For reprint orders, please contact: reprints@futuremedicine.com

Viral clearance for biopharmaceutical downstream processes

Viral clearance studies are mandated prior to entering clinical trials and for commercial launch of biopharmaceuticals. These studies are a key component of risk mitigation to reduce the potential for iatrogenic transmission of pathogenic viruses. This paper reviews regulatory guidance and practical strategies for designing viral clearance studies. Essential elements of a developmental phase-appropriate viral clearance package are detailed. These include scale-down model qualification, virus spike experiments and validation (clearance evaluation) of manufacturing process steps. Heuristics and learnings from available data are shared. Developments in this area including generic validation strategies, multiviral spiking strategies and use of newer model viruses for nonconventional substrates are also described. This review provides a framework for a comprehensive viral validation package for regulatory submissions.

Abhinav A Shukla*,[1]
& Hazel Aranha[2]
[1]KBI Biopharma, 1101 Hamlin Road, Durham, NC 27704, USA
[2]Catalent Pharma Solutions, 160 Pharma Drive, Morrisville, NC 27560, USA
*Author for correspondence:
ashukla@kbibiopharma.com

Need for viral clearance evaluation

The infectious capacity of viruses is well known; additionally, certain viruses cross the species barrier, and with current globalization as it relates to travel, viruses not indigenous to a particular location can enter into new environments [1,2]. Biopharmaceutical processes that produce product for human dosing need to be operated in a manner that significantly reduces the risk of viruses entering the final product. A variety of measures are taken to reduce this risk throughout the production process. A key aspect of risk mitigation is to conduct viral clearance spiking studies for several downstream purification steps to demonstrate the capacity and capability of the process to remove or inactivate known and unknown viruses. Contamination events in biomanufacturing are rare but can be catastrophic when they occur [3,4]; consequences of such events range the spectrum from impact on patient safety and drug shortages to legal, regulatory and financial implications [5,6]. The impact on manufacturing operations is significant; follow up on a contamination event includes investigation management, decontamination and other corrective actions [7]. Although viruses have been detected in bulk harvests and manufacturing environments [8-10], to date there have been no incidents of iatrogenic infectious virus transmission through cell line-derived biopharmaceuticals.

The International Conference on Harmonization (ICH) Q5A (R1) guidance [11] as well as the EMEA/CHMP/BWP/398498/2005 guidance [12] discuss the risk of potential viral contamination and approaches to be applied to ensure viral safety of biotechnology products derived from cell lines. Such contamination could arise from contamination of the cell line or from adventitious viruses introduced during production. The risk to cell culture products could arise from two sources: adventitious viral contamination either through contaminated raw materials or through introduction during processing or use of rodent cell lines known to express endogenous retroviral-like particles (RVLPs) although these are known to be noninfectious to humans. Usually, two types of RVLPs have been observed by transmission electron microscopy; C-type particles are most commonly observed in mammalian cell lines and the majority of these have been shown to be noninfective (e.g., in S^+L^- co-cul-

Key terms

Viral clearance: Elimination of target virus by removal of viral particles or inactivation of viral infectivity.

Risk assessment: A systematic process of organizing information to support a risk decision to be made within a risk management process. It consists of the identification of hazards and the analysis and evaluation of risks associated with exposure to those hazards.

Relevant virus: Virus used in process evaluation studies which is either the identified virus, or of the same species as the virus that is known, or likely to contaminate the cell substrate or any other reagents or materials used in the production process.

Specific model virus: Virus which is closely related to the known or suspected virus (same genus or family), having similar physical and chemical properties to those of the observed or suspected virus.

Process robustness: Ability of a process to tolerate variability of materials and changes to the process and equipment without negative impact on quality.

Nonspecific model virus: A virus used for characterization of viral clearance of the process when the purpose is to characterize the capacity of the manufacturing process to remove and/or inactivate viruses in general, for example, to characterize the robustness of the purification process.

tivation assays). C-type particles are commonly found in unprocessed cell culture supernatant. This baseline retroviral load is often used as a starting level for the calculation of excess logs of viral clearance required across a downstream process. Virus clearance is commonly expressed as $\log_{10}$ reduction value (LRV) units. **Risk assessment** is a cornerstone to ensuring viral safety for biotechnology products. Three complementary approaches have been adopted to prevent possible contamination of biotechnology products: Selecting and testing cell lines and other raw materials including cell culture media for the absence of viruses; viral clearance studies (a.k.a. virus spiking studies or viral clearance validation studies) which evaluate the capability of the downstream process to clear viruses and testing product at appropriate stages of the production for the absence of contaminating viruses. Table 1 highlights the factors to be considered in a risk assessment based on type of product, production system and raw materials used.

The cell line is regarded as a key risk for viral contamination either through the use of a contaminated cell line, through the use of contaminated reagents during cell line establishment or by issues during handling. Cell banks (master cell banks [MCBs] and working cell banks [WCBs]) are tested extensively prior to use in clinical production to document the absence of viral contamination. Additionally, cells are tested at the limit of their *in vitro* cell age that they would reach during production. Detailed guidance on the panel of expected cell line qualification tests is described in the ICH Q5A guidance. The acceptability of cell lines that contain viruses other than endogenous retroviruses is decided on a case-by-case basis. Unprocessed cell culture harvest bulks are required to be tested for adventitious viral contamination.

Viral clearance studies are a critical part of assuring safety of biopharmaceuticals. The following section describes regulatory expectations in terms of selection of a panel of viruses for the studies, process operations with potential to provide viral clearance, scale-down of the manufacturing process, ancillary studies that are conducted along with viral clearance and the calculation of LRV across a manufacturing process.

Regulatory expectations for viral clearance studies

Viral clearance studies assess the capability of the downstream purification process to clear **relevant viruses**. Unlike bacterial sterilization where an indicator organism is specified, there is no single indicator virus that can be used in virus clearance studies [13]. Hence a panel of viruses are used for viral clearance studies. The objective of viral clearance studies is to evaluate the ability of the manufacturing process to clear (inactivate/remove) known viral contaminants (**specific model viruses**), and to estimate **process robustness** by characterizing its ability to clear nonspecific 'model' viruses.
Specific model viruses are those that are closely related to the known or suspected virus (same genus or family), and having similar physical and chemical properties. **Nonspecific model viruses** are included to characterize the capacity of the manufacturing process to remove and/or inactivate viruses in general, for example, to characterize the robustness of the purification process to clear known and putative viruses.

