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ABSTRACT: The 2023 Viral Clearance Symposium (VCS) was hosted by Takeda on 24 and 25 May 2023 in Vienna, Austria. The present conference extended the structure of the previous biennial symposia held between 2009 and 2019. As recapitulated in the introductory session, the genesis of the VCS, as described in the Proceedings of the 2009 VCS was “the worldwide regulatory and industry recognition that challenges, gaps, and opportunities exist, that it formally addressed could benefit the field as whole.” This report provides a synopsis of the progress achieved at the conference resulting from detailed technical discussions and the pending questions that still require attention to address. The 2023 VCS was composed of nine individual sessions of short presentations followed by in-depth panel discussions from the presenters. Sessions included Regulatory Updates (with a focus on ICH Q5A(R2) efforts), including a summary of lessons learned from the 2019 VCS, and progress on these key areas mapped into 2023 VCS topics: Viral Clearance Strategy and Case Studies, New Modalities in Chromatography and Adsorptive Filters, Continuous Processing, Viral Clearance Strategy and Process Understanding, Virus Inactivation, Upstream and Downstream Virus Retentive Filtration and Cell Banks, and Advanced Technologies (advanced therapy medicinal products, next-generation sequencing).

KEYWORDS: Viral clearance, Viral safety, Viral clearance symposium, Upstream processing, Downstream processing, Resin lifetime, Facility risk mitigation, Submission, Strategy, Next-generation sequencing, Modular viral clearance, Depth filtration, Regulatory agencies, FDA, PEI, Regulatory perspective, Continuous processing.

Resolution of Key Questions from 2019 VCS

One strategic insight (1) from the 2019 Viral Clearance Symposium (VCS) was the value of prior knowledge that can be leveraged from both industry and health authorities to define worst-case conditions and gain understanding into mechanistic insights (specific examples highlighted in O’Donnell and Bolton [2]).

This concept is now incorporated into ICH Q5A(R2) Annex 6, which articulates specific examples applied to the definition of worst-case conditions for viral filtration and viral inactivation. A key concept of ICH Q5A(R2) that was discussed at length during the 2023 VCS was the use of a risk-based approach leveraging prior knowledge as applied to all aspects of viral safety (cell bank testing, viral clearance, use of new technologies, and new modalities with respect to control strategy). Two presentations and the associated discussions extended this approach via the combined application of mechanistic understanding and prior knowledge (e.g., extensive data sets) to a risk assessment to assess the potential impacts of process or facility changes on viral clearance and/or the need to perform requisite viral clearance studies. A third presentation leveraged the control strategy/risk assessment approach to a new modality beyond traditional proteins.

Another key question from the 2019 VCS was the need to better understand the mechanism of action (MOA) for virus removal by anion exchange (AEX), mixed-mode AEX (MMAEX), and hydrophobic interaction chromatography (HIC) to define a robust design space. This has partly been addressed via the potential to incorporate modular claims for specific chromatography steps and dedicated viral clearance steps in market applications (ICH Q5A(R2)).

A key area of discussion during the 2019 VCS was the measurement of retrovirus-like particles (RVLP) for a continuous process and the potential impact of process
The VCS reviewed this topic in detail with alignment on the measurement of RVLP in the permeate. Additional discussions are recommended on further applications of this approach at the 2025 VCS.

A detailed discussion on the potential to apply a similar approach of prior knowledge to obviate the need to perform resin reuse studies for modalities beyond protein A (e.g., AEX) was completed at the 2019 VCS. This flexibility was incorporated in the ICH Q5A(R2) guidance document. The 2023 VCS expanded this discussion to challenge the need to perform carryover studies given that no deleterious impact of resin reuse on viral clearance has been reported when appropriate cleaning procedures are employed for protein A and AEX resins (3). One unique presentation at the 2023 VCS extended this construct to viral filters with an initial promising evaluation of the reuse of viral filters demonstrating the lack of impact on viral clearance upon reuse when a detailed cleaning protocol was employed. An interesting topic because no virus is typically present in the manufacturing application, which certainly simplifies the assessment. Follow-up discussions on this topic at a future VCS are recommended to review potential assessments of the impact on process performance and viral clearance of multiple reuses in various systems.

**Next Steps (2025 VCS)**

Some key areas identified that require further discussion at the 2025 VCS are as follows.

1. The potential to utilize big data/modeling approaches to support prior knowledge and risk assessments

2. Case studies, gap analysis, and risk assessment associated with viral filter reuse (potential topic for industry consortia – BioPhorum, ISPE, etc.)

3. Case studies on implementing modular claims supported within the expanded scope of ICH Q5A(R2)

4. Application of risk assessment and prior knowledge to new modalities (e.g., cell and gene therapy) contained within the expanded scope of ICH Q5A(R2)

5. Lessons learned from the implementation of ICH Q5A(R2) (slated for finalization in 4Q2023), including prior knowledge and risk-based approach cases studies

6. Case studies highlighting the implementation of RVLP as a surrogate for adventitious agent risk assessment and viral clearance (including continued development of analytics)

7. Advancement/update of two ASTM efforts: i) claiming retroviral clearance on parvovirus retentive filters and ii) incorporation of new environmentally friendly detergents

8. Advances in the use of viral surrogates for viral clearance studies – additional examples presented at the subsequent 2023 ACS conference (4–6)

9. Strategies for claiming viral clearance utilizing continuous low pH inactivation systems

10. Continued evolution and considerations of the use of viral filtration for cell culture media

11. Advancement of strategies, case studies and leveraging of prior knowledge to support a risk assessment that obviates the need for viral clearance demonstration of cycled ion-exchange resins

12. Advancement of platform strategies to demonstrate the lack of viral carryover between resin cycles

13. Strategies and advancement on implementing next-generation sequencing (NGS) for adventitious agent testing supported within the expanded scope of ICH Q5A(R2)

**Conflict of Interest Statement**

The authors declare that they have no conflict of interest.

**References**


Proceedings of the 2023 Viral Clearance Symposium, Session 1: Regulatory Updates

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ABSTRACT: At the time of the 2023 Viral Clearance Symposium in Vienna, the ongoing revision of ICH Guideline Q5A(R1) Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin clearly was the dominant regulatory topic. At the symposium, the changes expected for Q5A(R2) to mirror advances of scientific knowledge, for example, the inclusion of new products, including viral-vector–derived ones, that can be subject to virus clearance, deliberations around continuous manufacturing processes, the use of prior knowledge to supplement or in part replace virus validation studies, and new molecular methods for detection of adventitious viruses, were discussed by a European and a US regulator as well as representatives from industry associations that had been involved with the drafting process.

Johannes Blümel, Virus Safety Section Paul-Ehrlich Institute

Setting the stage for the May 2023 edition of the Viral Clearance Symposium, the audience was presented with a Summary of the Current State of Viral Safety of Gene Therapy Medicinal Products at the PEI as well as some regulatory perspective on the then nascent ICH Q5A(R2) guidance.

As current virus safety concepts are largely derived from the learnings of early days with plasma-derived medicinal products manufactured from large pools of human plasma, he reminded the community that the selection processes for lower risk plasma donors, the testing for the absence of certain virus contaminants, and even the virus reduction methods embedded into production processes have over time all been shown to potentially have limitations. Specifically, not all viruses that may occur in blood are even tested for, and very resistant (hepatitis A) or extremely high-titer virus contaminants (B19V) may overwhelm the reduction potential of manufacturing. It is therefore imperative, and by now state-of-the-art, to have multiple virus inactivation and removal steps in place to safeguard these plasma products. The approach has proven successful throughout decades now, even against emerging viruses such as for example West Nile Virus in the US, in that even without testing the plasma supply for derivatives for this newly circulating virus they have been safe against virus transmission.

From these learnings, robust virus inactivation and removal steps are now part of all biological medicinal product manufacturing—unless some virus is part of the medicine’s active principle, such as for live-virus vaccines. The presentation challenged, however, whether this also needs to be accepted for gene therapy vectors now, in that smaller vectors such as adeno-associated viruses (AAV) can indeed be separated from contaminants by adequately sized virus filters that allow for vector passage yet retain larger adventitious virus contaminants. Equally, AAV vectors tolerate treatment with (solvent-) detergent combinations well, enabling effective inactivation of lipid-enveloped virus contaminants. In addition, virus filters are available that can remove viruses with larger particle sizes than AAV. The importance of implementing virus clearance steps for gene therapy has been re-emphasized by the first product of this class licensed in Europe, that is Glybera, where the production cell line Sf9 was found to contain an adventitious virus only after the product was available for use (1). Fortunately, a detergent treatment was part of the manufacturing process, and

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although originally implemented for a different reason, it can be expected to provide for inactivation of the lipid-enveloped Rhabdovirus contaminant.

The need to implement viral clearance is also reflected in the coming revision of the ICH Q5A guidance, in that the scope of the guidance is now defined as products that are amenable to viral clearance without negative impact on the product, and this includes genetically-engineered viral vectors and viral vector-derived products, which can undergo virus clearance. Beyond this guidance document, the regulatory landscape around gene therapy products is evolving rapidly, including European Pharmacopoeia monographs and other local regulations, which should be harmonized with ICH guidance documents.

Scott Lute, FDA/CDER/OPQ/OBP/DBRRII

The presentation provided for a Regulatory Update for Viral Safety, yet started emphasizing the foundational principles for the pharmaceutical industry in general, that is A quality product of any kind consistently meets the expectations of the user—drugs are no different. Patients expect safe and effective medicine with every dose they take. Pharmaceutical quality is assuring every dose is safe and effective, free of contamination and defects. It is what gives patients confidence in their next dose of medicine.

For innovations that can be expected from the Q5A (R2) guidance, which is still expected to be ready for sign off by November 2023, it was mentioned that a retrovirus-like particle safety margin of $\geq 4 \log_{10}$ can be considered acceptable and based on the FDA Virus Clearance database, such cases have been accepted in the past, too, based on solid justification. For clinical manufacturing—depending on particle titer and harvest volume required per final dose of the medicine—such safety margins can be achieved by only two highly effective process steps.

On the topic of chromatography resin reuse, it was mentioned that product-specific studies were not generally expected for Protein A columns. For other resins, justifications based on prior knowledge may be acceptable in lieu of product-specific studies, too. For prior knowledge, the principle to predict rather than matrix/specifically test process performance could be based on representative and justified in-house experience and has been used by many INDs, particularly for virus filtration, solvent-detergent or detergent inactivation, and low pH inactivation.

Finally, the Emerging Technology Program established at the Center for Drug Evaluation and Research was presented as a valuable industry partner, to discuss and obtain advice particularly on advanced manufacturing topics such as continuous processing or the use of novel nucleic acid tests such as next-generation sequencing, for example.

Marie Murphy, Eli Lilly, for the European Federation of Pharmaceutical Industries and Associations (EFPIA)

The presentation focused on EFPIA’s Industry Consortium Feedback Summary on the Published Draft Revision of ICH Q5A(R2) Virus Safety Guideline, with the general notion that the published draft revision of ICH Q5A(R2) has been welcomed by industry. During the public consultation period, the EFPIA industry consortium has taken the opportunity to provide specific feedback for consideration to the ICH Expert Working Group (EWG), with major themes and rationale from the main areas of revision within the guideline summarized in the following.

Widening the scope of the guidance to now include Advanced Therapy Medicinal Products was considered beneficial, yet a more precise definition of new modality products in or out of scope would provide additional value.

The opportunity to make use of advanced molecular methods for virus detection, for example, next-generation sequencing, potentially to replace in vivo and ultimately in vitro virus assays, was particularly welcome in the context of the 3Rs, yet more clarity would be beneficial around the detail of test substitutions.

With a growing body of virus reduction study results available across the industry, it is of interest to leverage the extensive in-house experience particularly for platform processes instead of repeating, for every new matrix and protein, the same studies over. Beyond being more cost-effective, the approach also allows therapies to reach patients more quickly. More clarity about how much product-specific information still needs to be provided would render the new guidance even more helpful.

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1 Replacement, Reduction and Refinement of laboratory animal use
The inclusion of virus safety guidance for continuous manufacturing processes, as well as alignment of terminology with ICH Q13, was also considered of importance, as the possibility of operating smaller manufacturing footprints as enabled by the technology will help to manage investments and ultimately the cost of goods for pharmaceuticals, an important trend with advances in medicine straining public health care spending already.

David Roush, MSD, for Pharmaceutical Research and Manufacturers of America (PhRMA)

PhRMA then provided a Summary of the Published Draft Revision of ICH Q5A(R2) Guideline and PhRMA Perspective on Viral Safety Clearance. PhRMA also applauded ICH’s efforts to provide harmonized approaches for testing and evaluating the viral safety of biotechnological products and guidance on what should be submitted for marketing applications. The aggregation of definitions and viral safety testing guidance is critical to help bridge differences between compendia and documents of ICH member regulatory agencies. PhRMA participates in ICH as a Founding Member. Feedback from the PhRMA Global Quality and Manufacturing Working Group (GQM WG) was routinely sought by the PhRMA Expert Working Group Lead and Deputy Lead throughout 2019–2022 (up to Step 2 sign off in September 2022), with the draft guideline circulated for PhRMA constituent review in October 2021. PhRMA feedback was subsequently addressed during regular meetings of the EWG including the Athens meeting in May 2022. Post release of the Step 2 version of the ICH Q5A(R2), a letter was submitted to the FDA consolidating the PhRMA comments on the Technical Document in January 2023.

PhRMA believes the draft guideline provides a comprehensive general framework for virus testing, strategies for assessing viral clearance, and a recommended approach for the design of viral tests and viral clearance studies. The risk-based approach to cell bank testing and adoption of prior knowledge to guide viral clearance strategies are key advancements in ICH Q5A (R2). Although ICH Q5A(R2) provides the framework, including associated training materials, industry consortia such as PhRMA and EFPIA can provide detailed perspective on implementation, for example, the EFPIA NGS White Paper. A key tenet of PhRMA is that a risk-based approach leveraging prior knowledge should be applied to all aspects of viral safety (e.g., cell bank testing, viral clearance, use of new technologies, new modalities).

Conflict of Interest Declaration

The authors are employees of Takeda Manufacturing Austria AG and have stock interests.

Reference

ABSTRACT: This session deals with the rational design of viral clearance studies for biopharmaceuticals including recombinant proteins such as monoclonal antibodies and, as new in scope of the symposium, also viral clearance for adeno-associated viral (AAV) vectors. For recombinant proteins, large datasets were accumulated over the last decades and are intended to be used for accelerated product process development and streamlining of viral clearance studies. How to utilize prior knowledge in viral clearance validation and how it can be used in a risk assessment tool to decide whether additional virus clearance studies are necessary during product development is being addressed by three of the presentations of this session. This also includes an a priori intended design and generation of validation data for a new kind of detergent such as CG-110, to build up a platform dataset to be used as prior knowledge in future marketing application. Another presentation investigates the virus removal mechanism of a newly developed hydrophobic interaction chromatography (HIC) resin and demonstrates for highly hydrophobic antibodies appropriate reduction for a retrovirus and impurities in a defined process range in contrast to the moderate to poor virus reduction of recent HIC resins. The last two presentations deal with virus clearance approaches for AAV, which will become mandatory with approval of the ICH Q5A revision. Appropriate virus removal and virus inactivation procedures can be implemented into the manufacturing processes of AAV vectors including viral filtration, viral inactivation (e.g., heat inactivation), affinity chromatography, and anion-exchange chromatography with which it seems possible to achieve a good clearance for helper and also adventitious viruses. The heat treatment step can be even a robust step for adenovirus helper inactivation for AAV products when product characteristics and process conditions are understood.


Background and Session Overview

Demonstration of adequate virus clearance in the manufacture of recombinant biologics such as monoclonal antibodies is a central aspect in the viral safety of the product and an integral part of product development. With increasing data knowledge and process understanding, industry desires a more streamlined approach for virus clearance studies, taking prior knowledge and respective data into account. This is a continuing goal and already had been addressed for different aspects at previous virus clearance symposia. In this session, presentations included a conceptual approach to conduct a reduced package of viral clearance studies using in-house knowledge, a proposal on a risk assessment tool to help decision-making for reevaluation of viral clearance in product development and process deviation management, and an approach to deliberately build up a platform dataset for a virus inactivation step using a new detergent. Because chromatography steps still bear some uncertainty in virus removal mechanisms, the removal characteristics of hydrophobic interaction chromatography (HIC) for viruses and host cell proteins has been explored, and a resin solution for highly hydrophobic antibodies is proposed. As a new issue in this symposium, two presentations on virus clearance for adeno-associated viral (AAV) vectors are included because the current revision of the ICH Q5A guidance...
may extend the scope to such viral vectors. Here, a comprehensive approach for adventitious and helper virus clearance for the AAV vector manufacturing process is demonstrated and, in the other presentation, heat treatment is explored as an option to inactivate helper adenoviruses for AAV products.

**Virus Safety: New Ideas and Concepts for Future Virus Validation Studies (Frank Kohne, Boehringer Ingelheim)**

Demonstrating virus safety is essential for the submission processes of biopharmaceutical products. In the last decades, various virus validation study approaches were applied for early and late-stage development phases. While ensuring patient safety, new concepts are of interest and therefore in evaluation to minimize internal and external costs and study time frames. In Investigational New Drug submissions, the robust virus clearance steps within the defined downstream processing will be assessed by a reduced number of virus validation study experiments.

For the virus filtration step, the result from only one test run using the small and very stable Minute Virus of Mice (MVM) as a model virus at low-pressure conditions is used as a worst case to demonstrate effective virus removal and to support the overall downstream process virus risk assessment instead of applying reduction factors of the long-time established Murine Leukemia Virus (MuLV) as a model virus for retrovirus-like particles. Additional process parameters (e.g., load volume, post flush volume) are kept at the upper limits of the defined process ranges.

In the virus inactivation step, the incubation time of the product-containing solution at low pH conditions is reduced from 65 min (process range: 60–70 min) down to 35 min (process range: 30–40 min) while additional time point samples before and after the defined time frame are tested with the large-volume plating procedure. The study is performed with two runs using the MuLV as a model virus at the lower and upper limit, respectively, of the defined process ranges for temperature respectively pH value.

Additionally, the column-based chromatography unit operations, often performed in flow-through mode as contributing steps for virus safety, are evaluated by a single and not a double run approach with two model viruses (MuLV and MVM). The process parameters (e.g., load volume, load flow rate, respectively residence time, product collection criteria, post flush volume, column bed height, buffer volumes) are applied at the limits of the defined process ranges.

This modified submission concept will be accompanied by use of the internal prior-knowledge database generated over the last decades.

**Viral Clearance Capability in HIC Resins (Shohei Kobayashi, Chugai Pharmaceutical)**

HIC is a purification mode that separates monomeric antibody from aggregates and fragments, as well as from host cell proteins (HCPs) and viruses, by the difference of hydrophobicity. It is also found to be effective for the removal of HCPs that cannot be removed by ion-exchange resins or multimode resins. The capability of virus and HCP removal and what parameters affect the virus removal are discussed following based on the historical data on the HIC process.

Phenyl Sepharose 6 Fast Flow resin showed effective HCP removal and moderate MuLV removal in many pipeline molecules with moderate hydrophobicity, whereas it showed limited MVM removal. As shown in Figure 1, summarizing in-house data, conductivity controlled by the amount of sodium sulfate is classified as a potential critical process parameter (pCPP) in virus removal, but operational pH and binding mode are not classified as pCPPs. Besides, resin cycling did not impact virus removal as observed for Protein A affinity and anion-exchange chromatography (Figure 2).

For highly hydrophobic antibodies for which conventional HIC resins and multimodal resins cannot be used, Eshmuno CMX, a weak cation-hydrophobic multimodal resin recently launched by Merck Millipore, was found to be effective in removing HCPs and MuLV (Table I).

