Biotechnology Advances 87 (2026) 108784

Contents lists available at ScienceDirect

Biotechnology Advances

journal homepage: www.elsevier.com/locate/biotechadv

Review article

Viral clearance in biopharmaceutical manufacturing: Current strategies, challenges, and future directions

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ARTICLE INFO

Keywords:
Downstream processing
Viral clearance
mAbs
LRV
Chromatography
Filtration

ABSTRACT

Viral safety remains a fundamental requirement in the manufacturing of monoclonal antibodies (mAbs), particularly due to the widespread use of mammalian cell lines susceptible to both endogenous and adventitious viral contamination. This review provides a comprehensive overview of current viral clearance strategies integrated into downstream processing (DSP), highlighting the mechanisms, performance, and practical implementation of key unit operations. Chromatographic methods, including Protein A affinity, ion exchange (CEX and AEX), hydrophobic interaction (HIC), and mixed-mode chromatography (MMC), contribute to virus removal to varying extents, depending on virus type, resin chemistry, and process conditions. Anion exchange membranes have demonstrated high log reduction values (LRVs), especially for small non-enveloped viruses, while mixed-mode resins enhance removal through dual-mode interactions. Dedicated viral inactivation steps, such as low-pH incubation and detergent treatment, remain effective against enveloped viruses, with the use of stabilizing agents like arginine and extremolytes increasingly adopted to preserve product quality. Virus filtration continues to represent the most robust barrier to small viruses, though its performance depends on parameters such as filter material, fouling tendency, and viral load. Emerging solutions, such as activated carbon filtration and membrane chromatography, offer scalable, orthogonal alternatives compatible with disposable and continuous processing formats. Notably, viral clearance strategies have been successfully incorporated into continuous downstream workflows, including multicolumn capture, inline inactivation, and extended-duration filtration. Collectively, these advances support the transition toward more flexible, efficient, and sustainable viral safety frameworks, paving the way for next-generation biomanufacturing platforms.

1. Introduction

Safeguarding microbiological integrity is a cornerstone of biopharmaceutical manufacturing, and nowhere is this more critical than in the consistent prevention of viral contamination, which poses significant risks to both patient safety and product integrity throughout the entire production lifecycle.

Viral safety begins upstream. During cell culture and solution preparation, risk mitigation begins with the use of virus-retentive filters for sterile filtration of media, buffers, and other process inputs. In parallel, raw materials, especially those of biological origin, are subject to stringent sourcing and characterization to prevent the introduction of adventitious agents. These precautions are further reinforced by strict adherence to Good Manufacturing Practices (GMP), including closed-

Abbreviations: AEX, Anion Exchange Chromatography; BEV, Bovine Enterovirus; BVDV, Bovine Viral Diarrhea Virus; CEX, Cation Exchange Chromatography; CPE, Cytopathic Effect; CPV, Canine Parvovirus; DDM, n-Dodecyl-β-D-maltopyranoside; DFF, Direct Flow Filtration; DM, n-Decyl-β-D-maltopyranoside; DSP, Downstream Processing; CDSP, Continuous Downstream processing; EC, Extremolyte Compound (e.g., EC4, EC5); EMCV, Encephalomyocarditis Virus; HCCF, Haversted Cell Culture Fluid; HCP, Host Cell Protein; HIC, Hydrophobic Interaction Chromatography; LDAO, *N*,*N*-dimethyldodecylamine-N-oxide; LRV, Log_{10} Reduction Value; mAb(s), Monoclonal Antibody(ies); MMC, Mixed-Mode Chromatography; MuLV, Murine Leukemia Virus; MVM, Minute Virus of Mice; NGS, Next Generation Sequencing; OG, n-Octyl-β-D-glucopyranoside; PPV, Porcine Parvovirus; PRV, Pseudorabies Virus; qPCR, Quantitative Polymerase Chain Reaction; SV-40, Simian Vacuolating Virus-40; $TCID_{50}$, Tissue Culture Infectious Dose 50%; TFF, Tangential Flow Filtration; TNBP, Tri-n-butyl phosphate; VSV, Vesicular Stomatitis Virus; X-MuLV, Xenotropic Murine Leukemia Virus.

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https://doi.org/10.1016/j.biotechadv.2025.108784
Received 29 July 2025; Received in revised form 18 December 2025; Accepted 19 December 2025
Available online 23 December 2025

system operations, environmental controls, and validated procedures that ensure traceability and minimize contamination risk. Collectively, these upstream safeguards establish a critical foundation upon which downstream viral clearance strategies are built (Kiss, 2011; Liu et al., 2000).

Downstream processing itself is widely recognized as one of the most demanding phases of biologics production. It is often the costliest segment of the manufacturing pipeline and must deliver exceptional levels of product purity, especially given the parenteral administration routes of most biologics. As such, DSP is not only a technical challenge but also a critical point of regulatory scrutiny and a potential bottleneck in production workflows (Matte, 2022).

In recent years, DSP protocols have been highly refined across various classes of biopharmaceuticals, especially those most relevant to the pharmaceutical market. These advances not only ensure exceptional product purity but also meet stringent safety criteria, incorporating robust viral decontamination procedures. A prime example is monoclonal antibodies (mAbs), which stand among the top-performing therapeutics worldwide. For this class, the entire workflow, from isolation and purification to final formulation, has been extensively optimized, giving rise to well-defined regulatory frameworks that place strong emphasis on viral clearance. Given that mAbs are produced using mammalian cell lines, they are particularly vulnerable to viral contamination, including the risk of propagating endogenous retrovirus-like particles that may compromise product quality (Strauss et al., 2009). As such, the implementation of validated viral clearance strategies is critical, given that viral contaminants can have serious clinical consequences. In this context, the ICH guideline effective since 2024 outlines the principles for assessing viral contamination risks and controlling potential viral sources. It further provides criteria for qualifying the viral clearance capacity of manufacturing processes, ensuring the safe production of biotechnology products derived from animal or human cell lines (ICH-Q5A(R2), 2024).

Therefore, a robust assessment of viral safety becomes an essential step in the validation of any production process. Each step claimed to reduce viral load must be rigorously evaluated, typically through dedicated viral clearance studies. These studies are not just a regulatory requirement but a key component of process validation. They provide indirect yet crucial evidence of the system's ability to inactivate or eliminate viruses, ensuring the safety and quality of biopharmaceutical products. Viral clearance is assessed under conditions that closely replicate those of the actual manufacturing process, through the controlled spiking of model viruses during DSP. The ultimate goal is to challenge the process under realistic conditions and demonstrate its robustness in removing or inactivating a limited number of representative model viruses selected through a risk-based strategy; illustrative examples of relevant viral agents are provided in Table 1.

For viral clearance studies, model viruses are carefully selected to represent the major classes of potential contaminants, based on differences in genome type, structure, envelope status, and resistance to inactivation. The effectiveness of each purification step is typically quantified using the $\log_{10}$ reduction value (LRV) also referred to as $\log_{10}$ reduction factor (LRF) or, in some contexts, $\log_{10}$ clearance units, which expresses the logarithmic decrease in viral load achieved during the process. These metrics provide a standardized measure of process robustness in eliminating viral particles across different stages of downstream processing.

It is now widely recognized that viral clearance is achieved throughout the DSP phase, through a series of steps that collectively contribute to the reduction of viral load in biopharmaceutical manufacturing. Overall, within the DSP workflow, it is possible to distinguish between unit operations primarily aimed at product purification, such as in the case of monoclonal antibodies, which also contribute to the partial removal of viral particles, and process steps specifically designed for effective viral inactivation or removal, as depicted in Fig. 1.