Additional considerations for the selection of model viruses for the viral clearance studies include the titers to which the virus can be grown to, the presence of a reliable assay for the detection of viruses and any health hazards that the viruses may pose to personnel conducting the studies. Table 2 provides an example of a panel of viruses that could be selected for a viral clearance study for a murine cell line-derived product. It must be emphasized that if novel cell substrates are used (e.g., avian cell lines), the panel of viruses to be used must be evaluated.

Murine retroviruses are a key concern in a mouse or hamster cell line, despite the lack of propensity of most RVLPs for replication. A model for a murine retrovirus that is often employed for viral clearance studies is xenotropic murine leukemia virus (X-MuLV). Another commonly used virus is mouse minute virus (MMV).

Table 1. Risk assessment for viral safety.

Risk factor	Source	Comments
Type of product	Directly mammalian sourced, e.g., plasma-derived coagulation factors, immunoglobulins, other proteins and enzymes	High risk
	Recombinant proteins/monoclonal antibodies	Low risk
Production system	Well characterized cell lines (CHO)	Low risk
	Advanced Therapy Materials: gene therapy products	Medium/high
	Transgenic systems	Low/medium
Raw materials	Animal-derived additives: bovine serum albumin, transferrin	High
	Indirectly animal-derived: e.g., recombinant protein ligands produced in a microbial system supplemented with additives such as beef/meat extract	Low/medium

MMV is a small highly resistant nonenveloped virus that is included as a nonspecific model virus to demonstrate process robustness. It has also been implicated in the reported contamination of CHO cell cultures.

In the case of human cell lines, while the approach is similar in terms of using specific and nonspecific model viruses, there are some differences in the actual viruses that are used. A typical panel for a human cell line could include: herpes simplex virus, HIV-1, hepatitis A virus, porcine parvovirus and possibly encephalomyocarditis virus. Additionally, when working with human cell lines, for example, HEK293 cells, besides standard viral safety issues, special precautions should be taken because this cell line is susceptible to infection by human viruses. Rubino [14] reported a viral contamination event (with Rhinovirus) that occurred with the HEK-293 cell line even after a number of precautions were taken; this was attributed to a breach in current GMP (cGMP).

At the Investigational New Drug application (IND) submission stage, viral clearance studies are required to be conducted with at least two model viruses. Typically, one model retrovirus (X-MuLV) and one parvovirus (MMV) are selected. These studies are conducted in duplicate to demonstrate reproducibility of the clearance obtained [12].

At the commercial licensure filing stage, it is common to conduct the viral clearance studies, in duplicate, with at least three to five model viruses. Table 2 presents a representative panel of viruses that is commonly included for a Phase III study. Additionally, it is necessary to establish the clearance mechanism for the viral clearance steps. For example, in the case of chromatography steps it is necessary to obtain data for additional fractions from the process beyond load and elution pool. Mass balance must be established; flowthrough, wash, strip and regeneration samples are also analyzed to determine where the virus tends to elute. Sometimes, fractions immediately preceding and following collection of the elution peak are also analyzed to demonstrate robustness of the claimed clearance in case peak width varies during process operation.

If a significant change is made to the process (either cell culture or downstream purification), the impact on viral clearance needs to be assessed. Depending on the change, for example, increased product titer, change in position of a process step, supplementary studies may be required.

Biopharmaceutical downstream processes & heuristics for viral clearance validation

A variety of unit operations are sequentially employed for downstream processing [15,16]. Typical downstream processes can be divided into harvest and clarification steps that remove cells and cell debris from cell culture, and downstream purification steps including chromatographic steps, membrane filtration, viral removal filtration and ultrafiltration/diafiltration. There are a number of hold steps in the process conducted in a variety of container types. Less typically, processes may include flocculation or precipitation steps. A number of these steps can serendipitously provide viral clearance for the downstream process in addition to serving their purpose as purification steps from other process- and product-related contaminants.

Harvest depth filtration

Cell culture harvest and recovery operations typically utilize centrifugation and depth filtration steps followed by one or more microfiltration steps to provide a clear harvest supernatant that can be loaded on to chromatographic processing steps in the downstream process [17]. Among these, depth filters provide a high surface area and an adsorptive surface. It has been shown that depth filters can adsorb impurities from cell culture supernatants and improve the performance of downstream chromatographic steps [18]. While depth

Key term

Critical process parameter: A process parameter whose variability has an impact on a critical quality attribute and therefore should be monitored or controlled to ensure the process produces the desired quality.

filters can clear viruses [19], issues related to reproducibility of process scale-down and virus clearance with depth filters usually preclude validating them as viral clearance steps. Since depth filters are very low pressure devices, it is not clear if the flow distribution in large-scale depth filters would match those in a scale-down model, leading to concerns about whether robust viral clearance could be claimed. Nevertheless, between 2 and 4 LRVs have been reported with depth filters such as Millipore A1HC and Cuno 90ZA from 3M that are commonly used for cell culture harvest [20].

Low pH treatment

Low pH conditions are known to successfully inactivate enveloped viruses. This is particularly compatible with monoclonal antibody (mAb) downstream processes in which Protein A capture columns are typically eluted at low pHs [21]. Very often this low pH elution is followed by a low pH incubation step to inactivate viruses.

Generally viral inactivation steps are conducted between pH 3.0 and 4.0. It has been shown that robust viral inactivation requires a pH of 3.8 or below [22]. Since manufacturing operation requires a pH range, a typical range of 3.7 ± 0.1 pH units is employed with the viral study being conducted at the high end of the range to constitute the worst-case condition for this particular step. A recent publication lists a preference to conduct viral inactivation at pH 3.6 or below [23]. Certainly a more conservative choice of pH for this step is dictated by the stability profile of the product being dealt with. A typical time period for this step can extend up to 1 h in duration. At pH 3.8 or below, viral inactivation proceeds very rapidly and is often complete within less than 10–15 min. For all inactivation studies (e.g., low pH, detergent inactivation) kinetics of inactivation must be established.

The low pH condition is typically achieved by addition of a weak acid to the protein solution (e.g., dilute HCl, or higher concentrations of citric acid or acetic acid). Higher concentrations of a strong acid can create localized low pH environments in a large tank and result in aggregation or other issues that could potentially impact product quality. Weak base solutions are typically employed for neutralization after the step is complete since prolonged exposure to low pH conditions is deemed undesirable.