**Virus Safety Strategies for CHO Cell-Derived Products Using ICH Q5A(R2) Guidance (John Fisher, Genentech)**

A second version of ICH guidance Q5A is currently in the public consultation phase of review. Many of the proposed changes reflect the collective experience and knowledge gained from the industry for cell line-derived products, advances in new technology, as well as the virus safety needs for emerging new product types and modern manufacturing processes. The new
version of the guidance enables the use of platform validation based on prior knowledge to streamline virus validation for marketing authorizations, similar to what many have been doing for years in clinical applications. To leverage a platform validation approach (e.g., claiming the virus clearance of one product/process for another), there must be a thorough understanding of the mechanism of action, the impact of process parameters, and the effect of variability in process intermediates. A case study was presented for the application of prior knowledge and platform validation using the example of a virus inactivation step using a relatively new non-ionic detergent.

High impact parameters for detergent inactivation were identified based on the literature and internal robustness data. The manufacturing conditions are based on the understanding of the mechanism of action. It has been widely accepted that detergents facilitate solubilization and disrupt the virus lipid envelope. Virus inactivation using the new detergent was shown to be highly effective using a detergent concentration at or above the critical micelle concentration. Inactivation is robust across a wide range of temperature (2°C–20°C), including the manufacturing process requirements (12°C–18°C), and inactivation is highly effective after 30 min. Variability in process and feedstocks can be evaluated using a combination of development and validation studies. In this case, Genentech has data for more than 18 products that cover a wide range for several parameters related to the cell culture process (e.g., solution matrix, product concentration, cell viability, packed cell volume, HCP, and deoxyribonucleic acid (DNA)) as summarized in Table II. Additional development studies were performed to study additional parameters/ranges (e.g., lipids).

The use of prior knowledge and platform validation requires a thorough understanding of the step at the manufacturing scale. Virus inactivation using detergent is well controlled in manufacturing. The harvested cell culture fluid is 0.2μm-filtered as part of the harvest.

Figure 1

Summary of MuLV and MVM removal in Phenyl Sepharose 6 Fast Flow. Top figures show the relation of the LRV with conductivity controlled by sodium sulfate concentration; bottom figure shows the relation of the LRV with operation pH. Green dots are from flow-through mode, blue squares are from bind and elute mode. LRV, log reduction value; MuLV, murine leukemia virus; MVM, minute virus of mice.
filter train. The $0.2 \mu m$ filter removes cell debris and/or aggregated virus particles that may protect viruses from detergent access. The detergent is viscous; therefore, a diluted stock solution is used to provide better control of an accurate addition. Homogeneity is verified during qualification runs.

Virus clearance studies were performed at worst-case conditions for the critical parameters (minimum detergent concentration, temperature, and time) known to impact virus inactivation. X-MuLV inactivation Log Reduction Value (LRV) for the 18 products demonstrate that detergent inactivation is insensitive to changes in feedstock/process variability, and potential interactions between virus and product do not affect inactivation (Figure 3). Therefore, the step is insensitive to changes in process conditions and product, and platform validation can be applied. To apply a platform validation LRV, claim revision 2 (currently draft) of ICH Q5A stipulates that one consider all LRVs from relevant platform data and recommends a conservative claim to avoid overestimating the reduction capability of the step. Since the implementation of the new detergent, seven quality-approved validation studies with replicate runs have been performed (Figure 3). All these validations were performed using consistent conditions (worst-case), large-volume testing, and the same assay dilutions (e.g., interference). Therefore, these seven validations are considered relevant data for a platform validation claim. The variability in LRV is primarily due to the variability of spike virus titer, although two products did show low levels of incomplete inactivation. Given the similarity of the conditions tested and the assay methods, three studies that represent the range of LRV expected are selected for use in the platform validation claim. A conservative LRV is claimed.

This dataset is relatively modern and was generated with platform validation for clinical trial applications in mind. Historical studies may not have been performed with the intent of leveraging the data in platform validation. One challenge is determining what data is relevant to include in the platform validation dataset. Historical LRVs may be limited by low virus stock titer, high assay limit of quantification (e.g., low

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**Table I**

<table>
<thead>
<tr>
<th>Mab</th>
<th>Sodium Sulfate in elution (mmol/L)</th>
<th>Elution pH</th>
<th>MuLV (LRV)</th>
<th>HCP (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mab B</td>
<td>125</td>
<td>8.5</td>
<td>1.4</td>
<td>80 - &gt;34</td>
</tr>
<tr>
<td>Mab C</td>
<td>80</td>
<td>6.8</td>
<td>5.4</td>
<td>130 - &gt;13</td>
</tr>
<tr>
<td>Mab D</td>
<td>100</td>
<td>8.0</td>
<td>2.2</td>
<td>65 - &gt;32</td>
</tr>
</tbody>
</table>

**Table II**

Prior Knowledge Ranges for Virus Inactivation by Detergent (Triton CG-110)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Evaluated Ranges (Development and validation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecule Type</td>
<td>rProtein, IgG (1,2,4), half Mab, bi-specific, Fc-fusion, enzyme</td>
</tr>
<tr>
<td>Solution Matrix</td>
<td>5 processes</td>
</tr>
<tr>
<td>Product Concentration</td>
<td>7.8-fold</td>
</tr>
<tr>
<td>Cell Viability</td>
<td>lysed to highly viable</td>
</tr>
<tr>
<td>Packed Cell Volume</td>
<td>2.6-fold</td>
</tr>
<tr>
<td>Host Cell Protein</td>
<td>3.6-fold</td>
</tr>
<tr>
<td>DNA</td>
<td>569-fold</td>
</tr>
<tr>
<td>Lipids</td>
<td>10–20× normal level in manufacturing</td>
</tr>
</tbody>
</table>

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Impact of column aging. No negative impact of resin cycling on virus removal is confirmed for Protein A affinity chromatography (Protein A), anion-exchange chromatography (AEX), and hydrophobic interaction chromatography (HIC).
volume tested in an infectivity assay), and high inter-
fERENCE dilution. Therefore, low reduction values in a 
historical dataset are not always indicative of actual 
impact. Another approach to address some of these 
issues is to supplement a historical dataset with new 
studies designed for the application of prior knowledge 
and testing the true capability of the step (e.g., using 
high titer virus stocks and large-volume testing).

Future virus validation strategies that are designed with 
prior knowledge in mind, such as those for the next 
generation of detergents or new virus filters, can benefit 
from well-designed and thorough development work. 
By understanding the mechanism of action through in-
ternal and external sources and studying a range of re-
presentative conditions, one can limit the number of 
future validation studies on which to base a platform 
validation claim (e.g., three validation studies).

Use of Prior Knowledge and Risk-Based Approach to 
Guide Viral Clearance Strategy to Accommodate 
Process Changes in Later Stages of Development 
(Krunal Mehta, MSD)

Viral clearance strategies for biologics from well-charac-
terized (e.g., CHO-derived) hosts continue to evolve 
due to the scientific advancements made in the field of 
biologics manufacturing, including mechanistic under-
standing of the viral clearance/inactivation of many 
unit operations. The viral clearance dataset is an essen-
tial component of regulatory filings. Often, these viral 
clearance studies are done before or in concurrence 
with process characterization studies, and most cer-
tainly before the process design space is finalized, 
resulting in proven acceptable ranges (PAR) being 
wider or outside of the worst-case conditions tested in 
the viral clearance study (Figure 4). Process changes 
are expected over the course of development due to 
changes in facility/scale or control strategies, which 
may necessitate reassessing the viral clearance capabil-
ities of the process as it evolves. In this presentation, a 
risk assessment tool is presented (consistent with the 
approach proposed in ICH Q5A(R2) [1]), leveraging 
prior knowledge/mechanistic understanding, to evalu-
ate the impact of process changes on the overall viral 
clearance capabilities. An overall risk score, which 
combines the severity/likelihood score and a prior 
knowledge score is determined to assess if the pro-
posed changes (facility fit, scale, process) would 
impact the overall viral clearance capabilities of the 
process and necessitate experimental assessment. 
Depending on the ranges tested during the viral clear-
ance study versus the PAR established for the process 
design space, different scenarios and proposed strat-
egies involving the proposed risk assessment tool are 
illustrated in Figure 5. This risk-based approach lever-
ges best practices from the literature (2) and enables 
streamlining and harmonizing of viral clearance strat-
egies, especially for late-stage programs or postlicen-
sure products that have already undergone extensive 
bioLOGICAL license application (BLA)-enabling viral 
clearance studies. The approach could also be 
employed for early stage (e.g., first-in-human (FIH) 
programs) to determine the necessity for experimental 
studies or the feasibility of leveraging modular claims 
based on prior knowledge. This approach could also be 
leveraged to manage process deviations that may have 
potential impact on viral clearance claims.
Advances in biotechnology have led to the emergence of recombinant AAV vectors as a novel modality for gene therapy. AAV is a nonenveloped parvovirus with single strand DNA with a size of about 25 nm. There are multiple manufacturing platforms to produce AAVs to support clinical trials, including transfection, producer cells with adenovirus type 5 (Ad5) as a helper virus, herpes simplex virus-based, system and baculovirus-based system (3).

As for recombinant proteins, viral safety and contamination controls of AAVs should be assured through the three-pillar approach: control of raw materials, including cell banks, virus testing at appropriate stages of the manufacturing process, and removal and inactivation of adventitious viruses and/or helper viruses in the purification process. The AAV purification process is normally designed to integrate viral clearance capabilities, as shown in Figure 6. Viral inactivation and viral filtration steps are designed as dedicated steps. Capture and polishing chromatography steps are used to contribute to viral clearance.

Evaluation of Viral Clearance Strategy for AAV Downstream Process (Junfen Ma, Sanofi)

Possible process ranges in product manufacture and viral clearance studies. The figure illustrates the risk of effect and failure of tested ranges during viral clearance studies in relation to product manufacture ranges at different steps of product development and process deviations. Viral clearance worst-case scenarios 1 and 2 are referenced to scenarios 1 and 2 in Figure 5. PAR, proven acceptable range; PQA, process quality attributes; VC, viral clearance; WC, worst case.

Risk scenarios for viral clearance settings and solution strategies. Three scenarios were discussed at the meeting: Scenario 1: Worst-case (WC) conditions span the updated proven acceptable range (PAR) (post-PC = post process characterization, post licensure CPV = post licensure continuous process verification). Even in the event of process setpoint change, the viral clearance results cover the PAR. Scenario 2: If the updated PAR falls outside the worst-case conditions tested in the viral clearance (VC) studies, the viral clearance strategy should be reassessed using a risk assessment tool, including prior knowledge to justify omission of additional experimental studies. Scenario 3: If the updated PAR falls significantly outside the worst-case conditions tested in the viral clearance studies, the viral clearance strategy should be reassessed using a risk assessment tool, including prior knowledge to guide supplemental experimental studies.
In this work, the viral clearance strategy for two different AAV manufacturing platforms is discussed, including a helper virus-dependent platform (producer cells with Ad5 as a helper virus) and a helper virus-independent process platform (transfection system). The ICH Q5A(R2) guidance (1) was used to design a risk-based approach for viral clearance.

For the transfection platform, it is proposed as “Case A”, where no virus, virus-like particle, or retrovirus-like particle have been detected in the cell bank or the unprocessed bulk. Viral clearance studies will be performed with nonspecific “model” viruses (1). Our proposed plan is to validate two orthogonal viral clearance mechanisms using two nonspecific model viruses, including viral filtration and anion-exchange chromatography steps. Both steps are considered robust steps (4).

For the producer cell platform with Ad5 as a helper virus, it is proposed as “Case F”, where a helper virus is used in production. Clearance of the virus should be demonstrated using the helper virus itself or a specific model virus (e.g., baculovirus, adenovirus, herpesvirus) (1). Our proposed plan is to validate 2–3 effective orthogonal viral clearance steps (≥4 LRV) with a cumulative log reduction supporting at least 2–5 logs of safety margin. The process steps will include viral filtration, viral inactivation (e.g., heat inactivation), affinity chromatography, and anion-exchange chromatography steps. Viral clearance studies will be performed with one process related virus, Ad5, and one model virus. In addition, viral safety will be assured by testing of the final AAV product for helper virus particles as well as for residual helper virus proteins and DNA.

With the proposed approach described prior, a case study was performed for the producer cell platform with Ad5 as a helper virus. In this study, two viruses were used, Ad5 as a process relevant virus and bovine viral diarrhea virus (BVDV) as a model virus. The characteristics of these two viruses are described in Table III. They are complemental to each other, thus creating a wide range of viral characteristics to evaluate the viral clearance capabilities of purification processes. A total of >18.6 LRV was shown across 4 steps for Ad5, and a total of >10.2 LRV was shown across 4 steps for BVDV.

To summarize, AAV process development and viral safety strategies have been evolving. However, a lot of biologics (including monoclonal antibodies and recombinant proteins) learnings and experience can be used with the consideration that AAV as a product is a viral vector.
Because different AAV manufacturing platforms are being used across the industry, a risk-appropriate approach can be used for the viral clearance and contamination control. The AAV purification process needs to be designed to integrate the viral clearance capabilities to include both dedicated steps and contributive steps.

Exploring the Operating Window for the Heat Inactivation Used in the AAV Downstream Process (Jason Morais, Sanofi)

Heat treatment is a reliable method for inactivating certain virus types. For enveloped viruses, heat incubation can solubilize the phospholipid membrane. For some nonenveloped viruses, it can irreversibly denature the protein structure of the viral capsid, rendering it incapable of infecting a host cell. As such, heat treatment can play an important role in the viral control strategy of a biologics manufacturing process, with particular relevance being demonstrated for helper virus-dependent AAV production systems. Wild-type Adenovirus 5 (wtAd5) is a common helper virus used in AAV production and is particularly susceptible to inactivation by heat exposure. WtAd5 is a midsized, nonenveloped virus with double-stranded DNA genome. Figure 7 demonstrates the inactivation kinetics of wtAd5 at incubation temperatures ranging from 49°C to 53°C.

Given that the AAV product can also be impacted by high-temperature exposure, the appropriate product quality attributes of AAV should be thoroughly evaluated when determining the optimal operating window for wtAd5 inactivation by heat incubation. Because AAV is a parvovirus, some temperature robustness is expected; however, heat lability can vary by serotype.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Envelope</th>
<th>Genome</th>
<th>Approximate Size (nm)</th>
<th>Shape</th>
<th>Physico-Chemical Resistance</th>
<th>LRV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad5</td>
<td>No</td>
<td>DNA</td>
<td>70–90</td>
<td>Icosahedral</td>
<td>Medium</td>
<td>≥18.7</td>
</tr>
<tr>
<td>BVDV</td>
<td>Yes</td>
<td>RNA</td>
<td>50–70</td>
<td>Pleomorphic/Spherical</td>
<td>Low</td>
<td>≥10.2</td>
</tr>
</tbody>
</table>

Figure 7

Heat inactivation kinetics for wild-type adenovirus. The heat inactivation kinetics for wild-type Adenovirus 5 (wtAd5) is investigated at different temperatures (color code). The control (pink dot) is wtAd5 at room temperature after 10 min; it also is the basis of comparison for the other temperature curves with respect to wtAd5 inactivation. The inactivation curves demonstrate 6 logarithmic reduction values of clearance within the first 20 min of exposure to temperatures ≥50°C.

Because different AAV manufacturing platforms are being used across the industry, a risk-appropriate approach can be used for the viral clearance and contamination control. The AAV purification process needs to be designed to integrate the viral clearance capabilities to include both dedicated steps and contributive steps.
and may not yet be fully understood for novel engineered capsids. Melting curve analysis, such as shown in Figure 8, can aid understanding of the relative heat stability of different AAV capsids but does not provide definitive data on those attributes that relate to capsid integrity or virion infectivity. For this, additional tools would need to be employed to understand the extent to which heat exposure has caused, for example, capsid aggregation, genome ejection, or deamidation, any of which could adversely impact AAV potency.

Other aspects that are critical to consider in the development of a heat incubation step for an AAV process involve manufacturability, scale-up, and virus validation. Such considerations could include assessment of the high-temperature impact on downstream process performance, understanding the equipment capabilities to enable controlled incubation temperature and ramp rates at the manufacturing scale, and thoughtful design of a suitable scale-down model with which to validate the unit operation for viral clearance, as well as worst-case conditions for conducting such a study.

In summary, by understanding and exploiting the differences in the thermal stability profile between AAV and wtAd5, an inactivation step with well-defined parameters can provide significant reduction of helper wtAd5, contributing to the overall patient safety of AAV products. For a heat incubation step to be robust, steps must be taken to understand the structure, infectivity, and potency of the AAV product of interest under the conditions being explored.

**Session Summary and Discussion/Conclusion**

This session, called Viral Clearance Strategies and Case Studies, illustrated various virus safety approaches in the life cycle of a biopharmaceutical product during the development and manufacturing phases.

In the past, a lot of viral clearance studies were performed to demonstrate the virus removal and virus inactivation capabilities of selected steps in the purification process. This large prior knowledge database from platform purification processes can be used to shorten downstream process development timelines, to support the experimental design of requested virus clearance studies, and to reduce the number of virus-spiked experiments ensuring sufficient virus safety of the biological product in clinical trial applications.

Besides filtration procedures, different chromatographic techniques are commonly used in downstream processes to guarantee the specified product quality in the drug substance. HIC using an established chromatographic resin showed only poor or moderate virus reduction factors. Thus, this developed process step was not often implemented in expensive viral clearance studies. Innovative HIC resins were designed by the vendors in recent years to compensate for this lack, providing not only robust removal of process-related impurities but also appropriate virus reduction factors of the endogenous model virus MuLV in a defined process range.

The virus inactivation step is important within the purification process to ensure robust inactivation of enveloped virions.
viruses. During process development activities, the procedure (e.g., acid treatment at low pH values, detergent treatment) and the respective process parameters are defined to not compromise the product quality on the one side and to assure sufficient virus reduction factors. Considering these specified process parameter ranges and historical process knowledge, worst-case conditions are applied in a dedicated viral clearance study.

Product and process specific knowledge generated during process development, scale-down model establishment, process characterization studies, viral clearance studies, and scale-up runs is key to set process ranges for production campaigns at the manufacturing scale to provide robust process performance and the specified product target profile in the final drug substance. With respect to virus safety, this entire database is extremely valuable for use in risk assessments during routine production. With this risk-based approach, it can be decided by the expert teams whether additional viral clearance studies are necessary to support product release.

The newly introduced purification strategies for AAV products also have to consider appropriate virus removal and virus inactivation procedures within the defined manufacturing processes. Virus filtration and heat treatment as robust process steps are accompanied by up to two chromatographic steps to ensure the virus safety of the final product. Some development programs showed that process ranges for selected process parameters have to be carefully defined, considering the setup and the given equipment capabilities of the intended production facility or that potential facility investments have to be initiated in a timely manner.

Conflict of Interest Declaration

The authors declare that they have no conflict of interest.

References


ABSTRACT: The session provided an update on the application and mechanistic understanding of intensified unit operations (e.g., mixed mode depth filters, mixed mode AEX) since the last conference in 2019. One of the key gaps identified in the 2019 Viral Clearance Symposium session on the topic was for more investigation required to achieve a clear understanding of the molecular mechanisms of virus removal and the relevance of different molecule’s interactions including resin, virus, and product. Further investigation into worst-case conditions for these unit operations is also warranted. One of the key outcomes from that 2019 discussion was also that multimodal anion exchangers can have robust and effective virus removal, depending on process and impurities—an observation that was recapitulated with more specific case studies and evidenced by broader application of these chromatographic resins in late-stage regulatory filings.

KEYWORDS: Mixed Mode Resins, Viral Clearance, Membrane Adsorbers, Mechanistic Understanding, AEX Chromatography, Process-Related Impurities, Detergent, Polysorbate 20, PS20, NMG, N-methylglucamide.