More specifically, the first group comprises purification steps primarily intended to isolate the target molecule, typically using column-based chromatographic techniques applied across the capture, intermediate, and polishing stages of DSP. These methods, including Anion Exchange Chromatography (AEX), Cation Exchange Chromatography (CEX), Mixed-Mode Chromatography (MMC), and Hydrophobic Interaction Chromatography (HIC), are essential for achieving the desired product purity and, at the same time, contribute to a measurable reduction in viral load. In contrast, the second group includes dedicated viral clearance steps specifically developed to ensure the inactivation or removal of viral contaminants. These steps are critical for meeting international regulatory standards and for safeguarding the viral safety of the final product. By strategically integrating both classes of operations, those aimed at purification and those designed for active viral

Table 1
Viruses used for viral clearance studies. For each virus, the table reports its family and genus, taxonomic classifications reflecting genetic and structural similarities, as well as its natural host, genome type (DNA or RNA, single-stranded (ss) or double-stranded (ds), and structured in a linear or circular form), and the presence or absence of a lipid envelope. Size and shape describe the virus's morphology, while resistance refers to its ability to withstand physical, biological, or chemical inactivation methods (ICH-Q5A(R2), 2024).

Virus	Family	Genus	Natural Host	Genome	Envelope	Size	Shape	Resistance
Adenovirus (Adeno) Type 2 or Type 5	Adenoviridae	Adenovirus	Human	DNA	No	70–90 nm	Icosahedral	Medium
Simian vacuolating (SV 40)	Polyomaviridae	Betapolyomavirus	Monkey	DNA	No	40–50 nm	Icosahedral	Very High
Bovine viral Diarrhea (BVDV)	Flaviviridae	Pestivirus	Bovine	RNA	Yes	50–70 nm	Pleo/ Sphere	Low
Reovirus type 3	Reoviridae	Orthoreovirus	Various	RNA	No	60–80 nm	Spherical	Medium
Encephalomyocarditis Virus (EMCV)	Picornaviridae	Cardiovirus	Mouse	RNA	No	25–30 nm	Icosahedral	Medium
Bovine Enterovirus (BEV)	Picornaviridae	Enterovirus	Bovine	RNA	No	25–30 nm	Icosahedral	Medium
Parainfluenza	Paramyxoviridae	Paramyxovirus	Various	RNA	Yes	100–200 nm	Pleo/ Sphere	Low
Pseudorabies (PRV)	Herpesviridae	Varicellovirus	Swine	DNA	Yes	120–200 nm	Spherical	Medium
Porcine Parvovirus (PPV) Canine Parvovirus (CPV) Minute Virus of Mice (MVM)	Parvoviridae	Protoparvovirus	Porcine Canine Mouse	DNA	No	18–24 nm	Icosahedral	Very High
Murine Lukemia (MuLV)	Retroviridae	Gammaretrovirus	Mouse	RNA	Yes	80–110 nm	Spherical	Low
Vesicular stomatitis virus (VSV)	Rhabdoviridae	Vesiculovirus	Equine, Bovine	RNA	Yes	70 × 150	Bullet	Low
Sindbis virus	Togaviridae	Alphavirus	Human	RNA	Yes	60–70 nm	Spherical	Low
Autographa california multiple Nucleopolyhedrovirus	Baculoviridae	Alphabaculovirus	Insect	DNA	Yes	250–300 nm	Polyhedral	Medium
Vesivirus 2117	Caliciviridae	Vesivirus	Unknown	RNA	No	27–40 nm	Icosahedral	Medium

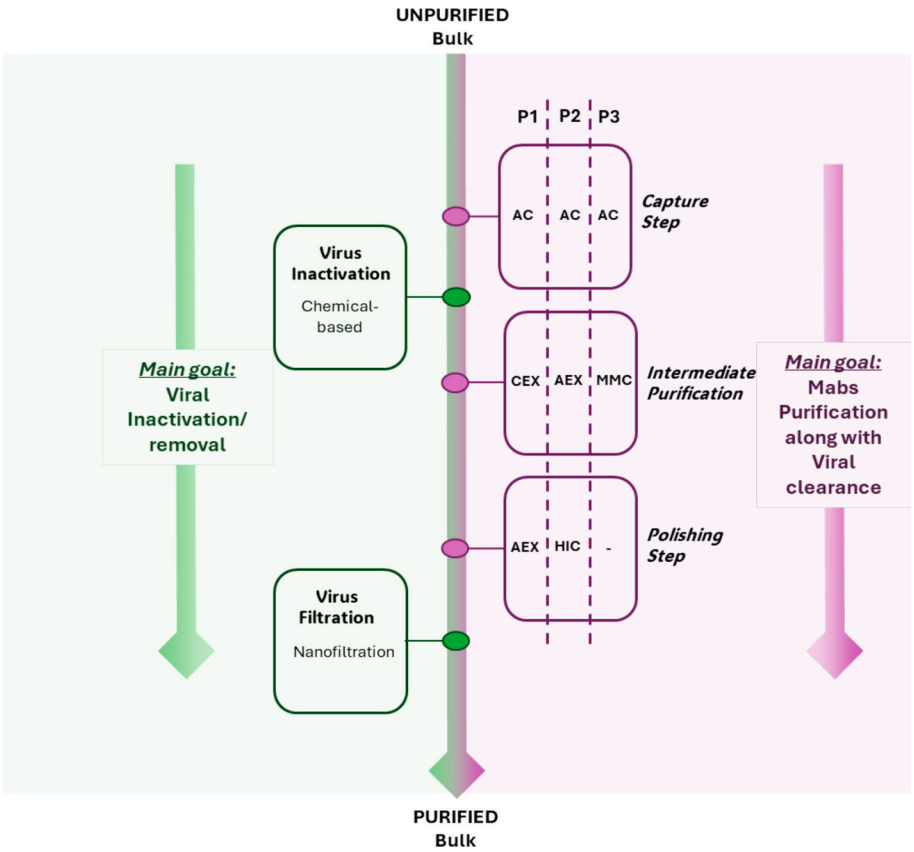

Fig. 1. Schematic overview of the main steps contributing to viral reduction during DSP in mAb purification. The left side of the figure highlights process steps specifically dedicated to viral inactivation and removal, while the right side illustrates viral clearance strategies involving different protocol combinations (e.g., Protocol-1 (P1), Protocol-2 (P2), and Protocol-3 (P3)).

inactivation, modern DSP workflows are able to deliver high manufacturing efficiency while ensuring compliance with the stringent safety requirements demanded in biopharmaceutical production.

The final outcome of the purification procedure must be a product that is safe for use and fully compliant with regulatory guideline (ICH-Q5A(R2), 2024). To this end, the removal of process- and product-related impurities is essential, including host cell proteins (HCPs), residual DNA, adventitious and endogenous viruses, endotoxins, aggregates, and other unwanted species, while ensuring an acceptable product yield. In addition to these intrinsic impurities, it is also necessary to eliminate contaminants introduced during purification, such as leached Protein A, extractables from filters and chromatography resins, buffer components, and virus-reduction agents like detergents (Liu et al., 2010).

