP and DV20 filters where the filters were not evaluated in pre-encapsulated holders. The viral breakthrough observed could therefore be a result of the system setup. Despite some indication of breakthrough, it is clear that a minimum of 4.0 $\log_{10}$ for MMV is achievable for these small-pore filters.

Investigating the unexpected

Inactivation and viral reduction filtration steps are generally considered to be 'robust' steps within the manufacturing process with respect to viral reduction. In our experience, there have been occasions where viruses have been detected unexpectedly. For example, there have been a number of cases where little or no inactivation of retrovirus has been achieved at low pH (pH 3.8). In addition, apparent breakthrough of infectious virus particles has been observed during filtration where the virus particle size is larger than the reported filter pore size [18]. In such cases, a systematic and detailed investigation should be implemented to assess both the process and analytical activities. The outcome of this investigation will confirm whether the unexpected result was an artifact from processing or analysis, or whether the observation was a true representation of the capacity of the step to remove or inactivate the model virus.

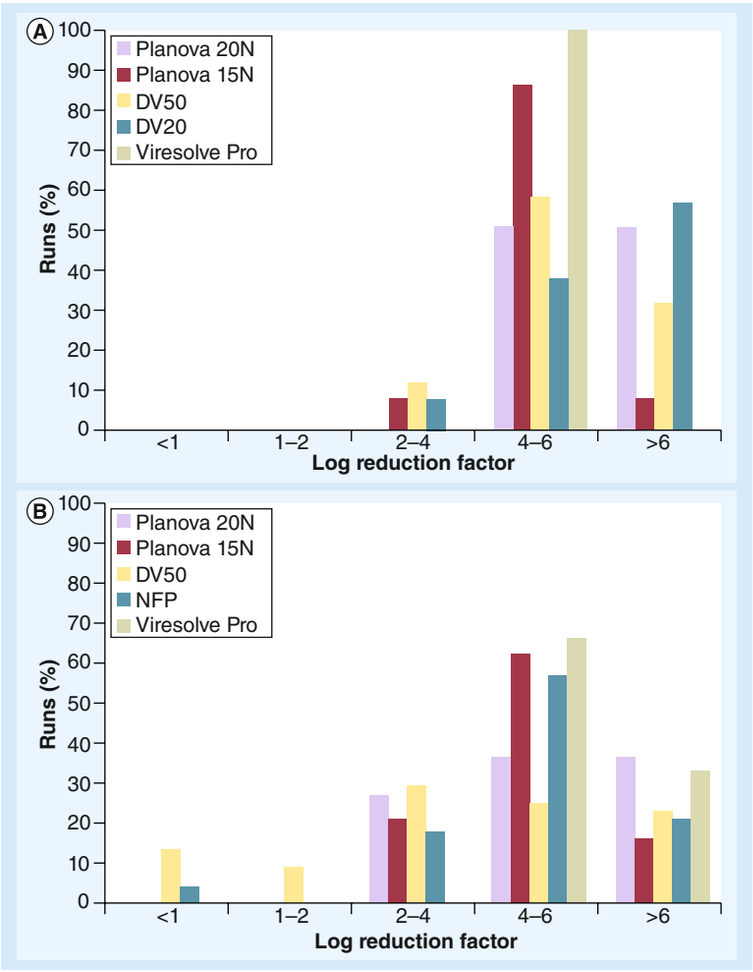

Figure 6. Summary of *Murine leukemia virus* and *Murine minute virus* retention by a selection of small-pore virus filters. (A) *Murine leukemia virus* and **(B)** *Murine minute virus.*

Conclusion

This article provides a summary of some of the key methods used toward achieving overall viral safety for biological products. While these approaches may not be considered novel, extensive characterization of these steps is being conducted within the industry to support the use of these steps as robust viral reduction steps.

Future perspective

It is likely that in the future with a greater understanding of the mode of action of these techniques; a more quality by design approach will be taken to viral clearance studies. This will facilitate the use of generic data to support the submissions of early-phase clinical products. As viral detection methods improve and new viruses are discovered, there is a changing landscape of potential viral contamination for which the classical methods of inactivation may not be as effective. We would anticipate the development of new physical or chemical inactivation techniques to be developed.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Executive summary

Study design

- Study design represents a critical stage in the evaluation of viral reduction. Studies should be designed to meet current regulatory requirements and to mimic the manufacturing process.

Proven methods of viral inactivation

- Viral inactivation steps should be included within the process design to meet with regulatory expectations.
- The choice of inactivation methodology will be based on application.
- pH extremes and treatment with solvent detergent have the potential to demonstrate robust viral reduction. However, consideration must be given to the influence of operational parameters and the effectiveness of the step and the stability of the product to the test conditions.

Risk mitigation & other methods of inactivation

- Routine testing and screening of substrates and raw materials can provide some level of assurance for viral safety. Detection is, however, limited by the sensitivity associated with these assays. Viral clearance studies are conducted on the downstream process to assess the ability of the method to remove viral contamination but increased focus has been placed on further reducing the risk of viral contamination by treatment of raw materials upstream in the manufacturing process by the use of inactivation techniques.
- Incorporation of upstream viral inactivation techniques can provide additional assurance of viral safety; however, it should be noted that some of these methods may not be suitable for all applications.

Virus reduction filtration

- Virus filters remove virus by size exclusion and have been demonstrated to remove $\geq$4.0 $\log_{10}$ of parvoviruses. (BioReliance data).
- Commercially available filters from different vendors have been demonstrated to perform in a similar manner with respect to virus retention; however, filter selection will be dependent on the specific application or requirements of the process.

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