Participants Contributions

Demonstration of Viral Clearance with Mixed Mode Resins and Membrane Adsorbers by Meta-Analysis of the CDER Viral Clearance Database (Scott Lute, FDA)

CDER’s Office of Biotechnology Products has recently updated its viral clearance database for therapeutic proteins to include data from all BLAs (commercial applications) submitted through January 2022 (3). As part of the meta-analysis of the data, we compared new anion exchange (AEX) chromatography modalities such as mixed mode resins and membrane adsorbers with the traditional packed bed single ligand resins. Based on the submissions collected in the database, mixed mode resins have a higher incidence of viral clearance claims compared with membrane adsorbers (N = 36 vs. N = 9); however, these are still significantly less than that of traditional AEX (N = 85). When comparing the viral clearance capabilities of these process steps run in flow-through mode, all three modalities reported an effective (>4 log_{10}) median log reduction value (LRV) for both retroviruses and parvoviruses, with retroviruses having about 1 log_{10} higher median LRV compared with parvoviruses. Although clearance values were effective, there were some differences observed between the traditional AEX resins and the new modalities, particularly in parvovirus removal. Firstly, membrane adsorbers demonstrated a lower median LRV compared with that of packed resin-based separations (4.1 vs. 5.3; however, this could be attributed to the low number of records for membrane adsorbers at this time. Secondly, mixed mode resins demonstrated similar median LRVs to those of AEX resins with less variability (5.1 [range 4.1–6.2] vs. 5.3 [range 0.5–8.2], respectively). The lower variability may be attributed to the ability of the mixed mode resin to maintain parvovirus clearance at a much wider buffer conductivity range (Figures 1 and 2). The database records demonstrated that, for AEX resins, as you increase conductivity up to 15 mS/cm the LRV of parvoviruses decreases from a median of 5.5 to 2. Conversely, for mixed mode resins, parvovirus clearance of >4 log_{10} was maintained up to a conductivity of 30 mS/cm, supporting the use of these resins in a wider buffer range.
This meta-analysis of BLA submitted data demonstrates that new modalities for AEX chromatography 1) are being implemented in commercial manufacturing and 2) provide relatively comparable viral clearance values dependent on the process conditions.

Viral Clearance Capacity of an Anion Exchange Membrane Adsorber (Chelsea Burgwin [Presenter], Ashley Reeder, Marissa Collins, Eileen Wilson, GSK)

Polisher ST presents a single-use, high-capacity alternative to packed bed anion exchange polishing chromatography in flow-through mode. Polisher ST is an AEX membrane with functional quaternary amine (Q) and guanidinium membranes. The functional Q portion of the membrane acts through electrostatic interactions to reduce turbidity and to remove negatively charged impurities such as deoxyribonucleic acid (DNA), host cell protein (HCP), and viruses. The novel guanidinium layer acts to bind HCP and virus through the formation of robust salt bridges (resulting from hydrogen-bonding and electrostatic interactions), providing additional impurities clearance functionality even under conditions of high ionic strength compared with conventional AEX resins. With the potential benefits of clarification, impurities clearance, and single-use technology, and because AEX chromatography can provide robust virus clearance, GSK performed an R&D viral clearance study to assess the impact of replacing AEX chromatography with Polisher ST on virus clearance in a monoclonal antibody (mAb) purification platform.

For the Polisher ST viral clearance study, a single IgG1 mAb was evaluated. The load material was protein A eluate, titrated to low pH for virus inactivation, adjusted to one of three load pH conditions: pH 5.5, 6.5, and 7.5. Conductivity was adjusted with NaCl to a consistent target of 5mS/cm to meet the recommended range of the device. To assess the worst-case viral clearance, we challenged the device to a load ratio of 20 kg/m², twice the vendor recommended load ratio. The load material was spiked with either minute virus of mice (MVM; 8.0 log₁₀) or xenotropic murine leukemia virus (XMuLV; 7.5 log₁₀), and virus concentration

Impact of buffer pH on MVM LRV in AEX-based separations. LRV, log reduction value; MVM, minute virus of mice.

Impact of conductivity on MVM LRV in AEX-based separations. AEX, anion exchange resin; LRV, log reduction value; MA, AEX membrane adsorber; MM, mixed mode AEX resin; MVM, minute virus of mice.
was assessed by infectivity and quantitative polymerase chain reaction (qPCR). For MVM, the flow-through was collected at every 5 kg/m² and a flow-through pool created for initial testing. For XMuLV, the flow-through was collected as a single pooled sample.

For the XMuLV runs (Figure 3), robust (>4.0 LRV) viral clearance to below the limit of detection was achieved by infectivity, with no infectious virus detected in the flow-through for all three pH conditions. Minor noninfectious XMuLV was detected by qPCR, likely due to nonfunctional XMuLV particles that were able to escape binding by the Polisher ST device.

For the MVM runs (Figure 3), robust or nearly robust viral clearance was achieved by infectivity; however, there was minor virus breakthrough observed at all three pH conditions by both infectivity and qPCR. To assess if the virus breakthrough was due to overloading the device to above the vendor recommended load ratio, the previously collected fractionated samples (every 5 kg/m²) were tested by infectivity. MVM breakthrough was observed in each individual fraction and was not exclusive to the fractions above the recommended load ratio.

Lastly, we compared the viral clearance performance of the Polisher ST device to a standard AEX column. In the AEX chromatography study, the load material was collected after low pH hold and neutralization adjustment, depth filtered, and the conductivity was not adjusted. The AEX chromatography study was operated at pH 7.5 so is compared to the Polisher ST pH 7.5 experiments. Porcine parvovirus (PPV) was the parvovirus used in the AEX column chromatography experiments but is similar enough to MVM to allow comparison to clearance in the Polisher ST experiments. In Figure 4, comparing the infectivity results, the AEX resin column and Polisher ST performed similarly for XMuLV, with each achieving robust clearance with no infectivity breakthrough detected. For parvovirus, the AEX column (PPV) outperformed the Polisher ST device (MVM), achieving robust non-breakthrough clearance of PPV whereas the Polisher ST device did have breakthrough of MVM, while still achieving robust clearance.

In conclusion, we found the Polisher ST membrane provided sufficient viral clearance to be considered as a valid alternative to standard AEX column chromatography.
Impact of Process-Related Impurities on Virus Clearance Capacity of Affinity Chromatography Steps—Implications for Process Development (Sven Schubert, Rentschler Biopharma)

In the initial capture and purification of various biopharmaceutical proteins (e.g., mAbs, bispecific antibodies, Fc fusion proteins), affinity chromatography, and in particular protein A chromatography, is a widely used downstream processing technique. Due to its high affinity, the protein A ligand is able to selectively capture the target protein directly from complex cell culture fluids containing process-related impurities (PRIs) such as HCPs and host cell DNA (HCD) as well as multiple media components. Therefore, one would expect high LRVs of viruses, because they should not be retained by the resin. However, data from numerous studies show that the reduction of (model) virus particles by this unit operation is rather moderate and varies significantly from 1 to 5 log10 (3).

The variability of observed virus LRVs appears to be based on complex interactions between the target protein, the viral particle, and PRIs as found in the harvested cell culture fluid (HCCF) (4). We confirmed this observation when process adaptations in the harvest filter train induced a significant reduction of PRIs. In particular, reduced PRI levels were achieved by the change of secondary harvest depth filters from non/low to highly functionalized membranes. As a result, the LRV of the corresponding protein A capture step decreased significantly regardless of the type of model virus used (i.e., MuLV of the Retroviridae family or MVM of the Parvoviridae family). As shown in Figure 5, further evaluation of the PRI level in the HCCF with change of the secondary depth filter revealed the highly reduced HCD, in contrast to the almost constant HCP level, as the main contributor to the variation in LRV reduction.

To further explore a possible correlation between HCCF, HCD level, and protein A virus removal capacity, a retrospective analysis of our internal virus and process database was performed considering 16 downstream processes for different classes of biomolecules (e.g., IgG, bispecific monoclonal antibodies, Fc-fusion molecules). In contrast to the previous work by Ayaji et al. (3) and Zhang et al. (5), which described a more general phenomenon of feedstock-specific virus removal variability, the data indicated a moderate and strong HCD level-dependent LRV for the evaluated model viruses MVM and MuLV, respectively.

Based on the obtained HCCF HCD level–LRV correlation, a hypothesis on HCD modulated virus removal was formulated in more detail for high and low HCD level scenarios. In the first scenario, at low HCD levels, the target protein carries a net positive charge or at least positively charged patches on the surface, under neutral pH conditions. Virus particles (pI < 6), on the other hand, have an overall negative charge at this pH. Both counterparts initially attract each other by long-range electrostatic forces and associate, leading to co-elution of viruses with the target protein. This interaction remains stable even with a wash buffer containing high concentrations of salt and chaotropic agents. In the second scenario, at high HCD levels, the positively charged protein or charged regions of the protein are shielded by the prominent HCD. As a result, any kind of electrostatic interaction leading to virus–protein assembly is prevented and does not occur due to the charge masking caused by the present DNA.

In addition to the database studies, virus clearance studies were performed on protein A chromatography steps with different modified starting materials. Three different mAbs with different isoelectric points in the most variable region, the light chain, were tested to cover a wide range of protein charge characteristics. In addition, the starting materials (HCCF) contained varying amounts of HCD, including a low HCD starting material spiked with CHO-extracted HCD. Each of the three mAbs shared a reduced LRV for the low HCD

Figure 4

Viral Clearance comparison of AEX and Polisher ST operated in flowthrough mode as measured by plaque assay.

<table>
<thead>
<tr>
<th>AEX Column</th>
<th>Polisher ST</th>
</tr>
</thead>
<tbody>
<tr>
<td>XMuLV</td>
<td>PPV</td>
</tr>
<tr>
<td>Run 2</td>
<td>Run 1</td>
</tr>
<tr>
<td>pH 7.5</td>
<td>pH 7.5</td>
</tr>
<tr>
<td>MuLV</td>
<td>MVM</td>
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</table>

<table>
<thead>
<tr>
<th>LRV</th>
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</thead>
<tbody>
<tr>
<td>&gt; 5.15</td>
</tr>
<tr>
<td>6.52</td>
</tr>
<tr>
<td>&gt; 6.41</td>
</tr>
<tr>
<td>4.53</td>
</tr>
</tbody>
</table>

Impact of Process-Related Impurities on Virus Clearance Capacity of Affinity Chromatography Steps—Implications for Process Development (Sven Schubert, Rentschler Biopharma)
level feedstock. In contrast, the application of conventionally treated HCCF containing high levels of HCD showed significantly higher LRVs. Finally, the LRV was almost restored for all the low HCD level HCCFs spiked with HCD compared with the conventionally treated HCCF. Accordingly, our hypothesis of a HCD level-dependent modulation of virus removal was confirmed.

In summary, we observed a wide range of LRVs for protein A-based chromatography, as reported in previous publications. Furthermore, the reduction of viruses was dependent on the feedstock composition. We identified the DNA content as one of the main modulators of the LRV based on historical in-house virus clearance and process data and mechanistic virus clearance studies with modulated HCD content. These results underscore the importance of considering the required virus removal capacity of the downstream processing capture step during the design of the upstream harvesting process.

Detergent-Based On-Column Activation—Regulatory Perspectives along a Mechanistic Case Study (Matthias Kron, Rentschler Biopharma)

Low pH or solvent/detergent treatment represents an essential step within the virus clearance strategy of biopharmaceutical production processes, as it guarantees efficient and robust inactivation of enveloped viruses. The classical low-pH approach for mAbs can be unsuitable when the target protein shows limited stability under acidic conditions, favoring a detergent-mediated approach. Typically performed in batch mode in large production vessels, detergent-mediated virus inactivation faces challenges in terms of protein stability, process control, and implementation of this unit operation into a standard purification process. On-column virus inactivation using a detergent-containing wash buffer provides several advantages over conventional batch-mode to overcome the aforementioned challenges. This intensified process design omits up to two unit operations, namely the virus inactivation itself as a separate unit operation and potentially an additional tangential flow filtration required for removal of the detergent. It is highly compatible with continuous processing and allows the exact control of the process conditions like incubation time and homogenous detergent concentration, which is challenging in batch mode operations. Furthermore, an immobilized protein on the resin might be more stable toward the detergent than in solution.

However, more sophisticated approaches for the corresponding virus clearance studies are required to reveal...
the mechanistic orthogonality of virus removal and inactivation in compliance with regulatory guidelines. To address these requirements, we have presented different experimental designs at the 2019 Viral Clearance Symposium, which are described in detail in reference 6 and are summarized in Figure 6A–C.

We have performed a case study cross-comparing these experimental designs, which were applied to a low pH sensitive non-antibody protein molecule using an eco-friendly N-methylglucamide (NMG) detergent. An affinity capture chromatography step with nonacidic elution conditions was chosen for the on-column inactivation. As a negative control, a wash buffer containing polysorbate 20 (PS20) as a non–virus-inactivating detergent (in the absence of Tri-n-butyl-phosphate) was included in the study. XMuLV was used as a model virus.

The simplified batch mode approach was directly compared with the loaded resin batch approach. For each approach, two runs were performed with NMG and PS20 (applying concentrations as present in the wash buffer), respectively. Samples were taken for median tissue culture infectious dose (TCID50) analysis after 0, 15, 30, 45, and 60 min of incubation at 16.5°C (including large-volume plating for 45 min and 60 min time points). Specifically for the simplified batch mode, clarified cell culture fluid was taken as a test item reflecting worst-case conditions regarding impurities present that might impact on inactivation efficiency. In contrast, unpacked product-loaded resin slurry (50% in wash buffer matrix) was used in the loaded resin batch approach. Despite these substantially different conditions, both approaches resulted in a fast and complete inactivation of XMuLV below the limit of quantification (see Figure 7). As expected, no inactivation was observed with PS20 in the simplified batch mode and only a minor reduction of around 1 LRV at the edge of significance in the presence of product-loaded resin beads. In conclusion, our results revealed a minor impact of the matrix conditions (buffer, impurities, resin beads) when a complete and efficient virus inactivation is expected. This needs to be kept in mind considering the technical challenge of

Figure 6

(A) Delta determination approach—Two virus-spiked chromatography runs are performed in parallel with or without detergent-containing wash buffer. The delta in virus titer determined in the eluate fractions can be attributed to the detergent effect. (B) Simplified batch mode approach—Protein dissolved in the respective detergent wash buffer (or another matrix containing the detergent) is spiked with the virus particles and inactivation kinetics are monitored by multiple sampling over time. Option: resin beads can be added prior to virus spike for a closer mimicry of actual process conditions. (C) Loaded resin batch approach—Chromatography column is loaded with protein under real process conditions. Resin is unpacked, transferred to a vessel, and detergent plus virus spike are added to start virus inactivation analogous to the simplified batch mode. Option: Loaded resin beads are equilibrated with detergent-containing wash buffer already before unpacking the column.
test item preparation and virus analysis for the loaded-resin batch approach.

The delta determination approach was investigated by three runs applying different wash buffers either without a detergent or containing NMG or PS20. The virus reduction in the collected eluate fractions was compared by different detection methods with TCID$_{50}$ and qPCR analysis allowing the discrimination of remaining infectious virus particles and virus genomes, respectively. Similar LRVs of about 3 were observed by TCID$_{50}$ analysis for all runs (considering a likely overdetermination of virus load titer for PS20, see Figure 8). Of note, although the residual infectivity was $<$ the limit of quantification (LOQ) for the run with NMG-containing wash buffer, the achieved LRV was only 3 and comparable to that of the control run where infectious particles were still detectable in the eluate. This finding is due to the high detection limit of the TCID$_{50}$ assay in this case when no large-volume plating is used to decrease the LOQ of the assay. In contrast, the higher sensitivity of qPCR analysis revealed a difference of almost 4 LRV between NMG and the control, demonstrating the detergent effect of NMG in inactivating virus on the column. In part, also a physical removal effect of a detergent-containing wash buffer in general is indicated by the results for PS20 for which no inactivation is expected based on the results from the batch mode experiments. However, the full mechanism and contribution of inactivation and physical removal under the different conditions could not be fully resolved in the chosen study design, because the analysis of the wash fractions was only performed by TCID$_{50}$ which delivered inconclusive results (data not shown). From this study, it can be learned that the delta determination approach should be set up with the full analytical panel (TCID$_{50}$ incl. LVP and qPCR) to resolve the full virus clearance mechanism and to achieve the highest claimable LRV.

Based on our findings from this case study and experience from the application of these different approaches, we provide a guidance for which of these approaches is to be preferentially selected with a special focus on regulatory implications. As shown in the decision tree in Figure 9, the choice depends on the process design, the (anticipated) virus clearance potential of the relevant chromatography step, the virus clearance potential of the overall process, and very much on the life cycle phase of the product (clinical phase vs. market approval). A key difference is whether or not an additional step for inactivating viruses at low pH is
integrated into the process. Having a low pH virus inactivation step in the process omits some of the regulatory expectations toward the detergent-mediated virus clearance effect on the column. This opens the door for the simplest approach to be applied for clinical phase to just validate the virus clearance of the whole chromatography step without mechanistically separating the detergent effect from the physical removal. For market approval, there is at least no need to show a kinetic of the detergent-mediated inactivation on the column, and the delta approach could be applied in a 2-run design (with and without detergent) at worst-case conditions (e.g., minimal contact time of the detergent wash buffer). In contrast, multiple 2-run designs applying different contact times by variable flow rates of the detergent wash buffer (incl. full analytical panel in all fractions) would be required to show both the kinetics and the mechanism of clearance with the delta determination approach if the detergent-mediated inactivation on the column represents the only mandatory and robust virus inactivation step of the process (Figure 9, very right branch). In general, the delta determination approach is recommended only when the chromatography step without the detergent has a low to moderate virus clearance potential or when the overall virus clearance of the process is uncritical, and you are not dependent on showing a high delta for the separate claim of the virus inactivation.

If the virus clearance potential of the chromatography step without detergent is already considered moderate to high, one of the batch mode approaches should be selected for the study. This allows a separate claim of the obtained LRV from the virus inactivation and adding the obtained LRV from an additional chromatography run without detergent. In our case study, this would sum up to a reduction value of $7.5 \log_{10}$ ($3 \log_{10}$ from chromatography run + $4.5 \log_{10}$ from the batch mode approach) compared with only $6 \log_{10}$ obtained from the chromatography run with detergent (Figure 8, qPCR data). The decision for the simplified batch mode or the technically more challenging loaded resin batch mode approach would depend primarily on the life cycle phase and how close the actual process conditions need to be reflected. A good compromise would be the simplified batch mode when adding native resin beads into the incubation vessel.

In summary, detergent-mediated on-column inactivation is a valuable tool to intensify bioprocess design and to overcome challenges in the overall virus clearance strategy. At the same time, the supporting virus clearance

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**Figure 9**

Decision tree for selecting the appropriate virus clearance study approach depending on the process design and regulatory requirements for clinical phase vs market approval. Reduction values in yellow boxes show the achieved virus clearance by the different approaches in this case study. qPCR, quantitative polymerase chain reaction; TCID$_{50}$, median tissue culture infectious dose; VC, viral clearance.
studies require a well-thought-out concept, tailor-made for the specific process and life cycle phase. Our different experimental approaches embedded into the proposed decision tree offer a valuable toolbox to design the best suitable strategy demonstrating the desired virus clearance and fulfilling all regulatory requirements.

Defining a Design Space for Viral Clearance by Mixed-Mode Chromatography (Greg McNamara, Regeneron)

Regeneron evaluated the effects of pH and NaCl concentration on clearance of XMuLV and MVM by mixed-mode chromatography (MMC) in the presence of a mAb or in a protein-free buffer environment. MMC offers additional selectivity compared with ion-exchange chromatography and may reduce the number of polishing steps in the manufacturing process of a mAb via the use of multiple chromatographic modalities in a single separation. The published literature demonstrated that MMC achieved comparable viral clearance to flow-through AEX chromatography when the steps were operated under conditions considered worst-case for virus removal.

Regeneron reported a 16-run D-Optimal design of experiments was performed at pH 6.5 and 8.5 and NaCl concentrations of 50 mM and 300 mM using Capto Adhere MMC resin. The responses measured for each run were protein yield (%) and Log10 Reduction Factor (LRF) determined by plaque infectivity assays for XMuLV and MVM, and reverse transcription–quantitative polymerase chain reaction (RT-qPCR) assay for XMuLV. The use of both RT-qPCR and infectivity for enveloped virus was used to distinguish between removal and/or potential chemical inactivation.

In the absence of protein, no significant effect on viral clearance was observed across all pH and salt concentrations within the ranges evaluated. Results are shown in Table I. Effective XMuLV and MVM clearance (>4 LRF) was observed when measured by infectivity. XMuLV clearance determined by infectivity was >1 log10 greater than clearance determined by RT-qPCR, indicating a combination of inactivation and virus partitioning was likely observed.