This review outlines the current landscape of viral clearance strategies employed in mAb manufacturing, with a focus on evaluating their effectiveness under process-relevant, industry-mimicking conditions. We compare a range of chromatographic resins, including Protein A, ion exchange, and hydrophobic interaction resins, in terms of binding capacity, selectivity, and structural features, to assess their suitability for virus removal. We also examine the performance of key viral clearance techniques, such as chromatography, filtration, and chemical inactivation, using LRVs as benchmarks, while ensuring that experimental conditions align with those of large-scale production. Different manufacturing formats, including fed-batch and continuous bioprocessing, are considered to understand their impact on viral clearance efficiency. Special attention is given to the role of stabilizers, such as arginine and polysorbates, in mitigating protein aggregation during low-pH viral inactivation steps. Finally, we explore high-potential technologies, including membrane chromatography and column-free approaches, such as activated carbon filtration and viral nanofiltration, to evaluate their potential integration into mAb purification workflows.

This review aims to provide a critical and up-to-date perspective on the optimization of viral clearance strategies, offering insights that can support the development of safer, more robust, and efficient biomanufacturing processes.

2. Mechanisms and stages of viral clearance in DSP

2.1. Steps contributing to viral clearance (chromatographic techniques)

Preparative chromatography stands as a pillar of DSP, offering remarkable versatility in combining high selectivity, loading capacity, and operational robustness. Its adaptability, through tailored selection of stationary phases, separation modes, and process conditions, enables the design of highly efficient purification strategies, particularly for mAbs. All the techniques described in the following sections can be applied within the DSP phase, following various operational combinations, as illustrated in Fig. 1. The optimal purification strategy depends on several factors, including the properties of the stationary phases, capital and operational costs, and, most importantly, the complexity of the medium. Striking the right balance between process efficiency, product safety, and economic sustainability is therefore essential.

In mAb purification, the DSP typically comprises three sequential chromatographic stages: the capture step, intermediate purification, and polishing. During the capture step, the target molecule is selectively isolated from the crude feedstock, and most host cell-derived impurities, such as proteases and oxidative species, are removed to preserve the protein's structural and functional integrity. The subsequent stages are designed to progressively eliminate finer contaminants, such as HCPs, residual DNA, aggregates, and viral particles, ultimately delivering a highly pure product that meets regulatory standards.

Beyond the sequence of operations, a key aspect lies in the mode of chromatographic application, particularly in the intermediate and polishing steps. Unlike the capture phase, which is most commonly performed in bind/elute mode, the latter stages can be implemented using different configurations depending on the target molecule and impurity profile. In bind-and-elute mode, the molecule of interest binds to the stationary phase, while impurities are washed away; elution is then achieved by modifying process conditions (e.g., pH or ionic strength) to disrupt the ligand-analyte interaction. Alternatively, flowthrough mode is employed when the stationary phase is designed to retain impurities, allowing the target molecule to pass unbound through the column, which is particularly advantageous when the molecule exhibits low affinity for the resin. A third strategy, referred to as overload mode, consists of loading the sample beyond the resin's capacity. In this setup, impurities with stronger affinity are retained, whereas the target molecule, because of its lower affinity or higher abundance, is recovered in the flowthrough. Importantly, regardless of the selected chromatographic mode, the elution phase can be further tailored using step or gradient elution strategies, enabling the differential release of product and, to some extent, co-bound impurities by controlled modulation of process parameters such as conductivity or ionic strength.

The rational integration of these operational modes, adapted to the specific features of the molecule and process, represents a critical element in building efficient, scalable, and regulatory-compliant purification workflows in the biopharmaceutical landscape.

2.1.1. Protein A affinity chromatography for therapeutic mAb purification

Affinity chromatography is a highly selective technique for the separation of biomolecules, based on the specific interaction between the target molecule and a ligand immobilized on the stationary phase. Owing to its high sensitivity, efficiency, and molecular specificity, it is widely used for isolating proteins, enzymes, antibodies, and other biologically relevant molecules (Hage and Matsuda, 2015). Among affinity ligands, bacterial Protein A and Protein G are commonly employed for the capture of immunoglobulins. While both exhibit high affinity for the Fc region of IgGs, they differ in their species and subclass specificity, with Protein G being more suitable for murine antibodies and certain human IgG subclasses with lower affinity for Protein A. In this review, the focus will be placed on Protein A-based chromatography, due to its widespread use as the gold standard in the clinical and commercial manufacturing of therapeutic mAbs. Protein A, a surface protein derived from the Gram-positive bacterium *Staphylococcus aureus*, displays strong and selective affinity for the Fc region of human IgG1, IgG2, and IgG4 subclasses (Ayyar et al., 2012). Its high selectivity, strong binding capacity, and compatibility with elevated flow rates make it particularly effective for the selective capture of human mAbs from complex feedstocks. In addition to its primary role in isolating the target molecule, Protein A chromatography also contributes significantly to the removal of process-related impurities, including HCPs, residual DNA, culture medium components, and both endogenous and adventitious viral particles (Liu et al., 2010).

From a virus-clearance standpoint, Protein A chromatography typically achieves LRVs between 1 and 4 for various model viruses, performances that are often less consistent than those observed with other unit operations, such as flow-through anion exchange or virus filtration (Zhang et al., 2014). During loading and post-load washing, most non-antibody components and viruses predominantly flow through the column, yet a small fraction may bind either via direct interaction with the monoclonal antibody or nonspecific contact with matrix constituents. Subsequent pH shifts during elution can dislodge some of these bound viral particles, leading to low but variable virus levels in the eluate, hence the fluctuating LRVs (Zhang et al., 2014). Critically, these variations in viral clearance are often attributable to differences in the mAb product itself and the characteristics of the harvested cell culture fluid (HCCF), rather than process parameters like temperature, pH, or wash buffer concentration. For example, Bach & Connell-Crowley (Bach and Connell-Crowley, 2015) demonstrated that using spent HCCF significantly altered clearance outcomes, suggesting that virus–impurity (HCP) and virus–mAb interactions, rather than chromatography conditions, play a dominant role in LRV variability. In the same study, the authors reported that the addition of wash-buffer additives such as arginine or urea disrupted virus–impurity/mAb interactions, thereby enhancing virus removal during Protein A chromatography. The behavior of XMuLV was shown to resemble that of HCPs, as both can associate with mAbs and coelute with the product, ultimately reducing removal efficiency during Protein A capture. Building on this work, Pan et al. (Pan et al., 2019) expanded the investigation by evaluating additional virus types, including retrovirus-like particles and MVM, using a different Protein A resin (Eshmuno A). Through a high-throughput screening approach, they confirmed that the mAb molecule type plays the dominant role in determining LRV, with HCP content and resin type exerting lesser but still significant effects. Their results also supported the contribution of hydrophobic and ionic interactions to virus–mAb association and identified a new excipient with promising performance.

These findings are consistent with observations summarized by Li (Li, 2022), who reviewed previous studies on wash buffer composition and virus–mAb interactions, highlighting the influence of additive type and concentration on viral clearance efficiency.

In summary, while Protein A chromatography is an essential step for capturing the target antibody, its virus removal efficiency largely depends on mAb–virus interactions and feedstock composition. Nonetheless, this step can be optimized through strategic wash buffer design, using additives that reduce undesired binding and thereby enhance viral clearance.