One key concern about this step is often termed as the 'hanging drop problem.' This refers to the demonstration that the entire batch of product intermediate that is to be inactivated goes to the low pH condition. Drops of liquid on the walls or roof of the vessel may not attain the low pH condition. As a result, it is preferred to pump the product intermediate into another vessel after pH has been lowered. This ensures that all the liquid in the second vessel attains the low pH required for inactivation.

Solution pH has been shown to be the **critical process parameter** for this step [22]. A robust inactivation of ≥4.6 logs of retrovirus was achieved when a pH of ≤3.8 was employed for an incubation period of ≥30 min at room temperature in a citrate or acetate buffer system with ≤500 mM NaCl and ≤40 mg/ml of protein. This step was employed in numerous IND and Biologics Licensing Application (BLA) submissions; however only 41% of these submissions claimed complete inactivation for this step [24]. It is presumed that less than optimal selection of pH conditions for this step led to validation studies that did not result in complete inactivation.

Solvent detergent or detergent inactivation

Products that cannot tolerate low pH conditions often employ solvent/detergent or detergent alone as chemical means of inactivating enveloped retroviruses [25]. Solvent/detergent treatment was one of the mainstay methods in the plasma-derived products industry; it is also used for inactivating enveloped viruses in recombinant protein products, especially if the product cannot tolerate low pH conditions. Combinations of tri-n-butyl phosphate (TNBP; 0.1–1.0%) with detergent (Triton X100 or Tween 80; 0.5–1.0%) are commonly employed although

Table 2. An example of a panel of viruses used for viral validation studies for murine cell line-derived products.

Virus	Genome	Enveloped	Size (nm)	Resistance
Murine leukemia virus (MuLV)	ssRNA	Yes	80–120	Low
Pseudorabies virus (PRV)	dsDNA	Yes	150–200	Low to medium
Reovirus 3 (Reo 3)	dsRNA	No	60–80	High
Mouse minute virus (MMV)	ssDNA	No	18–25	Very high

effective retroviral inactivation can be achieved with detergent alone as well. Detergent only inactivation was prominently featured in the 2011 Symposium on Viral Clearance held in San Francisco (CA, USA). Critical process parameters are temperature and time, with low temperature and short time being worst-case conditions [23,26].

Chemical inactivation requires the clearance of the agents employed for the inactivation step. As a result this step is conducted during the beginning stages of the downstream process so that multiple chromatographic and filtration steps can provide clearance of the inactivating agent. It is possible that residual amounts of detergent may remain bound with the product despite these clearance steps. A residual assay is typically conducted on drug substance to assess this. Another key aspect is the possibility of solvent and detergent interfering with the performance of a chromatographic step, particularly one that immediately follows the inactivation. This could manifest itself in the form of altered purification attributes for this step as compared with the same step conducted without detergent in its load or could manifest itself in the form of decreased resin lifetime due to build-up of detergent on the resin. Modes of chromatography that are most successful in resisting interference from detergent include ion-exchange and affinity modes of purification such as Protein A chromatography. The latter is commonly used for Fc fusion protein purification and can be preceded by solvent-detergent treatment in the cell culture supernatant. More hydrophobic resins such as hydrophobic interaction or mixed mode resins should typically be avoided immediately after the detergent-based viral inactivation.

Considerations similar to low pH inactivation in terms of the ‘hanging drop’ also apply to solvent-detergent based viral inactivation procedures.

Another precaution that needs to be taken while evaluation viral clearance for solvent/detergent steps is the potential for cytotoxicity to the reporter cell line used to assay for virus in the infectivity assay format. These cytotoxicity effects often necessitate significant dilution of these samples prior to analysis, resulting in a reduced reportable LRV from a solvent/detergent inactivation step.

If solvent/detergent treatment is conducted on harvested cell culture fluid, the presence of residual amounts of cell debris and lipids could potentially impact the efficacy of this step [26]. In general, variation in lipid content due to cell culture viability variations at harvest only impacts kinetics of the viral inactivation step and produces a small effect of <0.5 LRV. It has been shown that Triton X100 is typically the most efficacious detergent for viral inactivation.

Viral filtration

Filtration through virus retentive filters is currently a key unit operation during the production of biopharmaceuticals [27-30]. Careful process design and appropriate validation are critical for the successful implementation and performance of virus retentive filters [13]. Detailed information related to factors impacting virus clearance by virus retentive filters and performance attributes are provided in a comprehensive technical report [31] published by the Parenteral Drug Association.

While virus filtration has been demonstrated to be reliable for larger retroviral particles, removal of small viruses such as parvovirus by the small pore size filters (in the range of 15–20 nm) is less robust [32]. Studies have to be done to ensure that there is no bleed-through of parvovirus when virus filters are used [33]. Data for virus retentive filters have been summarized in a publication by Miesegaes *et al.* [34]. Process operating ranges and depressurization have been reported to impact virus bleed through for virus-retentive filters designed to remove parvoviruses [35]. Sustained pressure excursions had a greater impact on log reduction values than the pressure differential (dP).

In viral spiking studies, the quality of the virus stock solution has a significant impact on the performance of virus removal filters [36]. Highly purified stock solutions can ensure that the viral filters do not clog due to particulates from the spiked viral preparations and result in significant reductions in load filterability that does not represent what the viral filters are capable of filtering in terms of process intermediate. Since viral filters are operated solely to serve as a dedicated viral removal step, they are typically sized based on the volumetric loading during viral validation studies and not in terms of what is possible for the process intermediate alone. This can lead to a situation in which the filters need to be oversized in actual manufacturing operation reflecting the low volumetric throughput that was achieved in the validation study. A low volume spike (0.01–00.1% v/v) is generally preferred for parvoviral grade filters to avoid clogging the membranes and artificially reducing the volumetric loading on these filters per unit surface area. An alternative strategy is based on the mechanism of viral breakthrough on these membranes [37]. It is postulated that smaller pores clog first channeling the viral particles through the larger pores leading to viral breakthrough. This preferential clogging is reflected in the % flux decay as compared with the initial flux and it is recommended that the viral filters be loaded to a certain % flux decay in both viral validation and actual process operation. Since the same % flux decay is reached much later in actual process operation as compared with the viral spike

experiment, this enables process operation to continue to higher volumetric loadings. Operation of the viral filter to a specific % flux decay level has attracted some interest although most organizations still prefer a more traditional approach relying upon volumetric loading per unit membrane surface area.