In the presence of a mAb, no significant effect on viral clearance was observed across all pH and NaCl concentrations within the ranges evaluated (Table I). Viral clearance was diminished in the presence of protein, suggesting that protein–virus competition and/or protein–virus interaction may influence virus adsorption. XMuLV clearance was reduced from an average of 5.86 LRF in the absence of protein to 2.89 LRF in the presence of protein. Decreased clearance was more pronounced for MVM as negligible clearance (<1 LRF) was observed for all runs. Low protein yields of <40% were observed for each run, indicating that protein–virus interaction and/or competitive binding may be

### Table I

<table>
<thead>
<tr>
<th>Load Material</th>
<th>pH</th>
<th>NaCl Concentration (mM)</th>
<th>XMuLV LRF</th>
<th>XMuLV LRF (qPCR)</th>
<th>MVM LRF</th>
<th>Protein Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>6.5</td>
<td>50</td>
<td>5.98</td>
<td>4.41</td>
<td>6.10</td>
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<tr>
<td></td>
<td></td>
<td>300</td>
<td>&gt; 6.29a</td>
<td>3.59</td>
<td>&gt; 6.42a</td>
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<tr>
<td></td>
<td>8.5</td>
<td>50</td>
<td>&gt; 5.48a</td>
<td>3.39</td>
<td>&gt; 6.35a</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>300</td>
<td>&gt; 5.68a</td>
<td>3.65</td>
<td>&gt; 6.27a</td>
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</tr>
<tr>
<td>mAb A</td>
<td>6.5</td>
<td>50</td>
<td>3.02</td>
<td>1.74</td>
<td>0.42</td>
<td>36.8 34.4</td>
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<td>0.90</td>
<td>0.07</td>
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<td></td>
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<td>50</td>
<td>2.10</td>
<td>1.14</td>
<td>0.00</td>
<td>39.9 39.9</td>
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<td>3.68</td>
<td>1.67</td>
<td>0.06</td>
<td>39.9 38.8</td>
</tr>
</tbody>
</table>

LRF, log reduction factor; mAb, monoclonal antibody; MMC, mixed-mode chromatography; MVM, minute virus of mice; qPCR, quantitative polymerase chain reaction; XMuLV, xenotropic murine leukemia virus.

*aVirus infectivity reduced below detection limits of assay.

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Figure 10

Clearance of XMuLV for mAb A unit operations. AEX, anion exchange chromatography; HIC, hydrophobic interaction chromatography; LRF, log reduction factor; mAb, monoclonal antibody; MMC, mixed-mode chromatography; qPCR, quantitative polymerase chain reaction; XMuLV, xenotropic murine leukemia virus.

Removal of Carryover Studies Support by Amgen Prior Knowledge Assessment (XiaoXiang Zhu, Amgen)

Virus carryover assessments for chromatography operations are usually performed after viral challenge studies. The purpose of the carryover assessments is to demonstrate that cleaning and regeneration procedures inactivate and remove virus in chromatography steps and to provide assurance that no residual virus is retained. Although the ICH Q5A(R2) guideline (7) continues to suggest providing evidence to demonstrate effective column cleaning and regeneration procedures, carryover studies have been considered as standard practice, especially for viral clearance studies supporting marketing applications.

As base-stable resins become a norm in biopharmaceutical manufacturing applications, caustic solutions (e.g., sodium hydroxide) are commonly employed to clean the resins between use and can provide assurance of virus inactivation/removal during resin cleaning. A prior knowledge assessment of carryover studies is performed based on Amgen’s portfolio of biologic molecules, as shown in Figure 11. A total of 117 carryover assessments from 2008 to 2021 were analyzed, and no residual XMuLV or PrV (enveloped model viruses) was found in any of the assessments. Only 5 of the 117 instances detected residual virus carryover in the
product pool, all of which involved the most resistant viruses to inactivation, MVM and Reo-3 (nonenveloped model viruses). In all five instances, the detected residual viruses were of low level, and satisfactory LRVs (all ≥ 1.0) were accomplished in the unit operations, which would support sufficient excess clearance capability and ensure a safe level per dose. Four of the five instances were associated with a program that was terminated and not commercialized. Two of the five instances were not base-stable resins that did not utilize sodium hydroxide for cleaning. Among the 115 cases in which sodium hydroxide was utilized for resin cleaning, the concentrations ranged from 0.3 M to 1.0 M. This prior knowledge assessment supports the proposal to remove the requirement of product-specific carryover studies when caustic solutions (e.g., 0.3 M to 1.0 M sodium hydroxide) are employed for chromatography resin cleaning and regeneration. Viruses, if retained on the column, would be inactivated and removed during the cleaning and regeneration steps, thereby making product-specific carryover studies unnecessary. We believe the elimination of carryover assessment does not pose a risk to viral clearance assessments and adventitious agent control strategy.

**Summary and Discussion**

MMC appears to offer comparable or improved viral clearance performances (XMuLV and MVM) to AEX with a wider conductivity range. The presentation on the concept of a design space for MMC builds on prior work in this space for AEX (8). The impact of product, impurity, and virus partitioning in MMC as a function of operating conditions, potentially determined via high-throughput screening, is essential to understanding the robust operating space. Examples of prior research in this space that can be applied are Brown et al. (9). Impurities (e.g., DNA) and the sample matrices may also impact the LRV determined via qPCR and infectivity for the typical chromatography steps evaluated (protein A, AEX, MMAEX). Specifically, a significant impact of DNA was observed in protein A chromatography with lower LRV observed for purified mAb loaded on protein A vs. HCCF. Some potential alternative hypothesis is mixed mode interaction for virus, DNA, and mAb as there was not a clear trend using high, medium, and low pI mAbs. Previous research (10) on virus flow-through purification suggests that when the external surface area per unit volume on a resin is minimized combined with impurity binding being maximized (e.g., DNA in HCCF), virus will selectively partition into the flow through. For protein A chromatography, this would result in a higher LRV and would be consistent with the viral clearance results obtained with HCCF and purified mAb streams. Another potential explanation could be a combination of hydrophobic interactions among the species and competitive adsorption for protein A ligands. Recent approaches/new modalities (e.g., mixed-mode membrane adsorber) and process intensification (detergent in wash buffers) can contribute to both physical removal and inactivation, which requires deconvolution of orthogonality in viral clearance experimental designs. One specific example was a comparison of a new mixed-mode membrane adsorber that had 200× higher loading than AEX, resulting in a lower LRV than that for AEX. Potential reasons for the lower LRV could be the impact of loading and lot-to-lot variability. Determination of worst case for new modalities may need to be explored experimentally vs. prospectively (e.g., virus-retentive filtration, low pH inactivation in ICH Q5A(R2) appendix [7]).

One interesting focal point of discussion was the potential to apply prior knowledge to simplify viral clearance experiments, including the option of eliminating carryover studies from viral clearance studies for well characterized systems in which resin cycling has been demonstrated not to impact LRV (e.g., AEX or protein A). This proposal leverages ICH Q5A(R2) Sections 6.2.6 “Function and Regeneration of Columns” and 6.6 “Application of Prior Knowledge for Evaluation of Viral Clearance” (7). Specifically, “for protein A affinity capture chromatography, prior knowledge indicates...”
that virus removal is not affected or slightly increases for used (e.g., end of life) chromatography media/resin." Hence, if the mode of virus clearance for protein A is well defined in the literature (11) and there is not an impact of reuse on viral clearance, a logical extension is that there would be limited to no virus detected in carryover studies, which obviates the need for these experiments.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

References


Proceedings of the 2023 Viral Clearance Symposium, Session 4: Continuous Processing

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ABSTRACT: The continuous processing session at the 2023 Viral Clearance Symposium (VCS) focused on understanding how to effectively design viral clearance operations for use in continuous processes and methods to perform viral clearance studies. In this session, an approach to directly address control considerations with operating continuous-flow reactors for low pH viral inactivation was presented. Continuous-flow low pH incubation chamber design and implications for residence time determination were discussed. Additionally, viral clearance capability between batch operation and connected operation were demonstrated to be comparable for a connected bind-elute chromatography and flow-through chromatography step. Overall, this session provided additional scientific knowledge to support viral clearance strategies when implementing a continuous manufacturing process.

KEYWORDS: Continuous processing, Downstream processing, Upstream processing, Viral Clearance Symposium, Viral clearance, Multicolumn chromatography.

Session Background and Overview

As the biotechnology industry moves toward implementing continuous processing for protein therapeutics, a thorough understanding of the impact that extended processing, dynamic fluid flow, and novel equipment may have on the viral safety of the process is required. Successful implementation of continuous processing requires an understanding of how to incorporate viral testing and clearance/inactivation into the process via representative small-scale models. Interest in this topic has necessitated a dedicated session on continuous processing at the last two Viral Clearance Symposium (VCS) meetings in 2017 and 2019 (1, 2). Although significant progress has been made in adapting traditional batch viral clearance steps to continuous operation, challenges still exist. There are a variety of continuous viral inactivation system designs (e.g., tubular reactors, glass bead packed columns). Continuous viral inactivation design requires careful residence time distribution modeling, and the sensitivity of the tracers used for modeling should be carefully considered. The study of connected chromatography operations can benefit from previous advances using in-line spiking in the scale-down viral filtration model. In-line spiking between integrated process steps may help in the design of a more efficient small-scale study for a dynamic process stream instead of bracketing conditions in batch studies. Based on the discussion at the 2023 VCS, further advances in modeling manufacturing scale systems (e.g., continuous low pH viral inactivation systems) will continue to be necessary to ensure critical parameters for viral safety are controlled.

In this session, three presentations from academia and industry demonstrated the robustness of continuous processing unit operations and discussed alternative strategies for viral clearance validation.

The first talk of the session presented an approach to directly address control considerations with operating continuous-flow reactors for low pH viral inactivation. The operating pH and the residence time distribution are critical parameters in determining viral clearance and can impact product quality from overincubation or
excessive pH adjustment. The approach presented demonstrated a robust and reproducible method to measure and control pH and minimum residence time (MRT).

The discussion on continuous low pH inactivation continued with a talk regarding continuous-flow incubation chamber design, in which data were presented comparing viral inactivation in traditional batch operation with the inactivation continuous mode.

The third talk shifted the discussion to connected chromatography and discussed viral clearance study design and execution involving a bind-elute chromatography step connected to a flow-through chromatography step. Viral clearance capability between batch operation and connected operation were demonstrated to be comparable.

Additionally, continuous processing has become more common place, so that talks involving elements of continuous operation are also included in other sessions of this VCS conference. Refer to Session 5, Viral Clearance Strategy and Process Understanding, for a presentation discussing the challenges in retrovirus-like particle quantification in a continuous process and Session 7, Up- and Downstream Virus Retentive Filtration, for two presentations discussing the evaluation of viral filtration in continuous processes.

Participants Contributions

Participants were requested to provide a short presentation on recent research projects related to continuous processing. A brief summary of each of these presentations in order of appearance at the symposium is provided.

Model-Based Control for Column-Based Continuous Low pH Viral Inactivation of Biopharmaceuticals (Paul W. Barone, Massachusetts Institute of Technology)

Methods for adapting low-pH hold to continuous processing have involved cyclic batch operation, continuous-flow tubular reactors, and continuous-flow column-based reactors. None of the continuous-flow reactors, however, directly address control considerations with operating the systems. The operating pH and the residence time distribution are critical process parameters (CPPs) in determining viral clearance and can impact product quality from overincubation or excessive pH adjustment.

The Braatz lab at Massachusetts Institute of Technology (MIT) has constructed a low-cost, column-based continuous viral inactivation system constructed with off-the-shelf components. The system consists of a glass bead packed column for the low pH hold with inline pH adjustment before and after the column. The A_{280} absorbance is measured at the column inlet and outlet. Model-based control is used to control the pH using fundamental acid/base equilibria. Model parameters are updated in real time based on online measurement data. Specifically, the residence time distribution, and therefore the MRT, can be estimated based on the absorbance measurements at the inlet and the outlet. Variation in the inlet absorbance is required for accurate parameter estimation, as seen in Figure 1. This is possible with normal process variations, for example, concentration peaks inherent in multi-column chromatography elution. If the inlet is inherently devoid of variation, such as from a well-mixed tank, concentration variation would need to be introduced deliberately. In the system developed at MIT, a periodic injection of deionized water, which has negligible ultraviolet (UV) absorbance relative to that of protein-laden input solution, was used to introduce the necessary variability.

Several viral inactivation experiments were performed to assess the performance and control of the system. In all experiments described, Phi6 bacteriophage, as a model virus, was spiked into the test solution. The final Phi6 titer was evaluated using a plaque assay. Due to technical limitations, the solution in the low pH column did not contain a monoclonal antibody or model protein, but instead used tryptophan as a tracer. However, the approach to pH and MRT control demonstrated here will work in the presence of proteins as well.

Initially, viral inactivation as a function of pH was evaluated. The MRT was set to 5 min and the pH setpoint for the first mixing unit was changed from 3.5 to 3.8, 4.2, 4.5, and 4.8 at 60-minute intervals. Phi6 bacteriophage was below the limit of detection (log_{10} reduction value (LRV) >6.8) at pH 3.5, with the LRV decreasing with each increase in pH. Next, the impact of changes to the MRT set point was evaluated. The pH was set to 4.5, which resulted in a LRV of 2 with a 5 min MRT in the previous experiment, and the MRT set point was varied from 20 to 2.5 min (Figure 2). At a MRT of 20 min, the Phi6 bacteriophage was below the limit of detection of the assay (LRV >6.5). As the MRT decreased, the observed LRV also decreased to a value of ~1.5 at the MRT of 2.5 min.
Finally, the reproducibility of the system was evaluated. The pH was set to 4.5 and the MRT was set to 15 min and the system was run for 15 h. The system demonstrated stable control of both pH and MRT during the entire 15 h operation. Samples were taken periodically and evaluated for Phi6 LRV, with all samples showing $\sim$4 LRV across the 15 h.

The approach presented here demonstrated a robust and reproducible method to measure and control the low pH MRT, which is a critical process parameter in ensuring robust viral clearance and final product quality. The approach and data presented here was published in *Biotechnology and Bioengineering* (3).

**Figure 1**

 Minimum residence time (MRT) controller setpoint tracking. (A) Flow rates (mL/min) determined by the MRT controller. (B, C) Absorbance measurements at the inlet (blue) and outlet (red) of the column. Decreases in absorbance are due to injections of DI water as a ‘UV tracer’. (D) Estimated MRT at two different setpoints. The black dashed lines indicate the setpoints. The MRT setpoint was changed from 4 min to 5 min at 180 min and back to 4 min at 360 min. Setpoint tracking was achieved within a single step. The residence time distribution (RTD) model accurately predicts the concentration at the outlet. MRT, minimum residence time; RTD, residence time distribution. *Used with permission from* (3).

**Figure 2**

 Measured LRV (bottom) at varying estimated MRTs (top) in the continuous low pH column. (top) The black dashed line is the MRT set point plotted vs time in the experiment. Vertical dashed lines are time points at which the outlet was sampled to measure Phi6 reduction. MRT setpoints were 20, 15, 20, 5, and 2.5 min. (bottom) Measured LRV as a function of MRT set point. Phi6 was below the limit of detection of the assay at the MRT set point of 20 min. LRV decreases with decreasing MRT set point. LRV, log$_{10}$ reduction value, MRT, minimum residence time. *Figure adapted from* (3).
Considerations for Implementation of Continuous In-Line Virus Inactivation at Low pH (Dr. Sladjana Tomic-Skrbic, Sr. Associate Director, Head of MSAT Downstream GeSA, South-EU and MEA, Millipore Sigma/Merck KGaA, Darmstadt, Germany)

The evolution from batch to continuous processing for monoclonal antibody manufacturing operations is increasingly being explored, primarily to reduce costs and improve control of quality attributes during processing. Low pH viral inactivation is a dedicated viral clearance process step for enveloped viruses. Incubation chambers that enable incorporation of the traditional batch step into a continuous process have been developed for in-line viral inactivation. The efficiency of different chamber designs for viral inactivation was assessed using riboflavin and PhiX174 model systems. Residence time distribution was measured over a range of operating and design conditions to establish a process window that ensures adequate exposure time and enables robust, predictable performance across different process scales. Under these conditions, robust xenotropic murine leukemia virus (XMuLV) inactivation was achieved in continuous mode, and data on both static and in-line dynamic inactivation were presented (Figure 3). No loss of virus titer due to shear or adsorption to flow path were observed.

Data were presented to support the rationale for using small-scale static spike testing for scaling and clearance studies of in-line continuous viral inactivation step.

Continuous Manufacturing: In-Line Virus Spiking for Connected Chromatography Applications (Ulrich Breuninger, BSc Expert Science & Technology, Downstream Process Development, Novartis Pharma AG, Basel, Switzerland)

To demonstrate viral clearance is paramount for any biopharmaceutical production process to reduce the risk of virus-transmitted disease for patients. Modern continuous manufacturing processes are reality. We implemented a continuous biomanufacturing concept that minimizes underutilized capacity, enables multi-product flexibility, and is highly automated, resulting in a lower operational cost. A schematic presentation is shown in Figure 4. However, these new concepts require new approaches to test virus removal at a small scale. One of the challenges is to determine viral...
clearance of a flow-through column connected directly to a bind-elute chromatography step. One option would be a design of experiments (DoE) viral clearance study that would cover all potential differences in pH, concentration, and conductivity that could have an impact on viral removal. Such an extensive study would require a high number of executed runs and therefore a huge amount of protein solution, columns, and buffers without truly reflecting the dynamics of the reality. Therefore, we invested in a more elegant way utilizing in-line spiking technology previously developed for nanofiltration.

**In-Line Spiking for Chromatography**: Prior knowledge of in-line spiking for nanofiltration (4) was applied to our continuous manufacturing concept. We have taken that concept to chromatography applications to mimic the dynamic nature of connected chromatography processes. An illustration of the different approach can be found in Figure 5.

We compared in-line spiking to batch spiking experiments for two different protein solutions and two viruses, XMuLV and minute virus of mice (MVM). Experiments have been done starting from a pool (discrete mode) without connection of two chromatography systems. Both spiking methods showed comparable log removal factors. The results are presented in Figure 6.

In a second step, we successfully confirmed that the in-line spiking technique will generate comparable data to that of the batch spiking. To do that, we performed in-line spiking in a connected chromatography setup connecting a bind-elute chromatography directly to a flow-through chromatography. The virus spike was added constantly into the eluate fraction of the first chromatography column, which was loaded directly onto the second chromatography column (Figure 5). With this setup, we achieved comparable results to those of the traditional batch spiking method for the flow-through chromatography (Figure 7). The dataset in Figure 7 was updated following the presentation to reflect the current results.

The dynamic nature of product streams in a connected chromatography process needs different tools to test viral clearance. We successfully adapted the concept of in-line spiking for nanofiltration (4) to column chromatography. This offers an elegant way that requires significantly less experiments. In addition, this technique allows us to be more comparable to the large-scale process.

**Conclusion**

Understanding of how to maintain and demonstrate viral clearance using connected processes has advanced in recent years, although challenges still exist. For
example, the variety of continuous viral inactivation system designs being implemented may require more research and understanding. Continuous viral inactivation design requires careful residence time distribution modeling, and the sensitivity of the tracers used for modeling should be carefully considered. For a fully integrated continuous process, the study of connected chromatography operations can benefit from previous advances in viral filtration using in-line spiking. In-line spiking between integrated process steps may help in the design of a more efficient small-scale study for a dynamic process stream instead of bracketing conditions in batch studies. Further advances in modeling manufacturing scale

Figure 5

In-line spiking in connected chromatography. FT, flow-through chromatography; B/E, bind-elute chromatography.

![Continuous spiking for chromatography](image)

Figure 6

Comparison of batch spiking vs in-line spiking in a discrete mode testing XMuLV and MVM removal. LRF, log reduction factor; MVM, minute virus of mice; XMuLV, xenotropic murine leukemia virus.
systems are necessary to ensure critical parameters are controlled (e.g., residence time in continuous low pH viral inactivation).