2.1.2. Ion exchange chromatography (IEX)

Ion exchange chromatography (IEX) is a fundamental component of mAb purification protocols, offering high selectivity and scalability while maintaining cost-effectiveness, making it ideal for use at various stages of downstream processing. It is typically employed following Protein A affinity chromatography, either during the intermediate purification phase or as a polishing step, depending on the impurity profile and process requirements. The technique operates on the principle of electrostatic interactions between charged biomolecules and oppositely charged groups immobilized on the chromatographic resin. Based on this mechanism, IEX is generally classified into two complementary approaches. CEX employs a negatively charged resin to capture positively charged species (cations), making it suitable for the separation of basic protein isoforms or degradation products. In contrast, AEX utilizes a positively charged stationary phase to bind negatively charged species (anions), such as acidic protein variants, DNA fragments, and certain host cell proteins.

In standard platform processes, CEX is often used in bind-and-elute mode as the first chromatographic step post-Protein A, followed by AEX in flow-through mode. This configuration is primarily guided by the physicochemical properties of the mAb, including its isoelectric point (pI) and surface charge distribution. Notably, in some specific scenarios, such as antibodies with basic pI values, CEX has been successfully used as an initial capture step, effectively replacing Protein A chromatography (Liu et al., 2010). This alternative configuration can enhance platform flexibility and be especially advantageous when dealing with unconventional antibody formats or unique biophysical profiles. The resolution and robustness of ion exchange chromatography make it particularly valuable for fine-tuning product quality and ensuring process consistency across manufacturing batches. However, when viral safety is also a design objective, resin choice and operational mode must be tailored not only to product properties but also to the characteristics of the target virus.

Although IEX contributes significantly to the viral clearance profile of therapeutic proteins, its effectiveness relies on electrostatic affinity between the virus and the resin.

Interestingly, comparative studies between XMuLV and parvovirus

(e.g. MVM), two viruses differing in size and structure but with comparable pI, have revealed distinct binding behaviors. When both are negatively charged, XMuLV exhibits stronger binding to AEX resins than parvovirus. Remarkably, even under conditions where both virus and resin carry positive charge, XMuLV remains bound, while parvovirus dissociates. Similar trends have been observed with CEX resins, where XMuLV consistently shows stronger retention.

These findings suggest that localized surface charge distribution, rather than global pI alone, may drive robust virus–resin interactions. In particular, XMuLV may possess electrostatic features absent in small, non-enveloped viruses like parvovirus. Therefore, designing effective IEX-based virus clearance steps requires a nuanced understanding of both virus structure and surface electrostatics, extending beyond traditional charge-based assumptions (Cai et al., 2019).

2.1.2.1. Cation exchange chromatography (CEX). CEX is widely employed in the biopharmaceutical industry as an intermediate purification step, typically in bind-and-elute mode, for the production of recombinant proteins and mAbs, owing to its robustness, scalability, and high dynamic binding capacity (Shukla et al., 2007). In this configuration, the antibody binds to the negatively charged resin and is subsequently eluted either stepwise or through a linear gradient of increasing salt concentration. By carefully tuning the operating pH and elution conditions, CEX enables the effective separation of process-related impurities such as leached Protein A, product-related aggregates, HCPs, residual DNA, and, to a certain extent, viral particles, depending on their physicochemical properties and interaction with the resin matrix.

For instance, in a study evaluating viral clearance using Xenotropic Murine Leukemia Virus (X-MuLV), more than 4 LRV were consistently achieved at pH 5.0 with a sodium concentration of 350–375 mM. However, viral clearance efficiency substantially declined at pH 5.5 and 6.0, and was completely abolished at pH 6.5 (Connell-Crowley et al., 2012). In contrast, clearance of Minute Virus of Mice (MVM), a small non-enveloped parvovirus, remained consistently low (<2 LRV) across all tested pH conditions.

CEX has also been investigated as a potential capture step, offering a cost-effective alternative to Protein A resins (Ahamed et al., 2008). In a comparative study assessing parvovirus removal by Protein A and CEX, clearance levels ranged between 1 and 2 LRVs. Using a Design of Experiment (DoE) approach, a statistical methodology used to systematically evaluate the effect of multiple process parameters on product quality and performance, Miesegaes et al. (Miesegaes et al., 2012) observed that conditions associated with significant loss of product purity also led to poor clearance of porcine parvovirus (PPV), with LRVs falling below 1. Interestingly, their data revealed a correlation between PPV LRVs and impurity removal. Conversely, Connell-Crowley et al. (Connell-Crowley et al., 2012) reported no such correlation in the case of MuLV, suggesting that the relationship between virus clearance and impurity profile may be virus-specific.

Several lines of evidence suggest that electrostatic interactions between viral particles and the resin matrix play a critical role in virus removal by CEX. Interestingly, X-MuLV virus was shown to bind more strongly to CEX resins, such as Fractogel® SO_3^- (Merck Millipore), than the majority of mAbs tested at pH 5.0, a result that was initially unexpected given the general assumption that mAbs exhibit higher affinity under these conditions. Conversely, MVM, despite having a similar isoelectric point to X-MuLV (pI 6.2 vs. 5.8), exhibited minimal interaction with the resin at the same pH. This discrepancy led the authors to hypothesize that specific structural features, such as localized charge clusters on the viral envelope, may enhance the binding of X-MuLV to the negatively charged resin, features that appear to be absent in MVM. These findings underline the importance of operating at acidic pH (around 5.0) for achieving efficient X-MuLV clearance via CEX. Although the precise molecular mechanism remains to be elucidated, the data support the idea that virus–resin interactions are highly dependent on virus-specific surface properties, beyond just global charge or pI (Connell-Crowley et al., 2012).

CEX can also be operated in overload mode, as an alternative to the conventional bind-and-elute configuration. In a recent study, this approach was evaluated for the removal of X-MuLV virus by intentionally overloading the column with a ten-fold excess of monoclonal antibody relative to the resin's binding capacity. The experiments were conducted using POROS® XS resin (Thermo Fisher Scientific) under overloading conditions corresponding to 2000 g mAb/L resin, at pH 5.0 and a flow rate of 18 column volumes per hour (CV/h). Under these settings, X-MuLV clearance reached 6.09 LRV with mAb1 and greater than 4.5 LRV with mAb2 (Masuda et al., 2019). The same overload strategy was also tested for other model viruses. LRV of 2.62 for MVM, ≥ 5.72 for Pseudorabies Virus (PRV), and 8.03 for Reovirus Type 3 (Reo-3) were observed, demonstrating that this mode can be effective across multiple viral species. This alternative configuration is particularly appealing from a cost-efficiency perspective, as it enables significant resin volume reduction compared to traditional B/E mode, which typically requires large quantities of expensive CEX resin to achieve comparable levels of viral clearance (Masuda et al., 2019).

2.1.2.2. Anion exchange chromatography (AEX). AEX chromatography is a highly effective strategy for the removal of both process- and product-related impurities during mAb purification. By employing a positively charged resin, AEX enables the selective capture of negatively charged contaminants such as HCPs, residual DNA, endotoxins, and leached Protein A. It is also effective in removing product-associated impurities, including aggregates or dimers, as well as endogenous retroviruses and adventitious viruses such as parvovirus and pseudorabies virus (Norling et al., 2005).

AEX is typically implemented in two operational modes, depending on the purification stage and the nature of the target molecule. Most commonly, it is used in flow-through mode during the polishing phase. In this configuration, the process is conducted at a pH above the isoelectric point (pI) of the mAb, rendering the antibody negatively charged and enabling it to flow through the column without binding. Meanwhile, negatively charged impurities, including many viruses, bind strongly to the positively charged matrix. This selective retention enables efficient removal of charged contaminants while maintaining product recovery (Roush, 2015).