Chromatographic steps

Chromatographic steps can provide effective resolution of the product species from many different kinds of impurities and this can include viral particles that either flow through the column or are bound tightly on the resin. Mechanisms for viral clearance need to be established on chromatographic steps. While all chromatographic processing steps may provide some viral clearance, ones that provide the most clearance and are typically validated for viral clearance include affinity chromatographic steps (e.g., Protein A) and ion-exchange chromatography, particularly anion-exchange chromatography.

Protein A chromatography provides a high degree of selectivity for mAbs and Fc fusion proteins and is widely employed for their purification [21]. Being an affinity step makes this selective for product relative to a wide range of impurities including viral particles. Most viral particles have been shown to flow through Protein A columns without binding [38]. Protein A chromatography can routinely provide 4–5 logs of retroviral clearance [24] but significant variability has been noted with different products. The number of logs of viral clearance on chromatographic steps is known to be highly dependent on the viral titer and spike % used in the load material. For steps that are capable of providing good clearance, using a high titer spike in the load material can significantly increase the number of LRVs that can be claimed. Other factors that could influence the clearance may also relate to the number of column volumes of washes and their buffer composition. A key consideration for Protein A viral clearance validation is the fact that the low pH conditions used for product elution can also inactivate retroviruses. However, if low pH viral inactivation is being claimed as a separate step, inactivation obtained during low pH elution cannot be included as the mechanisms would be identical and the regulations stipulate that orthogonal unit operations only be included in claiming the cumulative viral clearance for the manufacturing process. In such a situation, it is important to utilize a viral assay that can detect viral particles irrespective of whether they are lysed or not due to low pH conditions. Quantitative PCR (q-PCR) is often employed as the analytical technique since it is based on the detection of viral nucleic acids rather than the presence of an intact viral particle capable of lysis, as is the case in a viral plaque assay [39]. Protein A chromatography has been shown to be a robust viral clearance step over a large number of operational cycles. It has been demonstrated that other performance attributes such as step yield and product breakthrough during the load step are more likely to decay before viral clearance begins to deteriorate [38]. This could potentially justify not having to conduct studies on this mode of chromatography before and after the resin has been used to the full extent of its lifetime.

Anion-exchange chromatography is known to be a robust means of retroviral clearance [40,41]. This step can also provide >4 LRV under the right conditions. It has been shown that LRV on this mode of chromatography is highly dependent on the load conductivity, with higher conductivities resulting in low clearance as the viral particles begin to elute from the column. As a result, this is a preferred viral clearance step particularly for mAbs, which are typically basic proteins that can flow through anion-exchange columns. Conditions for generic viral clearance on anion-exchange chromatography on Q Sepharose FF in flowthrough mode have been developed [40] which include operation between pH 7.0 and 8.5, a loading conductivity of 4.6–12 mS/cm, linear flow rates between 76 and 600 cm/h, use of a Tris-buffered system, bed height of ≥11 cm and column loading of ≤250 g/l. A large-number of cycles (up to 120) are possible on anion-exchange chromatographic media if appropriate regeneration and storage conditions are employed [42].

The concept of viral clearance by anion-exchange chromatography has been extended to membrane adsorbers as well [43]. Greater than (>)5 LRV of clearance was obtained for a panel of viruses including retroviruses and parvoviruses while using a Sartobind Q membrane chromatography module. Since Q membrane chromatography requires relatively low load concentrations to enable effective binding of impurities (DNA, virus, HCPs), salt tolerant membrane adsorbers have also been developed [44] and launched commercially as the STIC membrane adsorbers from Sartorius-Stedim. These membrane adsorbers were shown to achieve high LRVs irrespective of load salt concentrations of up to 150 mM. Additionally, adsorptive membrane chromatography where the matrix is composed of a filter rather than beads has been used; these often have anion-exchange chromatography functionality [45]. Monolithic columns in which the entire chromatography column is polymerized at one time instead of being packed with beads offer potential flowrate and throughput advantages [46]. Their viral clearance properties ought to be similar to those achieved on chromatographic resins and membrane adsorbers depending on the chemistry being used.

Other modes of chromatography such as cation-exchange and hydrophobic interaction chromatography have been shown to provide lower LRVs than affinity chromatography or anion-exchange chromatography. Depending on process conditions, typically LRVs of 1–3 logs are obtained [47].

Resin cycling

Licensure (BLA/MAA) filings require demonstration of performance on new and used resins in terms of their viral clearance capability. This is aimed at ensuring that resins that have been used for multiple cycles are still capable of providing suitable viral clearance for the downstream process. Since it is rare for large-scale chromatographic columns to have reached the end of their predicted lifetime in terms of the number of cycles they are used for during clinical manufacturing, an artificially aged resin is often generated by operating the planned number of cycles across it at a smaller scale. The viral clearance capability of the used resin is then compared with that obtained on an unused chromatographic resin. Several publications have addressed these comparisons [38,42,47-48]. In most cases no significant decrease in viral clearance was observed prior to the decline of other performance attributes. Since consistency of these attributes is typically what the process is designed for, the risk to viral clearance from column reuse appears to be low. Nevertheless, providing documented evidence for this is the norm for licensure filings.

Case studies for early- & late-stage viral clearance regulatory submissions

The objective of virus safety studies for biotechnological investigational medicinal products (IMPs) is to demonstrate an acceptable level of safety for use in clinical trial subjects. As mentioned in the 'guideline on virus safety evaluation of biotechnological investigational medicinal products' [12] testing should be conducted according to the principles of ICH Q5A [11]; however, a demonstration of robustness is not required for early phase studies.

Most commonly, for IMPs, virus validation studies are done with a specific model virus (e.g., X-MuLV if the cell line is murine) and a small virus, for example, MMV. At least 2 orthogonal unit operations are evaluated. Filings in the EMA typically include duplicate runs. Demonstration of robustness may not always be warranted at the IMP stage. Any clearance of less than 1 $\log_{10}$ is generally not included when calculating cumulative log clearance for the entire process.