Conflict of Interest Declaration

The authors declare that they have no competing interests.

References


Session Introduction & Goals

The Strategy and Process Understanding session was designed to present new approaches to validate viral clearance of biotech unit operations. Conventional spike/removal studies with model mammalian viruses, with input and output measurements using 50% tissue culture infectious dose (TCID50) counts, have been used for decades. Although these approaches are still valid, some newer ideas have been proposed to streamline this process. The first to gain widespread acceptance in the early 2000s was the use of real-time polymerase chain reaction (qPCR) assays instead of TCID50 counts. These nucleic acid-based tests have greatly accelerated the pace at which viral clearance studies can be performed. This session discussed additional concepts, like adaptation of American Society for Testing and Materials (ASTM) International standards for viral clearance operations and use of mock-virus surrogates instead of actual live virus. The latter can simplify the biosafety considerations inherent in these studies but also open the possibility for Design of Experiments (DoE) studies examining process ranges and interactions.

This session also covered strategic topics like outlier analysis, quantitation of very low virus titers, and how to react to unit operation bypass. When the Center for Drug Evaluation and Research (CDER) and industry have analyzed and published databases of viral clearance information, they have always noticed consistent trends toward robustness for many unit operations but also a small fraction of outliers (1–3). How to follow up on the outliers has been an issue. For example, determining whether they were due to unspecified lab errors or to some subtlety of the unit operation in question. Because these cross-industry databases contain cases that were often performed by other groups long beforehand, proving lab errors or identifying subtleties is almost impossible. Several case studies were discussed by the participants.

Virus Safety: Learning from the Past (Helmut Winter, Boehringer-Ingelheim)

Virus validation studies are mandatory to demonstrate the virus reduction factors of the dedicated virus clearance steps within the downstream processing of biopharmaceutical products. Historical experiments from various products support process understanding and give guidance in defining process parameters in early- and late-stage development projects. Additionally, these results are valuable to accompany routine manufacturing processes at full scale. Most of the purification steps are well-known and predictable regarding their capability to reduce potential virus contaminations. Nevertheless, unexpected results occurred and could not be explained. In this session, two examples were discussed.

Results from spiking experiments on the examined virus filtration step using a well-known virus filter at various inlet pressure values were used to define appropriate
worst-case conditions at low pressure for future virus validation studies. It was observed that, in some cases, reduced parvovirus removal was detected at an inlet pressure below 0.3 bar, whereas in other experiments, effective parvovirus removal was shown also below 0.3 bar. Therefore, it was decided to set the lower limit of the defined process range for this virus filter at \( \geq 0.3 \) bar (Figure 1).

In addition, only negligible log reduction values of the tested retrovirus were gained as results from clearance studies using an established membrane adsorber module at neutral pH values and low conductivity levels. Because the membrane adsorbers are not as predictable for virus reduction as the anion exchange (AEX) or the mixed-mode (MM) chromatography, it is recommended to perform preliminary virus clearance studies during process development activities as confirmation to implement the membrane adsorber technology into the final downstream process (Figure 2).

A Challenge in Viral Clearance Determination: Estimation of TCID\(_{50}\) for Low Virus Concentrations (Anca Arentsen, Novo Nordisk)

Performing viral clearance studies is an important safety element of manufacturing biopharmaceuticals expressed from mammalian cells. In these studies, viral titers can be determined using cell-based infectivity assays.

For viruses causing cytopathic effect (CPE), the viral titer can be calculated as the TCID\(_{50}\) using the Spearman–Kärber equation. In this method, replicate wells are inoculated with decreasing concentrations of test material, resulting in a transition from all wells being infected to no wells being infected. Based on the ratio of infected wells at different concentrations of test material, the TCID\(_{50}\) is calculated. However, the Spearman–Kärber method is only accurate when virus is detected in more than one dilution or in \( \geq 10\% \) of wells at the lowest dilution.

**Spearman–Kärber equation**

\[
\log_{10}(\text{TCID}_{50}) = \text{constant} + \sum \frac{r_i}{n_i}
\]

\( r_i = \text{number of positive wells in dilution } i \)

\( n_i = \text{number of wells tested in dilution } i \)

When performing viral clearance studies, low virus concentrations are occasionally observed, for example, by large-volume titration. For low virus concentrations, the maximum likelihood (ML) method is widely used for estimation of virus titer. In these situations, the SK method would be used for the input sample (high virus titer) and the ML method for the output sample (low virus titer). In order to calculate the log reduction value, a way to convert the ML output to the TCID\(_{50}\) is presented (4).

**Conversion of ML output to TCID\(_{50}\)**

\[
\sum_{j=1}^{k} g_j m_j (1 - \exp(-\lambda m_j)) = \sum_{j=1}^{k} t_j m_j
\]

Reducing this expression to virus detection in one dilution only and converting the ML output to TCID\(_{50}\) yields:
Boxplot Membrane Adsorber with xMuLV

<table>
<thead>
<tr>
<th>Log red. $\lambda(TCID_{50})$</th>
<th>Residence time</th>
<th>Buffer composition</th>
<th>Load density</th>
<th>Conc. $[g/L]$</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.37</td>
<td>2 sec.</td>
<td>Phosphate/NaCl 7.2/9.0 mS</td>
<td>13500 g/L</td>
<td>12.5</td>
</tr>
<tr>
<td>0.52</td>
<td>6 sec.</td>
<td>Acetate/Tris pH 7.5/2.7 mS</td>
<td>5000 g/L</td>
<td>9.7</td>
</tr>
<tr>
<td>0.04</td>
<td></td>
<td></td>
<td>1400 g/m²</td>
<td></td>
</tr>
<tr>
<td>0.08</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.36</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 2

Analysis of outliers of xMuLV clearance by Sartobind Q. xMuLV, xenotropic murine leukemia virus.

$$\lambda(TCID_{50}) = -2 \ln \left(1 - \frac{g_j}{t_j}\right) / m_j$$

$\lambda$ = concentration of virus in the original sample  
$k$ = number of dilutions tested  
$g_j$ = number of positive wells in dilution $j$  
$t_j$ = number of wells tested in dilution $j$  
$m_j$ = volume of original sample per well in dilution $j$

This formula gives a very easy to use method of estimating the TCID$_{50}$ in the low range when the Spearman-Kärber formula cannot be applied. This enables determination of the logarithmic reduction value (LRV) in cases in which only very low virus concentrations are observed after a process step.

**Enhancing Developability of Viral Clearance in Downstream Unit Operations by Leveraging a Toolbox of Virus Surrogate-Based Methodologies**

(Lukas Döring, Rentschler Biopharma SE and Karlsruhe Institute of Technology)

Due to the cost-intensive and time-consuming nature of virus spiking studies, profound optimization or characterization of viral clearance in downstream operations is currently not feasible. As indicated by recent publications, methodologies using retrovirus-like particles (RVLPs) and/or minute virus of mice virus-like particles (MVM-VLP) have the potential to overcome these bottlenecks for dedicated chromatography, inactivation, and virus filtration steps (5–8). Surrogate methodologies are especially beneficial during process development and characterization, as demonstrated in the following three case studies for different types of AEX and MM-AEX resins.

In the first case study, we evaluated an AEX chromatography step in bind/elute mode for its capability to clear viruses during early-phase development. High salt elution conditions were assumed to be critical for virus removal. In three MVM-VLP-based spiking experiments, the pH was incrementally increased by 0.5 units. MVM-VLPs were not detected in any of the experiments, resulting in LRVs $\geq 3 \log_{10}$. Based on these results, the process step was assumed to effectively clear viruses. This was confirmed by validation runs using infectious MVM (Figure 3).

In the second case study, the criticality of the exchange of a single buffer matrix component on the virus removal capabilities of an AEX membrane adsorber was assessed. The pH and salt concentrations were kept constant, and therefore the impact was assumed to be negligible. However, insignificant removal ($LRV < 1$)
\[ \log_{10}(\text{LRV}) \text{ was observed under these process conditions applying MVM-VLP spiking agents. These findings were verified by classical spiking studies with infectious MVM. As a result, the buffer exchange was abandoned to retain viral clearance (Figure 4).} \]

In the third case study, a MM-AEX chromatography step in flow-through mode was characterized using a DoE approach. Spiking with MVM-VLPs was easily integrated in an already planned experimental setup. The main challenge was the weakly acidic pH of the processing matrix. Robust and effective viral clearance could be confirmed throughout the entire design space (see Figure 5), while impurity removal and yield optimization readouts were not compromised by the MVM-VLP spike.

In conclusion, virus surrogate-based methodologies have the potential to make viral clearance a developable quality attribute, predicting effects of process changes and enabling characterization of virus clearance. Despite being of high value, VLP testing systems are rather new and their comparability to infectious model viruses needs to be further evaluated.

With the increasing knowledge about the comparability of VLPs and infectious viruses, prospective publications should focus on how data generated using virus surrogates could be implemented in regulatory filings, and how they could enable the development of viral clearance using Quality by Design (QbD) principles.

**The Importance of Virus Segregation: Sensitivity Analysis of Carryover (Soren Kamstrup, Novo Nordisk)**

The concept of viral clearance is a well described and quantitative determination of the reduction of virus by a unit operation, for example, a chromatographic step. The cumulative reduction of multiple steps ensures the virus safety of the final product.

Using a simple equation for estimating a critical potential carryover (CPCO) during production, the consequence of small amounts of material bypassing a unit operation step (with potential virus carried over from before to after the step) can be easily calculated (9):

\[ \text{CPCO} = \text{Volume} \times 10^{-\text{LRV}} \]

CPCO represents the volume of prestep material which—if bypassing the step and re-entering poststep—will reduce the virus reduction value by a factor of 2 (or 0.3 on a log scale).
For example, consider 1000 L of unprocessed bulk harvest (UPBH) containing $10^7$ RVLP/mL. The total load of RVLP in the process will then be $(10^7 \text{ RVLP/mL} \times 1000 \text{ mL/L} \times 1000 \text{ L}) = 10^{13}$ RVLP. If the LRV of the step is $3.0 \log_{10}$, the RVLP load postcapture will be reduced to $10^{10}$ RVLP. The CPCO will be $1000 \text{ L} \times 10^{-3} = 1 \text{ L}$. This means, that if 1 L of UPBH were to bypass the step, it would add another $10^{10}$ RVLP, bringing...
the total load to $2 \times 10^{10}$ RVLP. In that case, the effective LRV will be $\log(10^{13}/(2 \times 10^{10})) = 2.7 \log_{10} 2$ (Figure 6).

Having multiple or even all steps in the same room (ballroom type facility) greatly increases the sensitivity of the cumulative LRV toward carryover. For a typical monoclonal antibody (mAb) process with a cumulative reduction of $15 \log_{10}$, as little as 0.005 nanoliter of UPBH could compromise overall clearance if transferred to final purified bulk (Figure 7).

This calculation tool does not encourage or endorse carryover in any form or amount. It can be used to gauge the order of magnitude it would take to compromise

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**Figure 6**

Potential virus cross-contamination by small-volume carryover of one column. LRV, logarithmic reduction value; RVLP, retrovirus-like particle.

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**Figure 7**

Enhanced sensitivity of multicolumn train to virus cross-contamination by small-volume carryover. CPCO, critical potential carryover; LRV, logarithmic reduction value; RVLP, retrovirus-like particle.
virus reduction through carryover. When looking at a manufacturing process, a “CPCO map” can be an eye-opener as to how sensitive (or how robust) a process is toward, for example, aerosol-borne carryover.

To prevent carryover, appropriate mitigations must be put in place, such as a process closure, dedicated equipment and utensils, spatial or temporal segregation, air flow controls, or walls.

Thereby, the calculations presented can be used to select which mitigations are appropriate under which circumstances.

**Cleaning Agents that Effectively Inactivate Baculovirus on Manufacturing Surfaces (Sean O’Donnell, Eli Lilly)**

Gene therapy manufacturing using baculovirus vectors for producing recombinant adeno-associated viruses (AAVs) is an increasing trend among manufacturers that is being driven by process economics, scalability, and product quality considerations. The presence of live replicating virus in a manufacturing suite can pose risks to other products being manufactured in the same building/suite and especially pose a bigger risk to any other processes where baculovirus can replicate in the production cell line. It is important to understand what cleaning agents are capable of inactivating baculoviruses. In this study, we investigated common cleaning agents that are used in manufacturing and their effect on killing baculovirus. Specifically, we looked at virus killing with the following cleaning agents: concentrated bleach, 10% diluted bleach, Spor-Klenz, 0.8% Vesphene, pH-adjusted bleach, and 70% isopropyl alcohol (IPA) on both stainless-steel surfaces and epoxy-coated surfaces. Results showed that all cleaning agents robustly inactivated baculovirus within 10 min of contact time (Table I), suggesting that routine cleaning practices in manufacturing suites are adequate for inactivation of baculovirus.

**New Paradigms: Evaluation of Cygnus Alpha RVLP Kit against In-House Method (William Rayfield, Merck & Co.)**

RVLPs have been observed to be present in products produced using Chinese Hamster Ovary (CHO) as the host cell. These RVLPs are apparently defective and noninfectious; however, because of their morphology and biochemical resemblance to tumorigenic retroviruses, RVLP pose a theoretical safety concern. Hence, RVLP viral clearance validation is a regulatory requirement for worldwide product market registration (in accordance with ICH Q5A). The standard testing for RVLP is detection by electron microscopy (EM), which has low sensitivity and high variability in the data generation.

An alternative method that may be used is reverse transcription-based real-time polymerase chain reaction (RT-qPCR). The method has a fast turnaround time (in a day) and higher sensitivity and lower variability in data generation. Due to the lack of understanding regarding the RVLP genome in different CHO cell

<table>
<thead>
<tr>
<th>Sanitizing Agent</th>
<th>Time</th>
<th>PFU/mL SS</th>
<th>PFU/mL Exopy</th>
<th>Overall LRF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentrated Bleach</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>6.2</td>
</tr>
<tr>
<td>10% Bleach</td>
<td>5</td>
<td>0</td>
<td>1.7</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>6.2</td>
</tr>
<tr>
<td>Spor-Klenz</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>6.2</td>
</tr>
<tr>
<td>0.8% Vesphene</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>6.2</td>
</tr>
<tr>
<td>pH-Adjusted Bleach</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>6.2</td>
</tr>
<tr>
<td>70% IPA</td>
<td>5</td>
<td>6.2</td>
<td>3.3</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>6.2</td>
</tr>
</tbody>
</table>

IPA, isopropyl alcohol; LRF, log reduction factor; SS, stainless steel.
lines, how variability in the RVLP genomic sequences may affect the performance of RT-qPCR, and the standardization in the reagent used for this method of detection remaining undetermined (e.g., reference standard used to establish the standard curve), this method has not been widely used. Efforts from CYGNUS Technologies have led to the generation of an alpha RVLP kit containing RVLP stock solution and reagents for qPCR analysis. This technology provides the opportunity to support biologics process development through the use of a particle that could mimic the clearance of a retrovirus without the need for strict live adventitious agent cleaning and controls.

This work details the use of the alpha MockV RVLP kit compared with the in-house direct RT-qPCR method (without RVLP extraction) for spiking studies using in-process intermediates from two MSD programs. The discussion includes the comparison of methods including quantitation and detection limits and ease of analytical operations. A comparative case study of a typical bioprocess train assessed by both the traditional and MockV RVLP system is presented (Figure 8).

Further evaluation to establish a robust method for RVLP detection and quantitation (such as a program-specific method) and improve on analyst experience (e.g., automation) is currently being performed.”

**RVLP Burden Evaluation in a Continuous Process**
(Beth Larimore, Evotec)

Traditional methods of quantitating RVLP burden are geared toward batch production and do not accurately reflect the RVLP concentrations in a continuous harvest process stream. A proposed strategy for evaluating the RVLP burden in a continuous process was discussed, and three case studies from 500 L-scale manufacturing runs were shared.

**Case 1: RVLP Quantitation on Day 13–15 Is Worst Case (Host A, Product A) (Figure 9)**

In this case, RVLPs accumulate in the reactor supernatant, while the product is harvested from the permeate. Thus, measuring the retentate RVLP late in the production cycle would be a $>2 \log_{10}$ overestimate of the actual load for the purposes of the RVLP safety factor calculation. Although worst-case estimates are acceptable to regulators, it would be preferable to have a more realistic starting load estimate.
Case 2: RVLP in the Permeate Is Unaffected by Perfusion Filter Swap (Host A, Product B) (Figure 10)

In Case 2, the permeate filter itself is capable of capturing RVLPs. Again, the RVLP in the retentate exceeds that in the permeate. Swap out of the filter does not impact capture; this is expected as RVLPs are remove by a sized-base mechanism. This observation shows that the partitioning phenomenon is robust and consistent, suggesting that permeate sampling could be a more realistic sampling point for RVLP measurement.

Case 3: RVLP at End of Production Is Worst Case (Host B, Product C) (Figure 11)

In Case 3, a different host cell line produces particles that seem to pass through the permeate filter uninhibited. Thus, each product/process needs to be analyzed individually to truly know the ultimate RVLP safety factor.

ASTM Standard Initiative Update and Path Forward (John Schreffler, Vir Biotherapeutics)

ASTM is a not for profit, international organization that develops consensus standards with direct stakeholder involvement. At the 2009 Viral Clearance Symposium, this initiative was started to develop a series of viral clearance standards, within AS and with cross-industry collaboration, to help ensure viral safety and potentially help reduce early-phase resources expenditures on viral clearance studies while maintaining patient safety. To date, three standards have been created within ASTM; low pH inactivation, surfactant inactivation, and the latest standard approved in 2022 retrovirus filtration using small virus retentive filters.

Figure 9


Figure 10

Evotec Case 2: RVLP in permeate is unaffected by perfusion filter swap (Host A, Product B). RVLP, retrovirus-like particle.
The first two were released in the 2010’s and have proven useful to set industry expectations for these two common enveloped virus inactivation steps. The more recent viral filtration standard was discussed, highlighting some of the challenges encountered across the life cycle and the final procedural and parametric specifications arrived at in the approved standard.

Writing the “Standard for Process to Remove Retroviruses by Small Virus Retentive Filters” involved four ballots across nine years and three companies. There were many meetings with and in the working group, responding to negative balloters. The final ballot incorporated extensive feedback from multiple parties.

The first step in this process was a historic look at filtration data from two VC testing houses to support the standard. Based on this, foundational decisions were made, for example, MVM data were excluded, as depressurization was judged to be too complicated across different filters. There were significant, but data-driven, discussions about the LRV level to specify in the standard, but the final decision was 6.0 log\(_{10}\). Per ASTM rules, the standard cannot name specific filters.

Key elements incorporated in the method include:

1. Differential pressure maintained within the specified limits provided by the specific small virus retentive filter manufacturer.

2. Passing of an appropriate pre-use assurance filter integrity test performed by either the viral filter manufacturer (Certificate of Analysis for each unique filter lot used) or the biopharmaceutical manufacturer (pre-use integrity test result), or both.

3. Passing of an appropriate post-use integrity test.

4. Any additional specifications for operation provided by the small virus retentive filter manufacturer must also be adhered to during filtration. The end user of the small virus retentive filter and this standard practice must show that these parameters were within these specified limits during the filtration operation.

Additionally, the future path of the overall ASTM initiative for viral safety initiative was discussed, including potential inclusion of different therapeutic modalities into current standards as well as maintenance and/or revision of current standards. Examples include:

1. Low pH Inactivation
   - Expansion of ranges

2. Surfactant Inactivation
   - Additional surfactants (esp. in the context of new EU environmental regulations)

3. Viral Filtration
   - MVM claims

Finally, there was a discussion regarding the value in expanding to the Cell/Gene therapy space and the possibility of standards for additional unit operations.