A notable advancement in the mechanistic understanding of virus clearance via AEX is presented in the recent study by Kitamura et al., which introduces the first predictive mechanistic model to describe the elution behavior of minute virus of mice (MVM) during AEX in flow-through mode. This model provides a comprehensive phenomenological description of the chromatographic process, incorporating column fluid dynamics, mass transport, and binding interactions via the Steric Mass Action (SMA) isotherm. Notably, it can differentiate between intact virions and defective capsids, each exhibiting distinct physicochemical properties and binding behaviors. This highlights viral heterogeneity as a critical parameter influencing clearance efficiency. To assess model robustness in realistic scenarios, the authors tested it with different spiked mAbs, confirming its predictive accuracy even in complex product matrices. Supplementary in silico structural analyses using *AlphaFold* and electrostatic surface mapping revealed that hydrophilic cationic patches on mAbs can modulate virus–mAb interactions and co-elution profiles. Practically, the model facilitates rapid in silico screening of process parameters, such as buffer type, pH, and ionic strength, enabling accurate LRV predictions while reducing experimental workload. This work marks a paradigm shift in virus clearance development, moving from empirical trial-and-error toward rational, simulation-based process design, offering new avenues for robust and cost-efficient bioprocess optimization(Kitamura et al., 2025).

These mechanistic insights are reinforced by Cai et al.(Cai et al., 2024), who demonstrated that AEX flow-through consistently achieves

log reduction values (LRVs) of ≥5–6 for enveloped viruses such as XMuLV, provided critical parameters are maintained. Effective clearance was observed with protein loads ≤155 mg/mL resin and HCP levels ≤500 ng/mg product (≤80 μg/mL resin), within a pH range of 6.3–8.2 and conductivity below 14 mS/cm. However, elevated levels of acidic HCPs impaired virus removal, likely due to competition for binding sites on the resin surface. In contrast, the clearance of small non-enveloped viruses like Minute Virus of Mice (MVM) via AEX proved highly variable, with LRVs ranging from <1 to ≥5 (Cai et al., 2024). This variability, in line with previous studies, underscores that while AEX may contribute to MVM reduction under favorable conditions, it should not be considered a primary barrier, reinforcing the need for orthogonal steps such as nanofiltration.

In addition to flow-through mode, AEX can be deployed in bind-and-elute configurations during intermediate purification. Under tailored pH and conductivity conditions, the mAb binds to the resin and is subsequently eluted, facilitating removal of product variants and impurities, especially in challenging feedstocks.

In a study evaluating Q Sepharose® Fast Flow resin (Cytiva), Strauss et al. (Strauss et al., 2009) identified feedstock conductivity and salt concentration as key parameters positively correlated with virus removal efficiency. These factors directly modulate the electrostatic interactions that govern virus binding to the anion exchange matrix. When the conductivity was maintained within the range of 3 to 14 mS/cm, the resin achieved LRVs ranging from 4.0 to 5.8 for model viruses such as X-MuLV, SV-40, and MVM (Strauss et al., 2009). Building on these findings, subsequent investigations using POROS® HQ resin (Thermo Fisher Scientific) focused on validating the identified parameters and optimizing AEX performance. Specifically, the studies assessed MVM clearance across three different mAb preparations. Notably, the study revealed that co-elution of virus and antibody, resulting from specific virus–mAb interactions, was a key determinant of viral clearance efficiency. This mechanism appeared to override traditional predictors such as pI and global charge, suggesting that physicochemical properties like localized charge density and hydrophobicity distribution significantly influence virus retention and removal (Hung et al., 2020).

These mechanistic insights are further corroborated by recent findings from Leisi et al., who demonstrated that pI alone is insufficient to predict virus–resin interactions. Instead, the surface charge distribution of the virus offers a more reliable parameter for optimizing AEX-based viral clearance (Leisi et al., 2021).

2.1.3. Hydrophobic interaction chromatography (HIC)

HIC is a separation technique that exploits differences in surface hydrophobicity among biomolecules, including recombinant proteins, mAbs, and virus particles. The interaction between the analyte and the stationary phase is promoted under high salt concentrations and is reversible, allowing gentle recovery of the target molecule. In the context of mAb purification, HIC is primarily employed during the final polishing stage (Fig. 1). At this stage, it plays a critical role in removing closely related impurities, such as aggregates, charge variants, or hydrophobic degradation products, that are not fully resolved by previous steps. The separation mechanism relies on transient, non-covalent interactions between exposed hydrophobic regions of the biomolecule and hydrophobic ligands immobilized on the resin surface. The extent of interaction in HIC depends on the surface hydrophobicity of the molecule, which can vary among proteins and viruses.

Since most biomolecules exhibit moderate hydrophobicity, HIC is particularly well-suited for their separation and serves as an effective complement to charge-based methods during the final polishing phase of biopharmaceutical purification.

Phenyl- and ethyl-based HIC resins are commonly employed for the purification of biomolecules with moderate hydrophobicity. Buffers such as ammonium sulfate and sodium citrate are typically used at high salt concentrations due to their mild kosmotropic behavior, which enhances hydrophobic interactions (Ghose et al., 2013). A study investigating virus removal via HIC demonstrated a distinct separation pattern based on viral hydrophobicity, with elution profiles indicating a gradient from less to more hydrophobic viruses (ØX174 < PP7 < MVM < PR772 = Reo-3 < X-MuLV ≤ PRV) (Johnson et al., 2017). Enveloped viruses like X-MuLV displayed higher hydrophobicity, likely due to membrane-associated proteins embedded in the lipid envelope, in contrast to non-enveloped viruses such as MVM (Johnson et al., 2017). To further explore this, a comparative study evaluated three commercial HIC resins, POROS® Benzyl, POROS® Ethyl, and POROS® Benzyl Ultra, using two distinct mAb feedstocks. All resins achieved complete clearance of X-MuLV (>4 $\log_{10}$) in both bind-and-elute and flow-through modes, obtained by adjusting the salt composition and concentration of the HIC buffers to modulate hydrophobic interactions. Complete clearance under both conditions is consistent with the high intrinsic hydrophobicity of this enveloped virus. Conversely, MVM clearance remained modest, consistent with its lower hydrophobicity. These findings confirm that HIC performance in viral clearance is closely linked to the physicochemical characteristics of the virus, particularly the presence or absence of a lipid envelope (Thermo Fisher, 2021).

2.1.4. Mixed-mode chromatography (MMC)

MMC, also known as multimodal chromatography, is gaining increasing relevance in pharmaceutical and biopharmaceutical applications due to its unique selectivity profile. By combining multiple interaction mechanisms, such as ionic, hydrophobic, and hydrogen bonding, MMC enables the retention and separation of a broad spectrum of compounds, particularly polar and charged species. This integrated interaction framework significantly broadens the design space, allowing robust performance across diverse process conditions. A key advantage is the ability to directly capture target proteins at relatively high salt concentrations, eliminating the need for dilution or specific additives, which makes it especially attractive for complex feedstocks (Zhang and Liu, 2016).

In the study by Cai et al., MMC demonstrated strong viral clearance capabilities, particularly for enveloped viruses such as XMuLV. The study reported consistent log reduction values (LRVs) ≥5, on par with or exceeding those achieved by conventional AEX. Notably, the presence of hydrophobic interactions extended the optimal operating window, with robust viral removal observed across pH values from 4.9 to 7.5 and conductivity levels between 5.1 and 26 mS/cm. This flexibility enhances process resilience, making MMC especially valuable in handling complex feedstock or variable upstream conditions (Cai et al., 2024).