An example of typical viral clearance numbers seen for a CHO-derived mAb downstream process is shown in Table 3. Duplicate runs were conducted for each step with each of the two viruses to help provide an idea of robustness in the viral clearance observed. As can be seen in the table, the duplicate runs agreed well with each other in terms of the log virus removal (LRV) obtained. Overall, over 18 LRV was obtained for X-MuLV. MMV being a small nonenveloped parvovirus is usually more difficult to clear since low pH conditions do not inactivate it. Over 10 LRV was obtained for this virus.

An often asked question is whether a certain number of logs of clearance are adequate for moving a program into the clinic. The answer is that there is no firm guideline that addresses that on an unequivocal basis. The nature of the cell line, dose as well as patient population the drug is used for and the steps in the downstream manufacturing process all factor into that assessment. Nevertheless, a goal that is typically applied in the biopharmaceutical industry is to obtain at least 4–6 logs of excess clearance for a model retrovirus. The calculation is based on the total logs of RVLPs that are present in the cell culture harvest supernatant. Box 1 provides an example of how the safety factor can be calculated for a model retrovirus. The essential purpose of this calculation is to find the probability of a single viral particle getting into a single dose of a product. The 4–6 logs excess clearance essentially means that only one dose in 10^4–10^6 doses can contain any viral particles.

For marketing authorization (late stage), the objective of the studies is not only to document clearance of viruses that could potentially be present in the bulk harvest by the use of specific model viruses, but also, to demonstrate the robustness of the manufacturing steps to clear any adventitious viruses that could potentially gain access to the product during purification and handling. As a product enters Phase III clinical trials, it is typical to expand the number of model viruses that are evaluated in viral clearance studies. Typically, the panel includes four to five specific/nonspecific viruses and three to five processing steps are evaluated in order to demonstrate that there is an adequate safety margin from a virus safety standpoint. Unit operations evaluated include two to three chromatography steps (anion, cation, hydrophobic interaction), detergent inactivation/low pH and virus removal filtration. Regulatory agencies also typically expect data that speaks to the mechanism by which each step clears viral particles. As a result, it is typical to determine viral titer in more than the load and elution fractions alone. The testing is typically extended to column flowthrough, wash, postelution and strip fractions as well. This helps determine where most of the virus is present and establish whether a particular virus binds weakly or strongly to a chromatographic resin under the conditions it is operated under. Understanding the mechanism by which a given step clears viruses is important to be able to speak to its robustness. As an example,

Table 3. Typical table of viral clearance obtained across various process steps in a downstream process sequence for a monoclonal antibody.

Process step	XMuLV run #1	XMuLV run #2	MMV run #1	MMV run #2
Protein A chromatography	2.78	2.71	1.97	2.04
Low pH inactivation	5.81	5.39	Not tested	Not tested
Polishing step #1	1.97	2.03	0.69[†]	0.79[†]
Polishing step #2	≥3.22	≥3.34	4.10	4.17
Viral filtration	≥4.96	≥4.97	4.52	4.63
Total LRV	18.74	18.44	10.59	10.84

Two viruses (XMuLV and MMV) were tested for a typical IND filing package for entry into clinical trials.
Not included in total LRV calculation as clearance <1 LRV is not regarded as robust.
IND: Investigational new drug application; LRV: Log_{10} reduction value; MMV: Mouse minute virus; XMuLV: Xenotropic murine leukemia virus.

in a late-stage viral clearance study, one would often collect pre- and post-elution peak fractions and determine viral titer. This can help assess if the step will still provide robust viral clearance if the elution peak elutes earlier or tails resulting in an increase in the number of elution column volumes. Another key aspect of a viral clearance package for marketing authorization for a biopharmaceutical is the inclusion of viral clearance studies conducted on resin that has been aged to the maximum extent of its allowable number of reuses in the manufacturing process [48]. Aged resin to conduct such a study is typically derived from scale-down column cycling studies that establish the useful lifetime of chromatographic resins since it is unlikely that resin used in clinical manufacturing would have seen the maximum extent of its useful lifetime.

Innovations & opportunities in viral clearance & virus safety assurance

Multiple innovations are occurring in the field of biopharmaceutical downstream processing [49]. Several of these will clearly result in innovation in viral clearance evaluation as well.

A key challenge in bioprocessing continues to be optimization of product yields through upstream and downstream innovations without adversely impacting product quality and safety. While continuous processing has been applied in several industries (petrochemical, chemical, food) the pharmaceutical industry has so far been risk-averse to methodologies that do not lead to a clear designation of a batch. However, there is increasing interest in applying continuous bioprocessing to the manufacture of biologics. Hybrid systems (continuous upstream/batch downstream; batch upstream/continuous down-stream; continuous bioreactor and capture followed by batch (post-capture) downstream), as well as fully integrated continuous processes are being evaluated. It will be necessary to evaluate and establish robust viral clearance in processes that can operate continuously during manufacturing [50]. It is anticipated that steps such as viral inactivation and viral filtration would still be conducted in a batch mode for continuous processes. While viral clearance capabilities of a chromatography process operated in a continuous mode should not be substantially different from that of a batch process, such data will have to be generated to support regulatory acceptance of continuous purification processes.

Affinity chromatography with novel ligands that bind specifically to target viral particles have been developed and commercialized. Trimeric peptide ligands with significant selectivity for MMV have been developed [51]. We should note, however, that ICH Q5A specifies that virus clearance validation should include nonspecific-model viruses to evaluate the robustness of the process.

Multivirus spiking assays using q-PCR assays are increasingly being adopted for validation of unit operations that remove viruses (e.g., chromatography) but not those that inactivate viruses (e.g., pH/ detergent inactivation) [52]. Multiplex PCR assays allow simultaneous amplification and quantification of more than one viral template and are achieved using separate probe and primer sets for each target sequence. However, careful optimization of assay parameters and primer/probe design is essential [52]. Multiviral spiking can make the execution of viral clearance validation studies significantly more efficient in terms of time and the amount of protein that is needed to conduct them. This can be quite significant in the case of low titer, low production volume products (e.g., vaccine proteins) such that the amount sampled for viral clearance studies does not become a significant proportion of the total protein in a batch.