**Conflict of Interest Declaration**

The authors declare that they have no competing interests.
References


CONFERENCE PROCEEDINGS

Proceedings of the 2023 Viral Clearance Symposium, Session 6: Viral Inactivation

JUNFEN MA1,* and DAVID ROUSH2

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ABSTRACT: The use of detergents or low pH hold are commonly employed techniques in biologics downstream processing to inactivate enveloped viruses. These approaches have been demonstrated to be robust and are detailed in ASTM E2888 (low pH) and ASTM E3042-16 (Triton X-100), accordingly. One of the recent challenges is the need for a replacement of Triton X-100 with a more environmentally friendly detergent with similar log10 reduction value (LRV) achieved. The presentations in this session focused on a detailed assessment of a range of detergents. The most well characterized and potentially robust detergents identified were TDAO (n-Tetradecyl-N,N-dimethylamine-N-oxide) and Simulsol SL 11 W. Key performance factors assessed (in direct comparison with the industry standard Triton X-100) were viral inactivation kinetics (total elapsed time to achieve equilibrium), LRV achieved of enveloped viruses, toxicity, potential impact on product quality and process performance, clearance of residual detergent in subsequent downstream steps, assays to support assessment with appropriate limit of quantification, and commercial supply of detergent of the appropriate quality standard. Both TDAO and Simulsol SL11 had similar overall LRV as Triton-100. In addition, for the low pH viral inactivation, reduced LRV was observed at pH > 3.70 and low salt concentration (outside of the ASTM range), which is a cautionary note when applying low pH inactivation to labile proteins.

KEYWORDS: Viral inactivation, Detergent, Low pH viral inactivation, Triton X-100, TDAO, Simulsol SL 11W.

Background and Overview

The use of detergents or low pH hold are commonly employed techniques in biologics downstream processing to inactivate enveloped viruses. These approaches have been demonstrated to be robust and are detailed in ASTM E2888 (low pH) and ASTM E3042-16 (Triton X-100), accordingly. One of the recent challenges is the need for a replacement of Triton X-100 with a more environmentally friendly detergent with a similar log10 reduction value (LRV) achieved. This discussion was initiated at the 2019 Viral Clearance Symposium (VCS) (1) and was the primary focus in the 2023 VCS Viral Inactivation Session encompassing five of the six presentations. n-Tetradecyl-N,N-dimethylamine-N-oxide (TDAO) was a surfactant that has been previously evaluated in the literature (2) and was discussed in detail during the session. Another key topic of the session was the combined impact of salt level (ionic strength) with pH in low pH inactivation when operating at conditions slightly beyond the ASTM standard—a topic explored in more detail (3).

Two Novel Inactivation Agents as Triton X-100 Replacements in Different Biomanufacturing Feedstreams (Remo Leisi, CSL Behring)

Solvent/Detergent (S/D) treatment is a commonly used virus inactivation step in the downstream processing of biotherapeutics, targeting and disrupting the integrity of enveloped viruses. Biodegradation of the widely used Triton X-100 detergent, however, has been found to result in hormone-like substances of environmental concern, and therefore a replacement by a more eco-friendly compound is needed. We evaluated the suitability of n-octyl-β-D-glucopyranoside (OG) and TDAO as sustainable alternative inactivants targeting enveloped viruses in various plasma-derived or recombinant protein feedstreams. A comparison of the three detergents is presented in Table I. Both detergents showed rapid...
TABLE I
Alternative Detergents as Replacement for Triton X-100

<table>
<thead>
<tr>
<th>Name</th>
<th>CMC [mM]</th>
<th>MW [g/Mol]</th>
<th>Formula</th>
<th>Characteristics</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triton X-100</td>
<td>0.22–0.24</td>
<td>Avg 625</td>
<td>C_{14}H_{30}O(C_{2}H_{4}O)_{n} (n = 9–10)</td>
<td>Alkylphenolethoxylate Non-ionic detergent Substance of environmental concern</td>
<td></td>
</tr>
<tr>
<td>n-Tetradecyl-N,N-dimethylamine-N-oxide (TDAO)</td>
<td>0.24–0.29</td>
<td>257.5</td>
<td>C_{16}H_{15}NO</td>
<td>Alkyl N-oxide Zwitterionic detergent</td>
<td></td>
</tr>
<tr>
<td>n-Octyl-β-D Glucopyranoside (OG)</td>
<td>18–20</td>
<td>292.37</td>
<td>C_{14}H_{28}O_{6}</td>
<td>Alkyl glucoside Non-ionic detergent, commonly non-denaturing</td>
<td></td>
</tr>
</tbody>
</table>

CMC, critical micelle concentration; MW, molecular weight.

and effective inactivation of pseudorabies virus, vesicular stomatitis virus, bovine viral diarrhea virus, human immunodeficiency virus, and xenotropic murine leukemia virus (XMuLV) in most feedstreams when used at above the critical micelle concentration (CMC). As anticipated, inactivation of vaccinia virus—which is cloaked in a more stable double lipid bilayer—was generally less efficient, requiring longer incubation and higher detergent concentrations to achieve effective clearance. Furthermore, incubation conditions as well as feedstream composition were found to have significant impact on inactivation capacity and kinetics, emphasizing the importance of assessing the relevant parameters on a case-by-case basis. In summary, OG and TDAO were found to be suitable, eco-friendly alternatives for the replacement of Triton X-100, providing a comparable or more efficient inactivation of a broad panel of enveloped viruses in various biomanufacturing feedstreams.

Implementation of a New Environmentally Friendly Detergent Capable of Inactivating Virus into Manufacturing Processes of Biopharmaceutical Products (Sean Michael O’Donnell, Eli Lilly)

Triton X-100 detergent treatment is a robust enveloped virus inactivation unit operation included in biopharmaceutical manufacturing processes. However, the European Commission officially placed Triton X-100 on the Annex XIV authorization list in 2017, because a degradation product of Triton X-100, 4-(1,1,3,3-tetramethylbutyl) phenol (also known as 4-tert-octylphenol), is considered to have harmful endocrine disrupting activities. This has prompted biopharmaceutical manufacturers to search for novel, environment-friendly alternative detergents for enveloped virus inactivation. We have identified a new environmentally friendly detergent, Simulsol SL 11 W, that robustly inactivated enveloped viruses. Virus inactivation by Simulsol SL 11 W was effective across different clarified bioreactor harvests at broad concentrations, pH, and temperature ranges. Inactivation was not matrix dependent, and inactivation was robust at both 4˚C and 30˚C (Table II). Furthermore, impurities in the matrix also did not have an impact on virus inactivation by Simulsol SL 11 W (Table III). Removal of Simulsol SL 11 W was readily achieved by protein A chromatography, and product quality was not affected by detergent treatment. Even though Simulsol SL 11 W was effective at small scale; large-scale studies were also evaluated for its use in manufacturing. It was shown that this detergent was suitable for use in manufacturing.

Considerations and Approach to Selecting Alternative Detergents to Triton X-100 (James Berrie, Lonza)

Virus inactivation by detergent as a dedicated viral clearance step in the manufacture of biopharmaceuticals
is a well-established and characterized process step. A small number of commonly used detergents have delivered the key to downstream processes with respect to overall viral clearance demonstration. Significantly, over the last decade the ‘go to’ option has been t-octylphenoxypolyethoxyethanol (Triton X-100). Following approval of the addition of Triton X-100 to Annex 14 of the European REACH regulation in 2016, after many years of consultation, the restriction was adopted into European law and a sunset date of January 2021 applied. Post this date, the use of Triton X-100 has been banned under EU and UK law, and use is only permitted under a continued use license overseen by the relevant Environmental Authorities. The associated production waste monitoring and testing for residual Triton X-100 and collection of relatively large quantities of waste for incineration imposes significant negative facility constraints. The search for alternatives that meet quality and process parameters, address environmental concerns, and have the required viral clearance capability poses many obstacles. Not all alternatives under evaluation are as ‘plug and play’ as Triton X-100, and therefore significant challenges are faced by manufacturers and developers with respect to low pH sensitive biopharmaceuticals and viral inactivation. Here we consider the parameters required to meet inclusion of a detergent candidate in a toolbox development approach and an overview of a study to identify two detergent candidates with differing properties. An overview of the methodology for assessing alternatives to Triton X-100 is provided in Figure 1.

### Figure 1

Methodology for screening alternatives to Triton X-100. IP, intellectual property.

Almost all biomanufacturing processes use a virus inactivation procedure to reduce the patient risk of

<table>
<thead>
<tr>
<th>Time Point (Min)</th>
<th>H₂O</th>
<th>Media</th>
<th>DPBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>4.12</td>
<td>2.00</td>
<td>1.50</td>
</tr>
<tr>
<td>T60</td>
<td>≥4.52</td>
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<td>4.53</td>
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<tr>
<td>T120</td>
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<td>≥4.52</td>
<td>≥4.52</td>
</tr>
<tr>
<td>T180</td>
<td>≥5.31</td>
<td>≥5.31</td>
<td>5.63</td>
</tr>
</tbody>
</table>

DPBS, Dulbecco’s phosphate-buffered saline; LRF, log reduction factor.

### Table III

Impact of Impurities on Virus Inactivation by Simulsol SL 11 W

<table>
<thead>
<tr>
<th>Time Point (min)</th>
<th>Molecule A CFM</th>
<th>Molecule A Concentrated CFM</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>0.62</td>
<td>2.25</td>
</tr>
<tr>
<td>T10</td>
<td>2.50</td>
<td>2.00</td>
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<tr>
<td>T30</td>
<td>4.58</td>
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<tr>
<td>T60</td>
<td>5.43</td>
<td>5.06</td>
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<tr>
<td>T120</td>
<td>≥5.42</td>
<td>≥5.05</td>
</tr>
<tr>
<td>T180</td>
<td>≥5.88</td>
<td>≥5.51</td>
</tr>
</tbody>
</table>

CFM, cell free media; LRF, log reduction factor.

### Table II

LRF Achieved with Simulsol SL 11 W in Different Matrices

<table>
<thead>
<tr>
<th>Time Point (Min)</th>
<th>LRF Achieved (4°C)</th>
<th>LRF Achieved (30°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H₂O</td>
<td>Media</td>
</tr>
<tr>
<td>T0</td>
<td>4.12</td>
<td>2.00</td>
</tr>
<tr>
<td>T60</td>
<td>≥4.52</td>
<td>≥4.52</td>
</tr>
<tr>
<td>T120</td>
<td>≥4.52</td>
<td>≥4.52</td>
</tr>
<tr>
<td>T180</td>
<td>≥5.31</td>
<td>≥5.31</td>
</tr>
</tbody>
</table>

DPBS, Dulbecco’s phosphate-buffered saline; LRF, log reduction factor.
virus-transmitted diseases. Although low pH inactivation is widely used, some products may be negatively impacted by the low pH conditions applied. In the past, Triton X-100 was commonly used as a virus inactivation detergent. However, with Triton X-100 becoming restricted by European REACH regulation in 2019, industry is searching for alternative compounds to inactivate virus without negatively impacting product quality and process performance.

Table IV summarizes the detergents we have tested and the tested concentration in relation to the CMC. All detergents have been tested for the inactivation of XMuLV in the presence of a protein in cell-free cell culture harvest. The results are shown in Figure 3.

TDAO (Deviron, Merck Millipore) and Simulsol SL 11 W (Seppic SA) were selected for further experiments to determine inactivation kinetics as well compatibility with Simethicone, a common anti-foam mixture used in CHO cell culture processes.

Figure 2

Summary of viral inactivation development study results. PRV, pseudorabies virus; XMuLV, xenotropic murine leukemia virus.

TABLE IV
Overview of Tested Detergents and the Known Critical Micelle Concentration

<table>
<thead>
<tr>
<th>Detergent</th>
<th>CMC (%)</th>
<th>Concentration (%) Tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>TDAO (Zwitterionic) - Deviron®</td>
<td>0.0036–0.0075</td>
<td>0.0098</td>
</tr>
<tr>
<td>ODTG (non-ionic)</td>
<td>~0.28</td>
<td>0.3372</td>
</tr>
<tr>
<td>DDM (non-ionic)</td>
<td>0.0087</td>
<td>0.0124</td>
</tr>
<tr>
<td>Anapoe C12E8 (non-ionic)</td>
<td>~0.0048</td>
<td>0.0108</td>
</tr>
<tr>
<td>Triton CG-110 (non-ionic)</td>
<td>0.1748</td>
<td>0.2153</td>
</tr>
<tr>
<td>Ecosurf EH-9 (non-ionic)</td>
<td>0.1066</td>
<td>0.1283</td>
</tr>
<tr>
<td>Simulsol SL 11 W (non-ionic)</td>
<td>Not applicable</td>
<td>0.15</td>
</tr>
</tbody>
</table>

CMC, critical micelle concentration; DDM, n-Dodecyl-β-D-maltoside; ODTG, n-octyl-β-D-thioglucopyranoside; TDAO, n-tetradecyl-N,N-dimethylamine-N-oxide.

Figure 3

Log removal factors for XMuLV using different detergents in the presence of protein after 60 min incubation at 14°C ± 1°C. DDM, n-Dodecyl-β-D-maltoside; LRV, log10 reduction value; ODTG, n-octyl-β-D-thioglucopyranoside; TDAO, n-tetradecyl-N,N-dimethylamine-N-oxide; XMuLV, xenotropic murine leukemia virus.
TDAO showed a fast, reproducible, and robust viral inactivation independent of Simethicone addition (Figure 4).

We successfully replaced Triton X-100 with TDAO for one of our development projects. We achieved a higher log removal factor (LRF) compared with that of Triton X-100. Although the Triton X-100 inactivation was lower than expected (Figure 5), no additional investigation of the low LRF of Triton X-100 was performed because the overall virus removal of the process was sufficient at that time.

TDAO as well as Simulsol show rapid and robust virus inactivation for enveloped viruses. After extensive screening of different available detergents, we successfully implemented TDAO in one of our development processes as a replacement for Triton X-100. Simulsol SL 11 W is considered as an acceptable TDAO alternative to complement the virus inactivation toolbox.

**Efficient Virus Inactivation of Acid-Sensitive Proteins Using an Ecofriendly Amine N-Oxide-Based Detergent**

(Sabine Faust, Rentschler Biopharma)

In the investigation for a viable replacement for Triton X-100, the zwitterionic detergent TDAO was systematically examined. Becoming commercially available as a biodegradable and fit-for-purpose pharma grade raw material under the name Deviron C16 (Merck Life Science KGaA) in 2022, TDAO was assessed for its virus inactivation properties.

**Figure 4**

TDAO and Simulsol SL 11 W kinetics in the presence of simethicone. Large volume testing was performed for the 60 min sample. TDAO, n-tetradecyl-N,N-dimethylamine-N-oxide

**Figure 5**

Comparison of the inactivation kinetics of XMuLV using Triton X-100 and TDAO for a project in development. TDAO, n-tetradecyl-N,N-dimethylamine-N-oxide; XMuLV, xenotropic murine leukemia virus.
inactivation potential in the context of two acid-sensitive antibodies (IgG2 and IgG4) and a conformationally labile Fc fusion enzyme. More importantly, the product compatibility with respect to critical quality attributes of these proteins and the removal of TDAO as a process-related impurity through standard unit operations was confirmed.

Incubation studies in harvested cell culture fluid (HCCF) demonstrated comparable product compatibility between TDAO and Triton X-100. The monomer, aggregate, and fragment levels remained stable for all tested molecule formats independent of the TDAO concentration (0.5% vs. 1.0%) and incubation time (3 h vs. >12 h). Further, the enzymatic activity of the Fc fusion enzyme was maintained for both tested detergent concentrations.

Robust product quality was confirmed for process intermediates further downstream and by different analysis for each molecule (size-exclusion chromatography, capillary gel electrophoresis, and activity assay).

To determine the virus inactivation potential of TDAO with these protein candidates, kinetic studies (0 to 60 min) were carried out at different detergent concentrations (0.5% to 1.0%), temperatures (4˚C to 17˚C), and process intermediate levels (HCCF and protein A eluate) using the enveloped murine leukemia virus (MuLV) as the model and the tissue infectious dose assay (TCID50) as the detection analytics. Complete virus inactivation was achieved at TDAO concentrations ≥0.5% within 15 min incubation time for all tested conditions and molecule types (Figure 6). The claimable LRVs are reduced due to the high cytotoxicity and could potentially be increased by detergent removal before analysis or higher sample volume analysis.

Process compatibility was assessed for TDAO-mediated virus inactivation in HCCF and capture eluate by comparison with a classical low pH treatment in a typical 3-column monoclonal antibody (mAb) downstream process (Figure 7A).

TDAO application in HCCF resulted in similar host cell protein (HCP) and high molecular weight species (HMWS) reduction by subsequent standard purification steps compared with the low pH reference inactivation post protein A capture step (Figure 7B). Moreover, high-performance liquid chromatography with evaporative light scattering detector analysis (by Merck Life Science)
KGaA) of process intermediate samples revealed sufficient TDAO depletion after the final chromatography step (< the limit of detection, LOD) (Figure 7C).

In contrast, for TDAO-mediated virus inactivation in the protein A eluate pool, the HCP and TDAO levels remained unexpectedly high even after the polishing chromatography step (>1000 ppm). Noteworthy, interfering effects were observed during UV280nm measurements at various process steps, limiting the use of TDAO after the capture step.

Final ultrafiltration/diafiltration experiments proved that TDAO removal to 100% is achievable when the start concentration is below the CMC (<10 ppm).

In conclusion, the knowledge gained on TDAO’s efficacy as a virus inactivation substance provides the basis for a new and valuable orthogonal strategy of viral clearance in mammalian cell line-derived processes. TDAO can specifically act as a substitute for Triton X-100 in the manufacturing of next-generation recombinant proteins, which are low pH sensitive.

Characterizing the Effect of Ionic Strength on Low pH Viral Inactivation (Jena Daya, Regeneron)

Regeneron reported viral clearance studies evaluating low pH hold (LPH) in the purification process of mAbs under worst-case conditions of high pH and minimum hold duration. Per ASTM E2888, highly acidic conditions (i.e., pH < 3.60) provide robust and effective (> 4 log10 reduction factor) enveloped virus inactivation. However, effective viral inactivation may not be observed in viral clearance studies when viral inactivation occurs above pH 3.60. Such
conditions may be required for acid-labile products to ensure product stability. Regeneron has designed an approach to achieve robust and effective retrovirus inactivation at higher pH (pH 3.70 to 3.90) by controlling the sodium chloride (NaCl) concentration of the low pH starting material.

Regeneron conducted two sets of multivariate design of experiments to characterize the operating space for low pH viral inactivation of a model retrovirus, virus XMuLV, above pH 3.60. The first experiment evaluated the effect of mAb isotype, pH (pH 3.65–3.80), temperature (15˚C and 20˚C), acid titrant type, NaCl concentration (0, 50, and 100 mM added NaCl), virus spike timing, and post spike filtration on XMuLV inactivation. The resulting reduced linear regression model ($p < 0.0001$) demonstrated that NaCl concentration had the greatest effect on virus inactivation in the pH range studied. pH has a large effect when the load material has no additional NaCl. Aside from pH and NaCl concentration, all other factors evaluated in the design of experiment have no practically significant effect on XMuLV inactivation. The model accounts for 98% of the variation in the data set ($R^2_{adj} = 0.98$) with a root mean square error (RMSE) of 0.18 log10. Details of this experiment design and results are published (3).

An additional multivariate study was performed to further understand the effect of salt concentration between 0 mM and 50 mM, to extend the acidic condition to pH 4.00, and to evaluate sodium acetate as a conductivity modulating salt species. The reduced linear regression model ($p < 0.0001$) shows no statistically significant difference in XMuLV inactivation between adjustment with sodium chloride and sodium acetate in the studied pH range. The effect of salt concentration on virus inactivation diminished at higher concentrations (>20 mM), and there was no effect of salt concentration at pH 4.0. The model accounts for 97% of the variation in the data set ($R^2_{adj} = 0.97$) with a RMSE of 0.28 log10. The prediction profiler showing XMuLV inactivation at 30 min for the significant factors is illustrated in Figure 8, with 95% confidence interval shaded in gray.

The combined studies demonstrated that ionic strength substantially affects XMuLV inactivation at pH 3.70–3.90. XMuLV inactivation is insensitive to NaCl concentration when operating at lower pH (pH < 3.70) or higher pH (pH > 3.90) in the NaCl range studied (0 to 50 mM). It is hypothesized that increasing the ion concentration in the starting solution may disrupt electrostatic interactions and reduce the formation of virus aggregates, thereby exposing the virus to the acidic pH solution. A contour plot of the combined studies is illustrated in Figure 9 and can be used to predict XMuLV inactivation at any operating pH and NaCl concentration in the studied range. These studies contribute to the understanding of ionic strength as an influential parameter in low pH viral inactivation studies.