In another study conducted by the company Cytiva, viral clearance performance was evaluated using Capto™ Adhere resin, a mixed-mode chromatography medium, on mAb supernatant previously purified with MabSelect SuRe™ Protein A resin. The study assessed two model viruses: MVM and Murine Leukemia Virus (MuLV). Under flow-through conditions at a conductivity of 10 mS/cm and pH 6.75, the resin achieved LRVs of 5.8 for MVM and 4.5 for MuLV, demonstrating its effective performance in virus removal during the polishing phase (Cytiva, 2020).

A recent study employed a Design of Experiments (DoE) approach to evaluate viral clearance and impurity removal using the mixed-mode chromatography resin Nuvia™ aPrime 4 A (Bio-rad Laboratories, 2021). The results demonstrated effective clearance of both MVM and X-MuLV, achieving >4.7 $\log_{10}$ and > 5.07 $\log_{10}$ reductions, respectively. High NaCl concentrations combined with elevated pH conditions (up to pH 8) significantly enhanced viral removal. In particular, for MVM, improved clearance was attributed to the virus's capsid isoelectric point (pI ~6.1–6.2), which under high-salt and alkaline conditions promoted stronger interactions with the resin matrix (Bio-rad Laboratories, 2021).

Collectively, these chromatographic approaches, each with distinct separation principles and operational modes, constitute a robust and adaptable toolbox for achieving both high product purity and effective viral clearance in mAb manufacturing workflows.

2.2. Dedicated viral inactivation and removal steps

In monoclonal antibody manufacturing, viral safety is ensured through well-established, validated strategies that emphasize simplicity, robustness, and regulatory compliance. These processes typically incorporate multiple orthogonal steps, each targeting different classes of viruses, to ensure comprehensive risk mitigation. Following the initial capture step, dedicated viral inactivation procedures are implemented, complemented by virus filtration and additional clearance operations. Together, these layers of control routinely achieve log reduction values exceeding 4 ($\geq 10^4$-fold) for each model virus in validation studies, establishing a high level of assurance in product safety.

2.2.1. Chemical methods (e.g. low pH, detergent)

Chemical inactivation strategies primarily rely on low pH exposure or solvent/detergent treatments, which disrupt the lipid envelope of viruses, effectively neutralizing enveloped species such as retroviruses and herpesviruses. In contrast, non-enveloped viruses, most notably those from the Parvoviridae family, exhibit substantial resistance to these methods, posing a persistent challenge for viral clearance. (Miesegaes et al., 2010).

Recent studies have demonstrated that low-pH inactivation procedures can consistently achieve an average viral reduction of 5.4 to 6.0 $\log_{10}$ for enveloped viruses, particularly those belonging to the Herpesviridae and Retroviridae families. In the case of retroviruses such as MuLV virus, most reports converge on optimal inactivation conditions at pH values between 3.5 and 3.8, with incubation times of at least 60 min (Ajayi et al., 2022) Under these conditions, viral inactivation exceeding 5 $\log_{10}$ is routinely observed, confirming the robustness of this strategy for removing enveloped viral contaminants (Ajayi et al., 2022). Furthermore, Cai et al. demonstrated that low pH treatment at approximately pH 3.6 for a minimum of 30 min at temperatures $\geq$15 °C consistently achieved log reduction values (LRVs) $\geq$5.3 for XMuLV across all tested conditions. Notably, none of the evaluated variables, including pH, temperature, protein concentration, levels of HCP and DNA, or the presence of monomers and aggregates, significantly impacted inactivation efficiency, confirming the intrinsic robustness of this unit operation(Cai et al., 2024).

An alternative to low-pH inactivation is solvent/detergent (S/D) treatment, in which non-ionic detergents, typically Triton X-100 combined with tri-n-butyl phosphate (TNBP), disrupt viral lipid envelopes, achieving rapid viral inactivation (>4–6 $\log_{10}$ within <1 min at 22 °C) (Conley et al., 2017; Hunter et al., 2022; Roberts, 2008).However, environmental concerns arose when the European Chemicals Agency (ECHA) listed Triton X-100 as a Substance of Very High Concern by the European Chemicals Agency (ECHA) in 2012, leading to restrictions on its use in EU manufacturing from 2021 onward (Meingast and Heldt, 2020). Recent studies confirm that detergent-mediated virus inactivation may induce protein aggregation, influenced by factors such as detergent concentration, protein load, and detergent physicochemistry, similar to effects observed under low-pH treatment (Feroz et al., 2022). The choice of detergent depends on process-specific requirements and the molecule's propensity to aggregate. In light of regulatory constraints and safety considerations, several alternative non-ionic and zwitterionic detergents have been investigated, including n-octyl-β-D-glucopyranoside (OG), *N*,*N*-dimethyldodecylamine N-oxide (LDAO), n-decyl-β-D-maltopyranoside (DM), and n-dodecyl-β-D-maltopyranoside (DDM), along with proprietary biodegradable surfactants such as Ecosurf™ and CG-650. In addition, novel eco-friendly formulations such as Nereid and Virodex are currently under evaluation for their potential in virus inactivation processes. These compounds retain inactivation performance while reducing harmful byproducts (Feroz et al., 2022).

Yet, beyond viral inactivation efficacy, preserving the conformational and colloidal stability of monoclonal antibodies during these stress-intensive steps remains a critical concern. Such conditions can compromise product quality, making it essential to implement finely tuned strategies that safeguard both structural integrity and biological activity. The inclusion of chemically inert, low-molecular-weight excipients has proven effective in enhancing protein stability, purity, and functionality in aqueous formulations (Li et al., 2014). These excipients include a wide range of molecules like polymers such as polyethylene glycols (PEGs) and polyols, as well as sugars, salts and amino acids (Kamerzell et al., 2011). In particular, the use of uncharged extremolytes, small organic osmolytes derived from extremophiles, has been shown to stabilize mAb intermediates exposed to two critical DSP steps: low-pH viral inactivation and virus filtration (Ramos et al., 2019). In one study, two intermediate pools (denoted Intermediate A and B, with initial pH values of 5.0 and 6.2, respectively) were challenged under low-pH conditions (pH 3.2) for 1 h in the presence of 0.5 M of two distinct extremolytes, EC4 and EC5. The results were striking: Intermediate A exhibited a 27 % reduction in aggregate formation with EC4, while EC5 showed similar protective effects for Intermediate B. Crucially, these benefits did not come at the expense of structural integrity, as confirmed by electrophoretic analysis, which showed no detectable alterations to the primary structure of the antibodies. This evidence suggests that selectively chosen, uncharged stabilizers can significantly mitigate aggregation and preserve conformational integrity during harsh process steps, without interfering with viral clearance efficacy (Ramos et al., 2019).

Among the stabilizing agents investigated for use in viral inactivation, the amino acid arginine has shown promising potential for inactivating enveloped viruses during therapeutic protein production, owing to its ability to minimize protein denaturation under specific pH and temperature conditions. However, its widespread application in industrial settings remains limited by an incomplete understanding of its precise mechanism of action and the parameters that govern its efficacy. According to the literature, optimal inactivation conditions typically involve high concentrations of arginine (0.7–1 M) applied for at least 60 min, often in combination with synergistic factors such as elevated temperatures ($\geq$40 °C), acidic pH values ($\leq$4.0), or low concentrations of Tris buffer (5 mM) (Meingast and Heldt, 2020; Tsujimoto et al., 2010). Ultimately, the success of arginine and similar stabilizers in mitigating protein aggregation during low-pH treatment is influenced by multiple factors, including the solution pH, the concentrations of both protein and stabilizer, and the net charge and structural features of the interacting molecules.