Other innovations have occurred in the panel of viruses being used. High titer viruses can enhance the amount of viral clearance obtained for a given process step. Bacteriophage PR772 has been proposed as a high titer alternative to standardize pore sizes for large-pore viral filters [53]. In size-based applications such as virus

removal filtration, bacteriophages may be used to establish a product's **design space**. Some phages (e.g., bacteriophages PR772 and PP7) have been well characterized and possess physical properties (i.e., size, isoelectric point, etc) close to their mammalian counterparts. Design space conditions established with a phage is considered predictive of filter clearance of the corresponding mammalian virus. Note, however, that current regulatory expectations for filter validation studies for submission to regulatory authorities use mammalian viruses [34]. Bracketing or generic approaches may be appropriate for certain unit operations that provide robust viral clearance. American Society for Testing and Materials (ASTM) has also proposed standardized approaches for viral clearance. Such approaches can potentially obviate the need for viral spiking studies for certain product classes when certain steps are operated under conditions that are understood to be robust for viral clearance. For example, removal of SV40 by Q-anion exchange chromatography has been demonstrated [40]. Similarly, low-pH inactivation at pH ≤3.8 consistently achieves ≥4.6 LRV for XMuLV [38]. Since mAbs as a product class lend themselves to a **platform approach** for downstream processing, it is conceivable that significant efficiencies can be achieved as generic clearance is accepted by regulatory health agencies around the world.

A **quality-by-design (QbD)** regulatory submission has been advocated for a while; however, until recently no submissions were reported. Bell reported on the first US FDA approved viral clearance design space [54]. In general, Brorson *et al.* [55] recommend that one factor in successful acceptance of using a quality-by-design approach for virus clearance is to proactively raise the issue with the regulators.

The current approach to virus safety assurance has stood the test of time. Miesegaes *et al.* [24] in their process of data mining queried their database to determine whether viral clearance as measured by the metric of LRV has improved over time. They reported no specific uptrend for either retroviruses or parvoviruses clearance and cautioned that this not be interpreted as an absence of increasing process understanding or technological progress but rather that currently used unit operations provide the right strategy to address biopharmaceutical safety.

Key terms

Design space: The multidimensional combination and interaction of input variables (e.g., material attributes) and process parameters that have been demonstrated to provide assurance of quality.

Platform approach: The approach of developing a production strategy for a new drug starting from manufacturing processes similar to those used by the same applicant to manufacture other drugs of the same type (e.g., as in the production of monoclonal antibodies using predefined host cell, cell culture and purification processes, for which there already exists considerable experience).

Quality by design (QbD): A systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management.

Conclusion

Providing a definition for virus safety is nebulous at best. Zero risk is a myth. Anecdotal evidence and systematic assessments have attested to the fact that biotech products have had an excellent safety record. This can largely be attributed to three key strategies of: adequate sourcing and testing of materials and cell banks, documentation of viral clearance and in-process viral testing. This review describes regulatory expectations for viral clearance studies in a clinical development phase appropriate way. In addition, scientific principles and heuristics in the design of viral clearance studies for specific operating steps in downstream processing are described. Finally, a variety of innovations that can help improve viral clearance for bioprocesses are described.

Box 1. Example of a safety factor calculation for retroviral clearance.

- Retroviral like particle count for cell culture supernatant (typically highest of three measurements if that many runs have been conducted in manufacturing) = 8.6×10^8 retroviral-like type C particles/ml
- Dose = 10 mg/kg
- Average patient weight = 75 kg
- Dose = 0.75 g product
- Product titer = 1 g/l
- Downstream process yield = 50%
- Hence 1.5 l harvest supernatant translates into one dose of product
- 1.5 l harvest contains 1.29×10^{12} retroviral-like particles
- To obtain a 4–6 logs safety factor, one would need to obtain clearance of 1.29×10^{16} to 1.29×10^{18} retroviral-like particles
- Hence 16–18 logs of XMuLV clearance are needed
- The example shown in Table 3 meets this criterion for providing adequate retroviral particle clearance

Future perspective

Virus safety assurance of biologicals is far from being a stagnant field of endeavor. Methods for clearance and inactivation are evolving at an increasing pace. By 2020, a significant percentage of biopharmaceuticals available today will be off patent, but indicators of the accelerating development and approval of biopharmaceuticals strongly suggest that there will be a robust production of many newer biotherapeutics that will be on patent by that time [56]. Advances in safety procedures will proceed in lockstep with the continuing development of biopharmaceuticals. Such an effort will be necessary because newer biopharmaceuticals are likely to change in character and be associated with different sets of risks, especially since new contaminants are likely to be discovered. Of an estimated 150,000 viruses, only 5,000 have been detected to date, and viruses are constantly evolving [4]. Additionally, new blood-borne virus infections are being reported and each will need to be addressed in safety analyses and purification processes.

Executive summary

Background

- Demonstration of the ability of the manufacturing process to clear known and putative viruses is mandated prior to entering clinical trials and for commercial launch of biopharmaceuticals.
- These studies are a key component of risk mitigation to reduce the potential for iatrogenic transmission of pathogenic viruses.
- The viral risk profile of any biological is contingent on a variety of factors including: source of the biological, raw materials used, production systems, purification reagents and excipients.

Regulatory expectations

- Typically, the panel includes unit operations evaluated include two to three chromatography steps (anion, cation, hydrophobic interaction), detergent inactivation/low pH and virus removal filtration.
- For initiation of clinical trials (IND submissions), viral clearance studies are required to be conducted with at least two model viruses for biopharmaceutical products. Typically, one model retrovirus (X-MuLV) and one parvovirus (MMV) are selected.
- At the commercial licensure filing stage, viral clearance studies are commonly conducted with 3–5 specific/nonspecific viruses and three to five processing steps are evaluated in order to demonstrate that there is an adequate safety margin from a virus safety standpoint.
- It is necessary to establish the clearance mechanism for the viral clearance steps. For products in clinical development.

Biopharmaceutical downstream processes with potential to provide viral clearance

- Virus clearance methods are classified as either inactivation methods (physical/chemical methods) and removal methods (virus filtration, chromatography).
- A number of these steps can serendipitously provide viral clearance for the downstream process in addition to serving their purpose as purification steps from other process- and product-related contaminants.