**Summary and Discussion**

The presentations in this session focused on a detailed assessment of a range of detergents. The most well characterized and potentially robust detergents identified...
were TDAO and Simulsol SL 11 W. Key performance factors assessed (in direct comparison with the industry standard Triton X-100) were viral inactivation kinetics (total elapsed time to achieve equilibrium), LRV achieved of enveloped viruses, toxicity, potential impact on product quality and process performance, clearance of residual detergent in subsequent downstream steps, assays to support assessment with appropriate limit of quantification, and commercial supply of detergent of the appropriate quality standard. Both TDAO and Simulsol SL11 had similar overall LRV as that of Triton X-100. Commercial availability including Freedom to Operate with Simulsol SL11W was reviewed during the discussion.

One outcome of the discussion was a strong desire to update the ASTM standard to include viral inactivation using eco-friendly detergents that have been comprehensively assessed with the rigorous performance parameters summarized prior. One additional follow-up item from the discussion was to clarify the conditions of temperature and concentration where some residual XMuLV activity had been reported for Triton X-100, because this could serve as a guide for further characterization of eco-friendly detergents.

The conclusion of the discussion on the combined impact of salt/ionic strength and pH on low pH inactivation was that there was no impact to the current ASTM standard of low pH viral inactivation based on the data presented. The key point was that reduced LRV was observed at pH > 3.70 and low salt concentration (outside of the ASTM range), which is a cautionary note when applying low pH inactivation to labile proteins.

**Conflict of Interest Declaration**

The authors declare that they have no competing interests.

**References**


Conference Proceedings

Proceedings of the 2023 Viral Clearance Symposium, Session 7: Up- and Downstream Virus Retentive Filtration

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ABSTRACT: Session 7 of the 2023 Viral Clearance Symposium reviewed progresses in virus retentive filtrations applied to both upstream and downstream processing. Upstream topics included investigations and applications of media viral filtration for upstream cell culture viral risk mitigation. Downstream topics included evaluation of viral breakthrough in continuous processing using surrogate particles and demonstration of extensive viral filtration cycling with flow interruptions and long duration in connected process. Reuse of viral filters with proposed procedures was successfully demonstrated amid the supply chain challenge encountered during the pandemic. Discussions and additional considerations for the topics were also provided.

KEYWORDS: Virus filtration, Cell culture media, Pressure/flow interruptions, Viral filter cycling, Continuous/connected process, Viral filter reuse.

Background and Session Overview

The downstream processes of biopharmaceutical processes are expected to contain a viral filter capable of removing viruses as small as 20 nm in size. Virus removal by virus filtration is based on a size exclusion mechanism, with most viral filters having mean pore sizes of ~20 nm in size. Because of the size exclusion mechanism of virus removal, viral filtration is a robust viral removal step in the downstream process, and understanding the operating parameters of viral filters is a must.

Recently, upstream viral filters for media and feeds have been developed. These filters are similar to the downstream viral filters with pore sizes around 20 nm. However, because of cost and throughput concerns, these upstream filters are a modified version of the downstream filters usually containing only one layer of membrane or fewer hollow fibers. This allows for robust throughput of media and feeds while keeping the cost to a minimum.

In this session, the use of both upstream and downstream viral filters was discussed. Operating conditions in continuous processes were discussed for the downstream viral filters. Upstream viral filtration parameters and how they can be utilized in a manufacturing setting were also discussed. The session wrapped up with discussion of supply chain shortages of viral filters and alternative strategies for switching viral filters.

Participant Contributions

Detailed discussions on each of the five contributions in this session are provided as below.

Upstream Virus Barriers: Why and How (Andreas Wieser, Takeda)

Medical progress as enabled by early plasma products has also revealed biological safety challenges. The safety tripod combination of donor selection, donation testing, and virus reduction processes has effectively addressed these concerns, and today plasma products feature significant safety margins. The safety tripod concept has since been adapted to biotechnology manufacturing platforms and has ensured the safety of these products. However, cell-based manufacturing processes have occasionally been exposed to adventitious agents,
leading to manufacturing interruptions and thus to unstable supply situations. The rapid progress of advanced therapy medicinal products (ATMPs) also needs an innovative approach to ensure the learnings from more traditional biotechnology help to avoid any unwelcome reminder of the universal presence of pathogens.

The introduction of upstream virus clearance steps is valuable for any products too complex for downstream interventions in the sense of both assuring product safety and continuous supply. The gentlest method being virus filtration. Studies that investigated the feasibility of implementing culture media virus filtration were briefly presented. These studies have been summarized in Wieser et al. (1).

The focus of the studies was on the virus clearance capacity under extreme conditions, which have been indicated to be worst-case for virus removal, such as high process feed loading, long duration of filtration, and multiple process interruptions (2). Minute virus of mice (MVM) was chosen as a small challenge virus. This is because although culture media increasingly do not contain animal-derived components (3), viral contaminations of cell-derived bulk harvests have still occurred, for example, with MVM (4, 5). Therefore, MVM is a target virus. Furthermore, MVM represents small nonenveloped viruses, which are the main challenge for virus filters with a stipulated pore size of 20 nm, and is thus also a suitable model virus (6).

The equipment used consisted of an electronic recorder, a 5 L pressure tank, pressure transducers, temperature sensors, and the necessary tubing, as generally described elsewhere (7).

The filters investigated are grouped into first- (FG) and second-generation (SG) filters (8). The virus removal filters used were either FG filters (Asahi Planova 15 N, Millipore Viresolve NFP, Pall Ultipore VF DV20, and Pall Pegasus SV4) or SG filters (Asahi Planova BioEX, Millipore Viresolve Barrier, Pall Pegasus Prime, Sartorius Virosart CPV, Sartorius Virosart HF, and a developmental filter).

The laboratory scale experiments were designed to simulate the feeding of a perfusion fermenter with a fermenter feed media solution containing MVM as a contaminant (0.4% spike load). The spiked media was passed through a filter train consisting of a 0.2 μm pre-filter and a virus removal filter. The filtration was performed at constant transmembrane pressures for the virus filter. Filtration was performed until a flow decay of around 90% was reached or the duration of the experiment had exceeded 30 days, thus the maximum load was investigated. Furthermore, the impact of multiple interruptions during the loading process on virus clearance was also investigated. Each interruption lasted at least 1 h and one lasted at least 72 h to represent the pause between the first filling of the fermenter and the buildup of the inoculum before the actual perfusion fermentation started. The typical progress of such an experiment is shown in Figure 1.

It was found that most FG filters (gray lines in Figure 2) allowed virus breakthrough, and MVM reduction factors were below 4 logs. These filters are, thus, unsuitable for high-volume long duration applications. It is noted that these filters are designed for downstream manufacturing processes, which need virus filters designed to handle up to 1000 L/m² of an aqueous protein solution as rapidly as possible. Furthermore, FG filters were not typically designed to tolerate the impact of pressure interruptions or low-flow phases on their virus retention capacities.

SG filters generally have improved virus retention capabilities even if pressure interruptions occur. For some of these filters (SGI and SGII in Figure 2), even substantial virus removal capacities at very high loads (up to ~19,000 L/m²) were demonstrated.

In summary, the implementation of culture media virus filtration with respect to their virus clearance capacities under extreme conditions such as very high process feed loading (up to ~19,000 L/m²), very long duration (up to 34 days), and multiple process interruptions (up to 21, with cumulative interruptions of over 92 h) is feasible. It was found that a number of filters—especially of the newer second generation—are capable of effective virus clearance despite the harsh regimen they were subjected to. At the same time, the investigation of biochemical parameters for un-spiked control runs showed the filters to have no measurable impact on the composition of the culture media. Again, indicting this technology to be especially suitable for large-volume manufacturing process culture media preparation.

Characterization of Upstream Viral Filters: Throughput and Virus Retention Using Different Chemically Defined Media and Feeds (Luo Wen, Eli Lilly)

Biopharmaceutical manufacturers are constantly looking for ways to reduce the risk of a contamination of
their products or facilities. Methods such as high-temperature short time (HTST) can be used on cell culture media and feeds to mitigate the risk of contamination; however, HTST can often result in changes to the cell culture media and feeds that adversely impact cell growth. Until recently, HTST was the only option for reducing contamination in cell culture media and feeds, but now upstream viral filters are commercially available. These filters are capable of removing viral contamination with fast flux, little development, and no major changes to manufacturing facilities.

In this study, Millipore Viresolve Barrier (V Barrier) filters were evaluated for their capability to remove viruses using MVM and porcine parvovirus (PPV). Process parameters including loading capacity, temperature, flux rate, and pressure disruption were investigated for their impact on virus breakthrough. Chemically defined (CD) medias were evaluated without virus spike for overall throughput capacities. As shown in Figure 3, one media, CD C, performed well whereas the other two media showed overall lower capacities. This demonstrated that the filters may not be ready for implementation without some development work to meet throughput targets.

To assess the impact of virus spike on overall virus retention, MVM was spiked into CD C media at \( \approx 1 \times 10^6 \) median tissue culture infectious dose (TCID\(_{50}\))/mL. Three pressure disruptions were performed as indicated in Figure 2. In total, four fractions were collected before each pressure stop. A filtration pool was reconstituted from the four fractions. The virus titer in each fraction was determined by a cell-based TCID\(_{50}\) assay.

The TCID\(_{50}\) titer of Fraction 1 and Fraction 2 was \( \leq 0.99 \) log\(_{10}\) TCID\(_{50}\)/mL, indicating no viruses were detected after the pressure stop. When the capacity increased \( >2000 \) L/m\(^2\), virus breakthrough was observed in Fraction 3 with a titer at \(-0.62\) log\(_{10}\) TCID\(_{50}\)/mL. Overall, this MVM spiked run reached 2442 L/m\(^2\) loading capacity and achieved a MVM clearance of 5.83 logs (Figure 4).

Finally, to assess the impact of temperature on virus breakthrough of the viral filter, PPV-spiked material
Figure 2

Overview of the development of the logarithmic reduction factor ($R$) during representative runs for each filter investigated (Wieser et al. [1]). Arrows pointing up indicate the corresponding filtrate fraction titer was below the limit of detection. MVM, minute virus of mice.

Figure 3

Filterability comparison of three different chemically defined (CD) media.

Figure 4

Impact of loading capacity and pressure disruption on virus breakthrough. TCID50, median tissue culture infectious dose.
was run through the viral filter at 37°C. Two pressure disruptions were designed into the study, and three fractions were collected before each of the pauses. Robust PPV clearance of 5.90 logs and 6.14 logs were achieved for each Fraction 1. At the end of each run, the calculated total virus loading was 9.20 logs and 9.17 logs, respectively. Still, robust virus removal of 5.53 logs and 4.95 logs of PPV was achieved (Table I). At the higher temperatures, slightly more breakthrough was observed throughout the filtrations, potentially indicating that a raised filtration temperature may slightly increase virus breakthrough.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Filtrate</th>
<th>Loading Capacity (L/m²)</th>
<th>Virus Loading (log₁₀TCID₅₀)</th>
<th>PPV Clearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT</td>
<td>Fraction 1</td>
<td>231</td>
<td>8.78</td>
<td>5.90</td>
</tr>
<tr>
<td></td>
<td>Filtrate Pool</td>
<td>605</td>
<td>9.20</td>
<td>5.53</td>
</tr>
<tr>
<td>~ 37°C</td>
<td>Fraction 1</td>
<td>223</td>
<td>8.76</td>
<td>6.14</td>
</tr>
<tr>
<td></td>
<td>Filtrate Pool</td>
<td>572</td>
<td>9.17</td>
<td>4.95</td>
</tr>
</tbody>
</table>

PPV, porcine parvovirus; RT, room temperature; TCID₅₀, median tissue culture infectious dose.

Continuous Manufacturing: Implementation of Multiple, Deliberate Interruptions of a Virus Removal Filtration Process (Konstantin Zoeller, Novartis)

Virus filtration is one of the most effective process steps in the production of biopharmaceuticals to protect patients from virus transmitted disease. In small-scale studies, it was reported that small amounts of viruses that are in the size range of the filter pores can pass the filter membrane. Published data suggest that hydrodynamic force is a second mechanism of virus retention for small viruses by limiting particle movement in the filter membrane at high flow rates (9).

Interruption of flow during the nanofiltration process is regarded as one of the reasons for increased passage of small virus through the nanofilter. Although the virus passages reported did not severely reduce the viral clearance capacity of the overall process, the viral safety concern regarding process interruptions remains. Modern manufacturing concepts like those shown in Figure 5 require multiple filter interruptions to avoid frequent filter exchange and increased bioburden risk.

We evaluated the effect of filter cycling by interrupting 11 times with long interruption intervals of 5–6 h or 9–11 h, alternating using Viresolve Pro (Merck) and MVM spiking. We collected the filtrate after every cycle and analyzed for virus. As shown in Table II, no virus was detected in any of the tested fractions. High protein and low protein concentrations were studied without any detectable impact. Of note, the viral load remained stable when incubated at room temperature for a total process time of 99 h.

We successfully implemented that concept in a manufacturing process without a detrimental effect on viral clearance (Figure 6). We did not detect any difference between short interruptions (30 min) or long interruptions (6 h or 11 h, alternating). MVM viral clearance was claimed for larger viruses.

We successfully demonstrated that multiple process interruptions did not lead to measurable breakthrough of MVM, resulting in effective virus removal. In addition, other parameters like protein concentration, duration of interruptions, and number of interruptions did not lead to a detrimental impact on viral clearance. As demonstrated, the interrupted nanofiltration is regarded as safe in terms of virus removal.

Considerations for Viral Filtration in An End-to-End Continuous Process (Megan McClure, Caroline Mueller, Jodi Chien, Just-Evotec Biologics, Inc.)

Integrated end-to-end continuous processing presents challenges to viral filtration operation to maximize filter throughput while ensuring viral safety. Continuous virus filtration requires higher loadings to maximize filter utilization and minimize system manipulations, such as filter change-outs, to reduce process risk. However, high filter loadings and long process times also lead to filter flux and feed pressures that are much lower across these membranes than in a typical batch scale process. Table III shows potential process scenarios that could occur in a continuous process. As is well-known, low pressures across viral filters can lead
to virus breakthrough. Additionally, long processing times can create challenges in evaluating the viral clearance capabilities of viral filters, because the assessment may require studies spanning over several days as well as novel virus-spiking strategies. Such assessment is needed to adequately demonstrate a high level of viral clearance while not overloading filters with virus in a manner not representative of manufacturing operations. The purpose of this work was to identify virus filters that perform well in terms of antibody throughput while maintaining virus retention.

Multiple commercially available filters were tested for product throughput. Candidate filters with little to no plugging at high throughput were evaluated for virus retention. Much of the viral clearance testing was performed with a noninfectious MVM-like capsid containing no genetic material. Spiking studies were performed with monoclonal antibody (mAb) A on Filters A and E using the surrogate particle compared to some batch studies with MVM spiking. The spiking studies with the surrogate particle were performed at both high and low flow rates for comparison with the MVM data. Daily fractions were taken for studies that spanned multiple days. A 10-min pause was performed daily before initiating collection of the next day’s fraction. The initial study performed with only Filter A only compared the effect of flow rate and load challenge on the retention of the MVM-like particle. The results show that an 11-log particle challenge led to more virus breakthrough than a

### Table II

<table>
<thead>
<tr>
<th>Protein Concentration</th>
<th>High</th>
<th>Low</th>
</tr>
</thead>
<tbody>
<tr>
<td>Load</td>
<td>6.65</td>
<td>6.77</td>
</tr>
<tr>
<td>Hold (99 h)</td>
<td>6.65</td>
<td>6.29</td>
</tr>
<tr>
<td>Cycle 1</td>
<td>≤1.67</td>
<td>≤1.66</td>
</tr>
<tr>
<td>Cycle 2</td>
<td>≤1.67</td>
<td>≤1.67</td>
</tr>
<tr>
<td>Cycle 3</td>
<td>≤1.67</td>
<td>≤1.67</td>
</tr>
<tr>
<td>Cycle 4</td>
<td>≤1.67</td>
<td>≤1.67</td>
</tr>
<tr>
<td>Cycle 5</td>
<td>≤1.67</td>
<td>≤1.67</td>
</tr>
<tr>
<td>Cycle 6</td>
<td>≤1.67</td>
<td>≤1.67</td>
</tr>
<tr>
<td>Cycle 7</td>
<td>≤1.67</td>
<td>≤1.67</td>
</tr>
<tr>
<td>Cycle 8</td>
<td>≤1.66</td>
<td>≤1.67</td>
</tr>
<tr>
<td>Cycle 9</td>
<td>≤1.67</td>
<td>≤1.67</td>
</tr>
<tr>
<td>Cycle 10</td>
<td>≤1.66</td>
<td>≤1.66</td>
</tr>
<tr>
<td>Cycle 11</td>
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<td>≤1.66</td>
</tr>
<tr>
<td>Cycle 12</td>
<td>≤1.66</td>
<td>≤1.66</td>
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<tr>
<td>Combined Filtrate</td>
<td>≤2.75</td>
<td>≤2.75</td>
</tr>
<tr>
<td>LRF</td>
<td>≥3.90</td>
<td>≥4.02</td>
</tr>
</tbody>
</table>

LRF, log reduction fraction.
9-log particle challenge (Table IV). Additionally, a significant impact on flow rate was observed, with a large increase in surrogate particles retention at low flow rates. The 9-log load challenge of surrogate particles at the high flow rate showed complete retention in the first fraction and minimal breakthrough in the second fraction, similar to the MVM condition. The surrogate particle assay is less sensitive than the MVM TCID<sub>50</sub> assay, leading to a lower log reduction value (LRV) when no virus (or virus-like particle) is detected.

**TABLE III**
Batch versus Continuous Viral Filter Process Scenario

<table>
<thead>
<tr>
<th>Operating Mode</th>
<th>Filter Size (m&lt;sup&gt;2&lt;/sup&gt;)</th>
<th>Typical Process Flow Rate (mL/Min)</th>
<th>Expected Pressure (Psi)*</th>
<th>Filter Switch Frequency (Days)</th>
<th>Expected Loading (L/m&lt;sup&gt;2&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch</td>
<td>1.0</td>
<td>1000</td>
<td>14</td>
<td>NA</td>
<td>400</td>
</tr>
<tr>
<td>Continuous</td>
<td>1.0</td>
<td>60</td>
<td>0.9</td>
<td>4</td>
<td>346</td>
</tr>
<tr>
<td>Continuous</td>
<td>0.3</td>
<td>60</td>
<td>3</td>
<td>7</td>
<td>605</td>
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<td>4320</td>
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</table>

*based upon a permeability of 4.5 LMH/psi.

**TABLE IV**
Evaluation of MVM-like Particle Load Challenge and Flow Rate on Particle Retention of Filter A

<table>
<thead>
<tr>
<th>Condition</th>
<th>Log Load Challenge</th>
<th>Fraction 1</th>
<th>Fraction 2</th>
<th>Fraction 3</th>
<th>Fraction 4</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Loading (L/m&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>LRV</td>
<td>Loading (L/m&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>LRV</td>
</tr>
<tr>
<td>MVM Control - High Flow</td>
<td>9</td>
<td>200</td>
<td>≥ 6.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surrogate – High Flow</td>
<td>11</td>
<td>300</td>
<td>1.7</td>
<td>190</td>
<td>1.1</td>
</tr>
<tr>
<td>9</td>
<td>262</td>
<td>4.5</td>
<td>272</td>
<td>4.2</td>
<td></td>
</tr>
<tr>
<td>Surrogate – Low Flow</td>
<td>11</td>
<td>278</td>
<td>0.6</td>
<td>293</td>
<td>0.3</td>
</tr>
<tr>
<td>9</td>
<td>276</td>
<td>2.5</td>
<td>172</td>
<td>1.8</td>
<td></td>
</tr>
</tbody>
</table>

LRV, log reduction value; MVM, minute virus of mice.
A comparison of Filter A and Filter E is shown in Table V. All virus and virus-like load challenges were 9 logs. The standard curve for the MVM-like particle assay was shifted for the Filter E samples leading to a higher limit of quantitation (LOQ) and lower assay sensitivity. Filter E showed comparable MVM-like particle retention at both high and low flow rates, whereas Filter A had increased breakthrough at low flow rates.