Taken together, these insights underscore the need for integrated approaches that align viral clearance efficiency with protein stability preservation, thereby ensuring both therapeutic integrity and process sustainability.

2.2.2. Viral filtration

Viral filtration represents the final and most robust barrier against viral contaminants in biopharmaceutical manufacturing, offering size-based retention that complements upstream inactivation strategies. Endorsed by regulatory agencies such as the FDA and ICH (ICH-Q5A (R2), 2024), this method ensures high levels of viral safety across a broad range of particle sizes.

Widely applied in downstream processing, virus filtration operates via a size-exclusion mechanism capable of separating small viruses—such as parvovirus (18–26 nm)—from therapeutic proteins like monoclonal antibodies (~12 nm), typically achieving >4 $\log_{10}$ viral reduction with minimal product loss (Suh et al., 2024). Retentive filters are generally classified into two types: large-pore filters targeting viruses >60 nm, and small-pore filters capable of retaining particles as small as >20 nm. These membranes are typically composed of hydrophilic polymers such as polyvinylidene fluoride (PVDF), hydrophilic polyethersulfone (PES), or cuprammonium regenerated cellulose (Johnson et al., 2022).

Viral filtration is typically conducted under mild, near-physiological conditions, such as neutral pH and moderate ionic strength, which minimizes the risk of compromising product quality. The range of

commercial virus-retentive filters listed in Table 2 is effective in removing both large and small viruses from the process stream.

Parvoviruses, such as MVM, are commonly employed as spiking agents in worst-case validation studies because of their smaller diameter (~18–20 nm) and high resistance to inactivation. These characteristics make MVM particularly challenging to remove by size-exclusion filtration. Therefore, demonstrating effective removal of MVM serves as a stringent indicator of robust viral clearance performance. Viral reduction is quantified in the product pool following filtration with virus-retentive membranes. In a study conducted by Amgen, two filters, Viresolve® Pro and Viresolve® NFP (both from Merck Millipore), were evaluated for their ability to clear model viruses including MVM, Reo-3, X-MuLV, and PRV using two different monoclonal antibody preparations (mAb A and mAb B) (Gefroh et al., 2014).

When Viresolve® Pro was tested with mAb A, investigators monitored flux decay, a parameter indicating membrane fouling caused by pore blockage. Despite high flux decay, approximately 88 % for MVM and 90 % for Reo-3, complete viral retention was observed, with no detectable virus in the permeate, demonstrating the filter's high retention capacity even under challenging conditions.

In contrast, Viresolve® NFP exhibited a more complex behavior. The extent of flux decay significantly impacted LRV outcomes, particularly for smaller viruses. As the filter pores became increasingly clogged, the relative proportion of larger pores increased, allowing the passage of small viruses like MVM. Indeed, MVM was detected in several permeate samples under high flux decay conditions. Reo-3, however, did not penetrate the membrane, likely due to its larger particle size (60–80 nm), which limited breakthrough even as pore structure shifted (Bolton et al., 2005; Gefroh et al., 2014).

More recently, data presented at the Viral Clearance Symposium 2023 (Zhu and O'Donnell, 2024) investigated key parameters influencing virus filtration performance, including virus load, flow rate, and filter reuse. The findings confirmed that higher viral loads increase the risk of breakthrough events. Furthermore, reduced filtrate flux, often resulting from membrane fouling, elevated protein concentration, or process instability, can compromise viral clearance efficiency and process reproducibility, thereby increasing the likelihood of breakthrough depending on filter design and operating parameters (Peles et al., 2024).

However, it is important to distinguish between uncontrolled low flux caused by fouling and intentional low-flux operation under optimized process conditions. Controlled low-flux regimes, as demonstrated for certain filter types such as Planova BioEX, have been shown to support stable throughput and robust viral clearance even in continuous filtration setups, provided that product stability and inline prefiltration are carefully managed (Kozaili et al., 2024).

In addition, other studies have shown that filter reuse, when performed under well-controlled and validated conditions, does not adversely affect either product quality or viral clearance performance (Zhu and O'Donnell, 2024). Together, these findings underscore the importance of distinguishing process-driven variability from deliberate design choices and optimizing operating parameters accordingly to ensure consistent and reproducible virus filtration performance.

2.2.3. UV-C irradiation and ozone treatment as emerging strategies for viral inactivation

In addition to established chemical and thermal approaches, recent research has focused on identifying novel physical methods capable of improving viral safety while minimizing stress on the product and process. Among these, UV-C irradiation and ozone treatment have emerged as innovative strategies offering reagent-free, easily integrable solutions that align with the current shift toward continuous and intensified bioprocessing.

Although not yet widely implemented in large-scale bioprocessing, ultraviolet (UV-C) irradiation is increasingly being explored as an alternative or complementary approach for viral inactivation in downstream processing (Sadraeian et al., 2022). Unlike chemical treatments, UV-C provides a physical, reagent-free mechanism that directly damages viral nucleic acids. The germicidal wavelength range (200–280 nm),

Table 2
Filters used in viral removal studies. The filters are single-use membrane devices with defined pore sizes designed to retain viruses. The virus target indicates the specific virus tested for removal. The mode refers to the filtration setup, such as Direct Flow Filtration (DFF) or Tangential Flow Filtration (TFF). The layer describes the membrane structure, which influences retention and flow properties. The membrane chemistry defines the material composition, affecting virus binding, durability, and compatibility with process conditions. The manufacturer column specifies the company producing each filter. An asterisk (*) indicates that the filter is no longer commercially available, except for potential residual stock with unguaranteed availability. (Isu et al., 2022; Johnson et al., 2022).

Filter	Virus target	Mode	Layers	Membrane Chemistry	Manufacturer
Viresolve NFR	Retrovirus	DFF	Asymmetric triple-layer pleated sheets	PES	MilliporeSigma
Viresolve NFP	Parvovirus	DFF	Asymmetric triple-layer pleated sheets	PVDF	MilliporeSigma
Viresolve Pro	Parvovirus	DFF	Asymmetric double-layer flat sheets	Hydrophilic PES	MilliporeSigma
Viresolve 70*	Parvovirus	TFF	Single-layer flat sheet	Hydrophilic PVDF	MilliporeSigma
Viresolve 180*	Retrovirus	TFF	Single-layer flat sheet	Hydrophilic PVDF	MilliporeSigma
Virosart HF	Parvovirus, Retrovirus	DFF	Asymmetric single-layer hollow fibers	Modified polyethersulfone	Sartorius AG
Virosart HC	Parvovirus	DFF	Asymmetric double-layer pleated sheets	Polyethersulfone	Sartorius AG
Virosart CPV	Parvovirus	DFF	Asymmetric double-layer pleated sheets	Hydrophilic PES	Sartorius AG
Ultipor VF grade DV50	Retrovirus	DFF	Symmetric double/triple-layer pleated sheets	Hydrophilic acrylate-modified PVDF	Pall Life Sciences / Cytiva
Ultipor VF grade DV20	Parvovirus	DFF	Symmetric double-layer pleated sheets	Hydrophilic acrylate-modified PVDF	Pall Life Sciences / Cytiva
Pegasus grade LV6	Retrovirus	DFF	Asymmetric double-layer pleated sheet	Hydrophilic acrylate-modified PVDF	Pall Life Sciences / Cytiva
Pegasus grade SV4	Parvovirus	DFF	Symmetric double-layer pleated sheets	Hydrophilic acrylate-modified PVDF	Pall Life Sciences / Cytiva
Pegasus grade prime	Parvovirus	DFF	Pleated sheet	Polyethersulfone	Pall Life Sciences / Cytiva
Planova 35 N	Retrovirus	DFF	Asymmetric single-layer hollow fibers	Hydrophilic cuprammonium regenerated cellulose	Asahi Kasei Bioprocess
Planova 20 N	Parvovirus	DFF	Asymmetric single-layer hollow fibers	Hydrophilic cuprammonium regenerated cellulose	Asahi Kasei Bioprocess
Planova 15 N	Parvovirus	DFF	Asymmetric single-layer hollow fibers	Hydrophilic cuprammonium regenerated cellulose	Asahi Kasei Bioprocess
Planova BioEX	Parvovirus	DFF	Asymmetric single-layer hollow fibers	PVDF	Asahi Kasei Bioprocess
Planova S20N	Parvovirus	DFF	Asymmetric single-layer hollow fibers	Regenerated Cellulose (RC)	Asahi Kasei Bioprocess
Planova FG1	Parvovirus	DFF	Asymmetric single-layer hollow fibers	PES	Asahi Kasei Bioprocess