Innovations & challenges in virus safety assessment

- Biopharmaceutical downstream process development is far from being a stagnant field of endeavor. Methods for virus clearance and inactivation are evolving at an increasing pace.
- Bracketing or generic validation of viral clearance may be used for certain unit operations that provide robust viral clearance.
- Bacteriophages may be used as surrogates for mammalian viruses, especially to define the design space of a manufacturing process.
- Other advances in upstream and downstream processing include continuous bioprocessing and chromatography monoliths to increase efficiency. These willl need to be evaluate for their impact on virus clearance.
- Multivirus spiking assays using quantitative PCR assays are increasingly being adopted for validation of unit operations that provide virus removal but not methods that inactivate viruses.

Going forward

- Biopharmaceuticals, from a virological safety standpoint, have had an excellent safety record.
- This excellent safety profile has not been easy to achieve and can largely be attributed to the viral safety tripod strictly adhered to in the industry: adequate sourcing, documentation of virus clearance evaluation (virus validation studies) and in-process testing.
- New biopharmaceuticals produced in novel cell substrates are likely to change in character and be associated with different sets of risks, especially since new contaminants are likely to be discovered.
- Ultimately, the protection of patients is of paramount concern. Current indications are that as new threats to safety appear, the in-built flexibility and evolutionary nature of risk management strategies will be able to continue to ensure the safety of biopharmaceuticals.

The unrelenting potential for the appearance of new viruses is enhanced by the dissolution of global boundaries. Pathogens can now travel to locations that were not heretofore considered indigenous to them. Because of this globalization and the many viruses still undiscovered, vigilance and the ability to clear even the viruses we cannot yet detect must remain high.

Ultimately, the protection of patients is of paramount concern. Current indications are that as new threats to safety appear, the in-built flexibility and evolutionary nature of risk management strategies will be able to continue to ensure the safety of biopharmaceuticals.

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.

References

Papers of special note have been highlighted as:
•• of considerable interest

1 Arzt J, White W R, Thomsen BV, Brown CC. Agricultural diseases on the move early in the third millennium. *Vet. Pathol.* 47(1), 15–27 (2010).

2 Simon F, Savini H and Parola P. Chikungunya: a paradigm of emergence and globalization of vector-borne diseases. *Med. Clin. North Am.* 92(6), 1323–1343 (2008).

3 Bethencourt V. Virus stalls Genzyme plant. *Nat. Biotechnol.* 27, 681 (2009).

4 Pastoret PP. Human and animal vaccine contaminations. *Biologicals* 38(3), 332–334 (2010).

5 Aranha H. Current issues in assuring virological safety of biopharmaceuticals. *Bioprocess International* 10(3), 12–18 (2012).

•• **Provides an overview of current issues in virological safety.**

6 Aranha H. Virus safety of biopharmaceuticals: are we there yet? *Contract Pharma.* 13, 82–87 (2011).

7 Jones N. Identification and remediation of a cell culture virus contamination. *PDA J. Pharm. Sci. Technol.* 65(6), 615 (2011).

8 Kerr A, Nims R. Adventitious viruses detected in biopharmaceutical bulk harvest samples over a 10 year period. *PDA J. Pharm. Sci. Technol.* 64(5), 481–485 (2010).

9 Skrine J. A biotech production facility contamination case study – mouse minute virus. *PDA J. Pharm. Sci. Technol.* 65(6), 599–611 (2011).

10 Moody M. MMV contamination – a case study: detection, root cause determination and corrective actions. Presented at: PDA/FDA Adventitious Viruses in Biologics Detection and Mitigation Strategies Workshop. MD, USA, 1–3 December 2010.

11 ICH Q5A (R1). Note for guidance on quality of biotechnological products: viral safety evaluation of biotechnological products derived from cell lines of human or animal origin. In effect: October 1997. www.ema.europa.eu

•• **Regulatory document related to marketed products.**

12 EMEA/CHMP/BWP/398498/2005. Guideline on virus safety evaluation of biotechnological investigational medical products. In effect: 1, February 2009. www.ema.europa.eu

•• **Regulatory document related to investigational medical products.**

13 Aranha H. *'How To' Guide: Viral Clearance Strategies for Biopharmaceutical Safety'.* D & MD Publications, MA, USA (2004).

14 Rubino MJ. Experiences with HEK293: a human cell line. *PDA J. Pharm. Sci. Technol.* 64(5), 392–395 (2010).

15 Roque C, Lowe C, Taipa A. Antibodies and genetically engineered related molecules: production and purification. *Biotechnol. Progr.* 20, 639–654 (2004).

16 Shukla A, Thommes J. Advances in large-scale production of monoclonal antibodies and related proteins. *Trends Biotechnol.* 28(5), 253–261 (2010).

17 Shukla A, Kandula J. Harvest & recovery of monoclonal antibodies from large-scale mammalian cell culture. *Biopharm* 21(5), 34–45 (2008).

18 Yigzaw Y, Piper R, Tran M, Shukla A. Exploitation of the adsorptive properties of depth filters for host cell protein removal during monoclonal antibody purification. *Biotechnol. Prog.* 22, 288–296 (2006).

19 Tipton B, Boose J, Larsen W, Beck J, O'Brien T. Retrovirus and parvovirus clearance from an affinity column product using adsorptive depth filtration. *Biopharm* 9, 43–50 (2002).

20 Zhou J, Solamo F, Hong T, Shearer M, Tressel T. Viral clearance using disposable systems in mAb commercial downstream processing. *Biotechnol. Bioeng.* 100(3), 488–496 (2008).

21 Shukla A, Hubbard B, Tressel T, Guhan S, Low D. Downstream processing of monoclonal antibodies – application of platform approaches. *J. Chromatogr. B* 848, 28–39 (2007).

22 Brorson K, Krejci S, Lee K, Hamilton E, Stein K, Xu Y. Bracketed generic inactivation of rodent retroviruses by low pH treatment for mAbs and recombinant proteins. *Biotechnol. Bioeng.* 82, 321–329 (2003).

23 Chen Q. Viral clearance using traditional well-understood unit operations: low pH. *PDA J. Pharm. Sci. Technol.* 68, 17–22 (2014).

24 Miesegaes G, Lute S, Brorson K. Analysis of viral clearance unit operations for mAbs. *Biotechnol. Bioeng.* 106(2), 238–246 (2010).

25 Horowitz B, Bonomo R, Prince A, Chin S, Brotman B, Shulman R. Solvent/detergent treated plasma – a virus

inactivated substitute for fresh frozen blood plasma. *Blood* 79, 826–831 (1992).

26 Miesegaes G. Viral clearance by traditional operations with significant knowledge gaps: CEX and detergent treatment. *PDA J. Pharm. Sci. Technol.* 68, 30–37 (2014).