In conclusion, MVM-like surrogate particles can be used to study filter sensitivity to virus breakthrough. Higher load challenges can lead to increased breakthrough; thus, viral load challenge should be carefully considered. Lower flow rates can lead to virus breakthrough on some filters; however, some newer generation filters are more robust in terms of virus retention at low flow rates. Future work includes confirming these results with MVM spiking at low flow rates as well as testing more commercially available filters.

Supply Challenges of Virus Filters and Potential Alternatives (Nora Schroeder, Novartis)

During the COVID-19 pandemic, there was a high risk for supply constraints for certain raw materials and consumables including virus removal filters that could impact biopharmaceutical drug supply. To mitigate the challenges, a new approach was explored by identifying conditions that would allow possible reuse of virus filters.

A prerequisite of virus filter reuse is to identify an appropriate cleaning procedure that ensures virus filter membrane integrity and removal of residual impurities at the same time. We determined the capability of different cleaning agents to maintain or restore the process performance (e.g., flow rate, flow decay) of the virus filter. Meanwhile, it was evaluated that the use of a used and regenerated virus filter did not impact the quality of the product. Additionally, the cleaning agent needs to be removed to an extent that does not negatively impact the product. It was defined which cleaning agent concentration and incubation time is needed to achieve the desired effects. Furthermore, it was tested which storage solution is suitable for long-term storage of the virus filter after reuse.

It could be shown that the performance of the virus filters stayed consistent after reuse and is reproducible (Figure 7). Additionally, it was demonstrated that the product quality was not affected by reuse of the virus filter. No binding or altering of the protein by potentially present residual cleaning agent could be observed analytically. The protein carryover was significantly below the limit defined for acceptable protein carryover when reusing chromatography materials or ultrafiltration/diafiltration (UF/DF) membranes.

A panel of different viruses (i.e., xenotropic murine leukemia virus [XMuLV], pseudorabies virus [PRV], reovirus type 3 [Reo-3], and MVM) were included in the virus clearance study. The virus filter membranes were reused several times, and the last reuse of the virus filter was challenged with the respective virus spike. The log reduction achieved with the last reuse cycle was compared with that of a new virus filter under the same conditions. Comparable viral removal capacity was demonstrated after reuse of the filters. Several worst-case scenarios were tested in the viral clearance study, such as high load capacity and low pressure with pressure interruption (see results in Table VI).
In all runs, a log reduction of at least $4 \log_{10} \text{TCID}_{50}$ could be demonstrated, which confirms that regeneration of the virus filter does not impair its capability to remove the panel of tested model viruses. Only one run of the tested conditions for MVM showed a virus breakthrough or residual infectivity in the filtrate. However, the demonstrated viral clearance of at least $4 \log_{10} \text{TCID}_{50}$ made the assessed probability of residual viruses in the end formulation highly unlikely and provides a high safety margin.

In conclusion, regeneration of the virus filter is feasible. Here we demonstrated that the filter performance after repeated reuse was maintained, the product quality was not impaired by the regeneration, the protein carryover could be defined within acceptable limits for other unit operations (e.g., chromatography steps, UF/DF membrane), and virus safety was not impaired by repeated reuse of the virus filter.

### Summary and Discussion

In this session, we reviewed and discussed a wide range of investigations of virus retentive filtration relevant to both upstream and downstream applications across different process formats, as well as considerations for supply chain and reuse of virus filters.

For upstream, special interests were raised toward using viral filtration for cell culture media, to enable virus risk mitigation early on in the upstream process and also to alleviate some of the challenges encountered in the conventional means (such as HTST treatment). The two pieces of work presented by Takeda and Eli Lilly gave promising evidence that the newer generation of virus filters can now support the media viral filtration applications more effectively and achieve higher loadings that are practical for upstream media batching requirement, while navigating through the process.

### Performance of reused virus filters.

In all runs, a log reduction of at least $4 \log_{10} \text{TCID}_{50}$ could be demonstrated, which confirms that regeneration of the virus filter does not impair its capability to remove the panel of tested model viruses. Only one run of the tested conditions for MVM showed a virus breakthrough or residual infectivity in the filtrate. However, the demonstrated viral clearance of at least $4 \log_{10} \text{TCID}_{50}$ made the assessed probability of residual viruses in the end formulation highly unlikely and provides a high safety margin.

In conclusion, regeneration of the virus filter is feasible. Here we demonstrated that the filter performance after repeated reuse was maintained, the product quality was not impaired by the regeneration, the protein carryover could be defined within acceptable limits for other unit operations (e.g., chromatography steps, UF/DF membrane), and virus safety was not impaired by repeated reuse of the virus filter.

### TABLE VI

<table>
<thead>
<tr>
<th>Log$_{10}$ Reduction Factor of MVM after Maximum of Reuses</th>
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<tbody>
<tr>
<td><strong>High Pressure + High Spike Ratio</strong></td>
</tr>
<tr>
<td>Run 1</td>
</tr>
<tr>
<td>Filtrate + Flush</td>
</tr>
<tr>
<td>Carryover</td>
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</table>

MVM, minute virus of mice.
interruptions successfully and accomplishing satisfactory reduction factors. Possible considerations were discussed around the interest and necessity to perform cell culture studies to demonstrate the cell culture performance of the media before/after filtration and the absence of impact on the culture performance. Another point for consideration is to understand more around implementation of media viral filtration in large-scale manufacturing operations (e.g., at 10 kL or even larger facilities).

For downstream, a lot of interesting investigations were rooted from the requirements of continuous and/or connected processing, where considerations of high loading, interruptions, and viral filter cycling are similarly necessary. The study by Novartis provided encouraging insights that continuous viral filtration could tolerate frequent interruptions of varying duration and numbers without any adverse impact on the virus removal. In the work of Just-Evotec Biologics, utilization of surrogate particles is shown to be effective to aid evaluation of virus filters and their likelihood of breakthrough, and helpful insights could be gained to enable a suitable target for the viral load challenge.

The evaluation at Novartis for viral filter reuse provided comprehensive considerations of the procedures and requirements. Although the work demonstrated the feasibility of regenerating the filter for use and maintaining the performance (no impact to product quality and virus removal capability), considerations are raised regarding the implementation in manufacturing and implications with regulatory, which would be valuable to further understand.

Conflict of Interest Declaration

The authors declare that they have no competing interests.

References


ABSTRACT: The Cell Banks, Advanced Technologies (ATMPs, NGS) session at the 2023 Viral Clearance Symposium (VCS) focused on the assurance of high virus safety profiles of advanced technology medicinal products (ATMPs) by implementation of advanced virus detection methods using rapid and sensitive technologies, such as next-generation sequencing (NGS). All presentations in this session made the need to replace in vivo testing for viruses by new technologies that have been demonstrated to be incomparably broad in their detection capabilities and can even detect unknown viruses. An evaluation of historical data collected by the Consortium on Adventitious Agent Contamination in Biomanufacturing (CAACB) from their members’ in vivo and in vitro adventitious virus test experience as well as on using NGS was presented. The data convincingly supported the necessity to replace in vivo testing with faster, broader, more sensitive, more accurate, and more specific virus detection methods. Additionally, a collaborative study—initiated by the CAACB—with the goal to revisit traditional adventitious agent testing by using targeted NGS to replace in vivo and in vitro tests for well-known and broadly used Chinese hamster ovary (CHO) cells was presented, including the planned risk-assessment approach using prior knowledge and historical data. Overall, this session demonstrated that the use of new virus detection methods, such as NGS, represents a great opportunity to provide sufficient viral safety margins, specifically, for ATMPs, where downstream virus clearance is not possible. This path forward is also supported by the final ICH Q5A(R2) guideline.

KEYWORDS: Adventitious agent testing, Virus safety, Next-generation sequencing (NGS), High-throughput sequencing (HTS), Advanced technology medicinal products (ATMPs), Cellular therapy products, In vivo adventitious virus test, In vitro adventitious virus test, Upstream virus barrier, Viral Clearance Symposium.
Recently, new virus detection technologies have been developed with increased virus detection capabilities, that is, next-generation sequencing (NGS), also referred to as deep sequencing or massively parallel sequencing. NGS is designed as a nonspecific technique with the potential to detect both known and unknown adventitious agents, including viruses (4–6). In January 2010, porcine circovirus type 1 (PCV1) deoxyribonucleic acid (DNA) was unexpectedly detected in the oral live-attenuated human rotavirus vaccine, Rotarix (GlaxoSmithKline [GSK] Vaccines) by an academic research team using NGS. This highly sensitive analysis was successful in detecting the contamination of that vaccine, whereas all in vitro and in vivo adventitious virus tests had passed the licensed specifications of the approved vaccine (7).

Introduction of the NGS method for routine adventitious virus detection as a replacement for in vivo testing for adventitious viruses was discussed in the first two presentations of this session.

Chinese hamster ovary (CHO) cells are well-characterized, as approximately 70% of approved biotech products are manufactured using CHO cells (8). The excellent virus safety history with CHO-derived products combined with the extensive accumulated industry knowledge from CHO cell virus testing and virus clearance studies over the past 40 years has led to the desire for a more consistent global recognition of the advancement of science and technology and systematic risk assessment and for modernization of CHO testing, for example, by using NGS. Thus, the Consortium on Adventitious Agent Contamination in Biomanufacturing (CAACB) has initiated a collaborative project with biopharmaceutical industry representatives with the goal to revise the CHO bioprocess virus testing strategy to reflect current knowledge of virus risks and new cell testing methods. This CAACB project was presented in the last talk of this session.

Next-Generation Sequencing (NGS) instead of In Vivo Testing in Cell-Based Therapies (Jens Modrof, Takeda)

The virus control strategy of allogeneic, banked cell-based therapy products is of particular importance as often a variety of animal-derived media components are used during their development and production, and traditional downstream viral clearance operations are not feasible. In order to enhance viral detection capabilities, the replacement of the traditionally employed in vivo assay that involves inoculation of suckling and adult mice as well as embryonated eggs by agnostic NGS represents a great opportunity, because NGS has the potential to detect any viral contaminant at a sensitivity comparable to that of targeted polymerase chain reaction (PCR) (9), even yet unknown viruses (10), whereas for in vivo testing, the detection capabilities have been shown to be limited (11, 12). Further advantages of NGS can be its far shorter assay turnaround time and several times lower sample volume requirement, especially when the value of the sample is significant.

A specific challenge to use NGS for virus detection in the routine quality control context may be the comparably high complexity of the assay and the requirement for extended use of computerized systems for the bioinformatics analysis. Therefore, NGS should be employed as a validated analytical method in line with ICH Q2 (13) and 9CFR part 11 (14) as well as EU Annex 11 (15) requirements. In addition to overall suitability criteria, such as negative controls and positive controls, specific acceptance criteria should be defined considering each step in NGS, including sample preparation, library quality, and sequencing quality. However, a head-to-head comparison between NGS and in vivo testing is not considered necessary or appropriate.

In line with the global objective to replace, reduce, and refine animal testing (which is designated as the “3 Rs strategy”), the replacement of in vivo testing for viruses by NGS may help to reduce the use of laboratory animals as well as greatly enhance virus detection capabilities. This possibility is already recommended in regulatory guidance for the viral safety of vaccines (European Pharmacopoeia 9.3 Chapter 5.2.3 [16] and 9.4 Chapter 2.6.16 [2]) and for general biotechnological products in the current revision 2 of the ICH Q5A (17).

Historical Evaluation of the In Vivo Adventitious Virus Test and Its Potential for Replacement with High-Throughput Sequencing (HTS) (Paul W Barone, Consortium on Adventitious Agent Contamination in Biomanufacturing [CAACB], MIT)

Despite their broad use and history in the biotechnology industry, there is interest to move away from the in vivo adventitious virus test and in some cases the in vitro virus test. A publication from Gombold et al. in
2014 (11) compared the performance of the in vivo adventitious virus test to that of the in vitro virus test for the detection of 11 different viruses. Aside from this study, there is little publicly available information on the historical utility of the in vivo virus test.

The CAACB is an academic–industry consortium housed at the Massachusetts Institute of Technology. To address the aforementioned gap in knowledge, the CAACB collected historical data from 20 biopharmaceutical industry members, through a 36-question survey, on their experience with the in vivo adventitious virus test, the in vitro virus test, and the use of high-throughput sequencing for viral safety. The CAACB members report continued use of the in vivo virus test with 94% of members using it for cell line characterization and 50% of members using it for virus seed testing and/or lot release testing. Since 2000, CAACB members have performed more than 10,000 in vivo adventitious virus tests (using more than 84,000 animals) and more than 67,000 in vitro adventitious virus tests (Table I).

The CAACB members were asked if, over the lifetime of their use of the in vivo adventitious virus test, they had experienced a positive in vivo adventitious assay test that was not also detected in another supporting assay. For the three positive in vivo adventitious virus assays reported to the CAACB, all were also detected using another concurrent assay (the in vitro adventitious virus assay in all cases). The CAACB also collected data on the reliability of both the in vivo and in vitro adventitious virus assays. Results on reported false positive in vivo tests are presented in Tables I and II.

In vivo false positive events (Tables I and II) took months to resolve, with the mean taking 1–3 months (10 events) and one event taking longer than 1 year to resolve. It is also important to note cases in which there was a known virus contamination, but the adventitious virus test reported a negative result. This occurred in more than three cases (0.03% of the total number of tests) for the in vivo adventitious virus test and in more than six cases (0.009%) for the in vitro virus test. Finally, the in vivo adventitious virus test may need to be repeated to obtain valid results due to technical issues, such as injection site trauma, injury to the animal, cannibalism, or nonviable eggs. In vivo adventitious virus tests needed to be repeated more than 21 times for these reasons. These repeats can take significant time, 18 days to months depending on the animal and test protocol. Of the repeats reported to the CAACB, 37% took less than 1 month with the remaining 63% taking 1–3 months to complete.

Based on the preceding data and the fact that the in vivo adventitious virus test has not been reported to detect a virus contamination that was not also detected in another concurrent assay, the CAACB has concluded that alternatives are needed for the in vivo adventitious virus test. Toward that end, CAACB member companies demonstrate a clear interest in NGS as an alternative method, with 19% of member companies already using NGS and 62% “exploring” NGS as an adventitious virus safety tool. Additionally, 69% of CAACB members report that they are exploring replacing the in vivo adventitious virus test with NGS, and some members have included NGS in their Investigational New Drug (IND) filings. Finally, the data and conclusions reported here are published in *Biologicals* (12).

### TABLE I

<table>
<thead>
<tr>
<th></th>
<th>In Vivo Adventitious Virus Test</th>
<th>In Vitro Virus Test</th>
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<tbody>
<tr>
<td>Number of tests</td>
<td>More than 10,000</td>
<td>More than 67,000</td>
</tr>
<tr>
<td>False positives (# / rate)</td>
<td>More than 21 (0.2%)</td>
<td>12 (0.02%)</td>
</tr>
</tbody>
</table>

### TABLE II

Number of False Positives Reported to the CAACB by In Vivo Animal Model. Data Adapted from Barone et al. (12)

<table>
<thead>
<tr>
<th>Animal Model</th>
<th>Adult Mice</th>
<th>Suckling Mice</th>
<th>Eggs</th>
<th>Guinea Pigs</th>
<th>HAP</th>
<th>MAP</th>
<th>RAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of false positives</td>
<td>2</td>
<td>4</td>
<td>5</td>
<td>0</td>
<td>2</td>
<td>7</td>
<td>0</td>
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</table>
Modernization of CHO Virus Testing by Leveraging the Industry Knowledge and Using NGS (Bin Yang, Genentech/Roche; Paul Barone, MIT)

Hundreds of biopharmaceutical products were approved over the last several decades, and biopharmaceuticals are dominated by recombinant proteins, which are predominantly manufactured using CHO cells. Despite the extensive applications of CHO cells in biopharmaceutical production, there have been no reported safety problems related to viral contamination of the products as stated in ICH Q5A(R1) Section V (1). In addition, biopharmaceutical product development and commercialization have grown rapidly with advanced adventitious virus detection technologies, better virus safety understanding, and control. The current CHO Bioprocess virus testing approach does not reflect the wealth of knowledge gained over previous decades of process and product histories as well as the current state of analytical testing capability. ICH Q5A has been revised (17) to reflect such advances in biotechnology product development and manufacturing, and the time is right to modernize the CHO Bioprocess virus testing approach based on a comprehensive risk assessment and the application of modern testing capabilities as a means of updating testing efficiencies while ensuring product safety.

Conversely, new and emerging biotechnology products are using cell lines for which the biopharmaceutical manufacturing industry has much less experience with regards to viral safety. For these new products, a risk-assessment methodology to guide companies in determining the risks, including which viruses are the biggest risk to products manufactured with those cell lines, would be valuable. Therefore, performing a comprehensive and systematic assessment of viral risk to CHO cells, leveraging the industry’s decades of experience, and using that exercise to establish a framework to guide companies in assessing the viral risks to new and emerging cell lines would be a highly valuable exercise. The CAACB represents a significant cross-section of the biopharmaceutical industry, with a wealth of viral safety subject matter experts, and is an excellent resource to mobilize in pursuing this goal of modernizing the CHO Bioprocess virus safety risk assessment and establishing a framework to guide companies in assessing the viral risks associated with new and emerging cell lines. The CAACB has begun a collaborative study with the goal of ultimately improving virus safety control through i) reviewing the knowledge gained over the last 30+ years, ii) reassessing the virus risks posed from CHO Bioprocesses (including identifying viruses with potential high risks), iii) proposing risk- and knowledge-based CHO Bioprocess virus testing approaches that incorporate modern virus testing technologies such as NGS, and iv) using the CHO virus risk assessment as a case study to develop a general risk-assessment framework for assessing which viruses pose the greatest risk to any cell culture manufacturing process.

Conclusion

The need to replace in vivo testing for viruses by new technologies such as NGS that have been demonstrated to be highly sensitive and to also detect unknown viruses has become obvious from all the presentations of this session. Specifically, for cell therapy products, where downstream virus clearance is not possible, i) upstream barriers such as virus filtration of cell culture media can be implemented to protect such processes from virus contamination (18) and ii) the use of NGS represents a great opportunity to provide sufficient viral safety margins. Due to its advantages regarding broad virus detection, NGS is increasingly supported by regulatory guidance to be used as an alternative to in vivo virus testing—which is also in line with animal welfare objectives.

The Transcriptomic NGS method for detection of viral contaminants needs to be thoroughly controlled by multiple parameters on different stages of the assay to ensure robust and reliable assay performance in line with regulatory requirements per ICH Q5(R2)(17).

Based on i) the study results from a historical evaluation of the in vivo adventitious virus test and its potential for replacement with HTS (also referred to as NGS) performed by the CAACB (12) and ii) the fact that the in vivo adventitious virus test has not been reported to detect a virus contamination that was not also detected in another concurrent assay, the CAACB has concluded that alternatives are needed for the in vivo adventitious virus test, and the majority of CAABC members are exploring replacement of the in vivo adventitious virus test with NGS.

Also, in the discussions after this session, it was strongly agreed on a path forward to update the overall virus safety pillars for biopharmaceutical products through replacing the in vivo adventitious virus testing approaches with modern methodologies that maintain...
or are highly likely to substantially improve the final safety margins of the product.

Based on 40 years of safe use of CHO, the CAACB has initiated a collaborative study with the goal to revisit traditional adventitious agent testing by using targeted NGS to replace in vivo and in vitro tests, based on a risk-assessment approach using prior knowledge and historical data. This approach is supported by the current revision (R2) of the ICH Q5A guideline (17), stating that: “In vivo testing is not necessary for extensively used well-characterized cell lines such as CHO, . . ., based on prior knowledge.”

Conflict of Interest Declaration

The authors are employees of Takeda Manufacturing Austria AG and have stock interests.

References