27 Ireland T, Bolton G, Noguchi M. Optimizing virus filter performance with prefiltration. *Bioprocess International* 3(10), S44–S47 (2005).

28 Kim I, Choi Y, Kang Y, Sung H, Sohn K, Kim Y. Improvement of virus safety of an antihemophilic Factor IX by virus filtration process. *J. Microbiol. Biotechnol.* 18(7), 1317–1325 (2008).

29 Bakhshayeshi M, Zydney A. Effect of solution pH on protein transmission and membrane capacity during virus filtration. *Biotechnol. Bioeng.* 100(1), 108–117 (2008).

30 Marques B, Roush D, Goklen K. Virus filtration of high-concentration mAb solutions. *Biotechnol. Prog.* 25, 483–491 (2009).

31 PDA Technical Report 41: Virus retentive filtration. Parenteral Drug Association, MD, USA (2008).

•• **Overview related to issues to address in virus filtration.**

32 Brorson K, Sofer G, Aranha H. Nomenclature standardization for large-pore size virus retentive filters. *Bioprocess International* 3, S21–S23 (2005).

33 Wu Y, Ahmed A, Waghmere R *et al.* Validation of adventitious virus removal by virus filtration. *Bioprocess International* 5, 54–59 (2008).

34 Miesegaes G, Lute S, Aranha H, Brorson K. Virus retentive filters. In: *Encyclopedia of Industrial Biotechnology.* Flickinger M (Ed.). John Wiley and Sons, NC, USA (2009).

•• **Provides overview related to virus filtration.**

35 Woods M, Zydney A. Effect of pressure release on virus retention with the Ultipor DV20 membrane. *Biotechnol. Bioeng.* 111(3), 545–551 (2014).

36 Cabatingan M. Impact of virus stock quality on virus filter validation. *Bioprocess International* 3(10), S39–S43 (2005).

37 Bolton G, Cabatingan M, Rubino M, Lute S, Brorson K, Bailey M. Normal-flow virus filtration: detection and assessment of the endpoint in bioprocessing. *Biotechnol. Appl. Biochem.* 42, 133–142 (2005).

38 Brorson K, Brown J, Hamilton E, Stein K. Identification of protein A media performance attributes that can be monitored as surrogates for retrovirus clearance during extended re-use. *J. Chromatogr. A* 989, 155–161 (2003).

39 Shi L, Chen Q, Norling L, Lau A, Krejci S, Xu Y. Real time q-PCR as a method to evaluate XMuLV removal during pharmaceutical protein production. *Biotechnol. Bioeng.* 87(7), 884–896 (2004).

40 Curtis S, Lee K, Blank G, Brorson K, Xu Y. Generic/matrix evaluation of SV40 clearance by anion-exchange chromatography in flowthrough mode. *Biotechnol. Bioeng.* 84, 179–186 (2003).

41 Strauss D, Gorrell J, Plancarte M, Blank G, Chen Q, Yang B. Anion exchange chromatography provides a robust, predictable process to ensure viral safety of biotechnology products. *Biotechnol. Bioeng.* 102(1), 168–175 (2009).

42 Norling L, Lute S, Emery R, Khuu W, Voisard M, Xu Y, Chen Q, Blank G, Brorson K. Impact of multiple re-use of anion-exchange chromatography media on virus removal. *J. Chromatogr. A* 1069, 79–89 (2005).

43 Zhou J, Tressel T. Basic concepts in Q membrane chromatography for large-scale antibody production. *Biotechnol. Progr.* 22, 341–349 (2006).

44 Riordan W, Heilmann S, Brorson K, Sheshadri K, He Y, Etzel M. Design of salt tolerant membrane adsorbers for viral clearance. *Biotechnol. Bioeng.* 103(5), 920–929 (2009).

45 Hubbard B, Roush D. Emerging unit operations (session III), hydroxyapatite, mixed-mode and adsorptive membrane chromatography. *PDA J. Pharm. Sci. Technol.* 68(1), 51–65 (2014).

46 Ghose S, Cramer S. Characterization and modeling of monolithic stationary phases: applications to preparative chromatography. *J. Chromatogr. A* 928, 13–23 (2001).

47 Breece T, Gilkerson E, Schmelzer C. Validation of large-scale chromatographic processes part 2. *Biopharm* 15, 35–42 (2002).

48 Kelley B, Jakubik J, Vicik S. Viral clearance studies on new and used chromatography resins – critical review of a large dataset. *Biologicals* 36(2), 88–98 (2008).

49 Gottschalk U, Brorson K, Shukla A. The need for innovation in biomanufacturing. *Nat. Biotechnol.* 30(6), 489–492 (2012).

50 Cooney CL and Konstantinov KB. White paper on continuous bioprocessing. Presented at: *International Symposium on Continuous Manufacturing of Pharmaceuticals*. Cambridge, MA, USA, 20–21 May 2014. https://iscmp.mit.edu/white-papers/white-paper-4

51 Heldt C, Gurgel P, Jaykus L, Carbonell R. Identification of trimeric peptides hat bind porcine parvovirus from mixtures containing human blood plasma, *Biotechnol. Prog.* 24, 554–560 (2008).

52 Valera C, Chen J, Xu Y. Application of multivirus spike approach for viral clearance evaluation. *Biotechnol. Bioeng.* 84, 714–722 (2003).

53 Lute S, Aranha H, Tremblay D, Liang D, Ackermann H, Chu B, Moineau S, Brorson K. Characterization of coliphage PR772 and evaluation of its use for virus filter performance testing. *Appl. Environ. Microbiol.* 70(8), 4864–4871 (2004).

54 Bell C. Bringing QbD to life - The first FDA approved viral clearance design space. Presented at: *2014 PDA/FDA Virus and TSE Safety Conference*. Rockville, MD, USA, 9–11 June 2014.

55 Brorson K, Miesegaes G, Tounekti O, Skene J and Blumel J. 2014. Conference summary: gaps, lessons learned, and areas for improvement. *PDA J. Pharm. Sci. Technol.* 68(1), 83–89 (2014).

56 Ludwig B. High-development costs, complex manufacturing and legal hurdles are holding back generic drugmakers seeking to copy pricey biotech medications nearing the end of their patents. Reuters. http://uk.reuters.com