particularly 254 nm, induces pyrimidine dimers and other lesions in viral DNA or RNA, thereby preventing replication while largely preserving protein structure. Because proteins absorb far less energy at 254 nm than nucleic acids, UV-C treatment can inactivate viruses with minimal impact on antibody integrity (Li et al., 2005).

UV-C irradiation has shown particular promise against small, non-enveloped viruses, which are generally resistant to conventional low-pH or solvent/detergent (S/D) inactivation methods. For example, studies have demonstrated that parvoviruses such as Minute Virus of Mice (MVM) can be effectively inactivated by moderate UV-C doses (100–300 J/m^2), achieving >4–6 $\log_{10}$ reductions without measurable effects on monoclonal antibody quality (Bergmann, 2014). Laboratory-scale data further indicate that IgG monoclonal antibodies maintain their structural integrity and biological activity under UV-C exposure sufficient for virus inactivation, with only minor increases in aggregation, oxidation, or charge variants (Li et al., 2005). These findings support the technical feasibility of UV-C as a gentle, non-destructive inactivation method, offering an additional layer of viral safety, particularly for non-enveloped viruses that are otherwise difficult to neutralize chemically.

In biopharmaceutical manufacturing, UV-C has been evaluated as a continuous, in-line viral inactivation step compatible with single-use systems, typically positioned between two chromatographic operations (e.g., following Protein A or ion-exchange chromatography). This configuration enables flow-through exposure of the protein solution while allowing subsequent polishing steps to remove any UV-induced variants.

A notable example of innovation in this field is the development of a dedicated UV-C viral inactivation reactor by ZETA GmbH, in collaboration with SES-Tec. The system was specifically engineered to address one of the main challenges in downstream processing: the inactivation of small, non-enveloped viruses. The reactor design, combining computational fluid dynamics (CFD) with experimental validation at the ZETA TechCenter, ensures controlled and uniform UV exposure under process-relevant flow conditions. This technology exemplifies the growing drive toward continuous, in-line, reagent-free viral inactivation that can be directly integrated into biopharmaceutical manufacturing. By avoiding harsh chemical or thermal treatments and minimizing hold times, UV-C reactors have the potential to enhance viral safety while preserving product quality and supporting next-generation continuous processing strategies.

Despite these advantages, UV-C inactivation remains an emerging technology. As of 2025, it is not yet a routine unit operation in licensed monoclonal antibody manufacturing, but rather a promising option under active evaluation. Several companies are investigating its implementation in continuous bioprocessing platforms and as an additional safety barrier in established production schemes.

Alongside UV-based approaches, other emerging physical methods are also under investigation for viral inactivation. Among these, ozone treatment has attracted increasing interest due to its broad-spectrum oxidizing properties and ability to disrupt both viral envelopes and capsid proteins. While ozone is currently employed mainly for surface and water decontamination, controlled exposure is being explored as a means to enhance viral safety in bioprocessing without introducing chemical residues. Though still at an early stage of development, such technologies represent promising directions for the future implementation of non-traditional, reagent-free viral inactivation strategies in biopharmaceutical manufacturing.

3. Methods for virus detection

In the context of biopharmaceutical manufacturing, viral safety hinges on the reliable detection and quantification of infectious agents. Since the ultimate concern is the presence of replication-competent viruses, infectivity-based assays remain the cornerstone of detection strategies. Classical methods such as $TCID_{50}$ (Tissue Culture Infectious Dose 50 %) (Fig. 2, panel A) and plaque assay (Fig. 2, panel B) are routinely used to assess viral infectivity with high biological relevance. These assays must be fully validated when used in regulatory-grade viral clearance studies, as they define the functional endpoint of inactivation or removal processes. To complement these bioassays, molecular techniques have revolutionized the field of viral detection. Tools such as quantitative PCR (qPCR) (Fig. 2, panel C) and next-generation sequencing (NGS) (Fig. 2, panel D) provide unparalleled sensitivity, enabling precise detection and quantification of viral genomes, even at trace levels. These approaches not only enhance the resolution of viral safety assessments but also support the identification of non-cultivable, emerging, or low-abundance viral species. Together, infectivity assays and molecular platforms form a robust, multilayered strategy to ensure the virological integrity of biopharmaceutical products.

3.1. $TCID_{50}$ assay

The $TCID_{50}$ assay is considered the "gold standard" for detecting viruses in viral clearance studies (Fig. 2, panel A). In this assay, a viral sample is serially diluted and each dilution place on replicate cultures of susceptible, adherent cells in wells of a flat-bottomed plate. In this quantal assay, the infected cultures are incubated for several days and then wells are scored positive or negative, based on the presence or absence of virus induced cytopathic effects (CPE) which are visible changes caused by viral infection. Based on the percentage of wells showing CPE at each dilution, the viral concentration required to infect 50 % of the cultured cells is determined. Thus, the number of $TCID_{50}$ units per millilitre can be calculated typically using the Spearman-Kärber or Reed-Muench formulas (Cen, 2019).

$$\textit{Negative logarithm of the } 50\%\textit{endpoint} = \textit{Negative logarithm of the highest virus concentration used} - \left[\left(\frac{\textit{Sum of \% affected at each dilution}}{100} - 0.5\right) \times (\log \textit{of dilution})\right]$$

The $TCID_{50}$ value represents the viral concentration necessary to infect 50% of the cells in the culture, providing a measure of the viral titer in the sample.

The $TCID_{50}$ assay quantifies the viral concentration required to infect 50% of the cultured cells, thereby providing a measure of the infectious viral titer in a given sample. A principal advantage of this method lies in its ability to specifically detect infectious virus particles, offering a functionally relevant assessment of viral infectivity that is critical for evaluating the effectiveness of viral clearance processes. Due to its robustness and widespread acceptance, the $TCID_{50}$ assay remains a standard technique in virology for quantifying infectious virus across diverse viral families. Nevertheless, the assay has notable limitations which includes inherently time-intensive, with incubation periods that may extend from several days to weeks depending on the virus. The requirement for specialized cell culture infrastructure and technical expertise further adds to the resource burden. Additionally, the assay's sensitivity is limited by its reliance on visible CPEs, which may not be

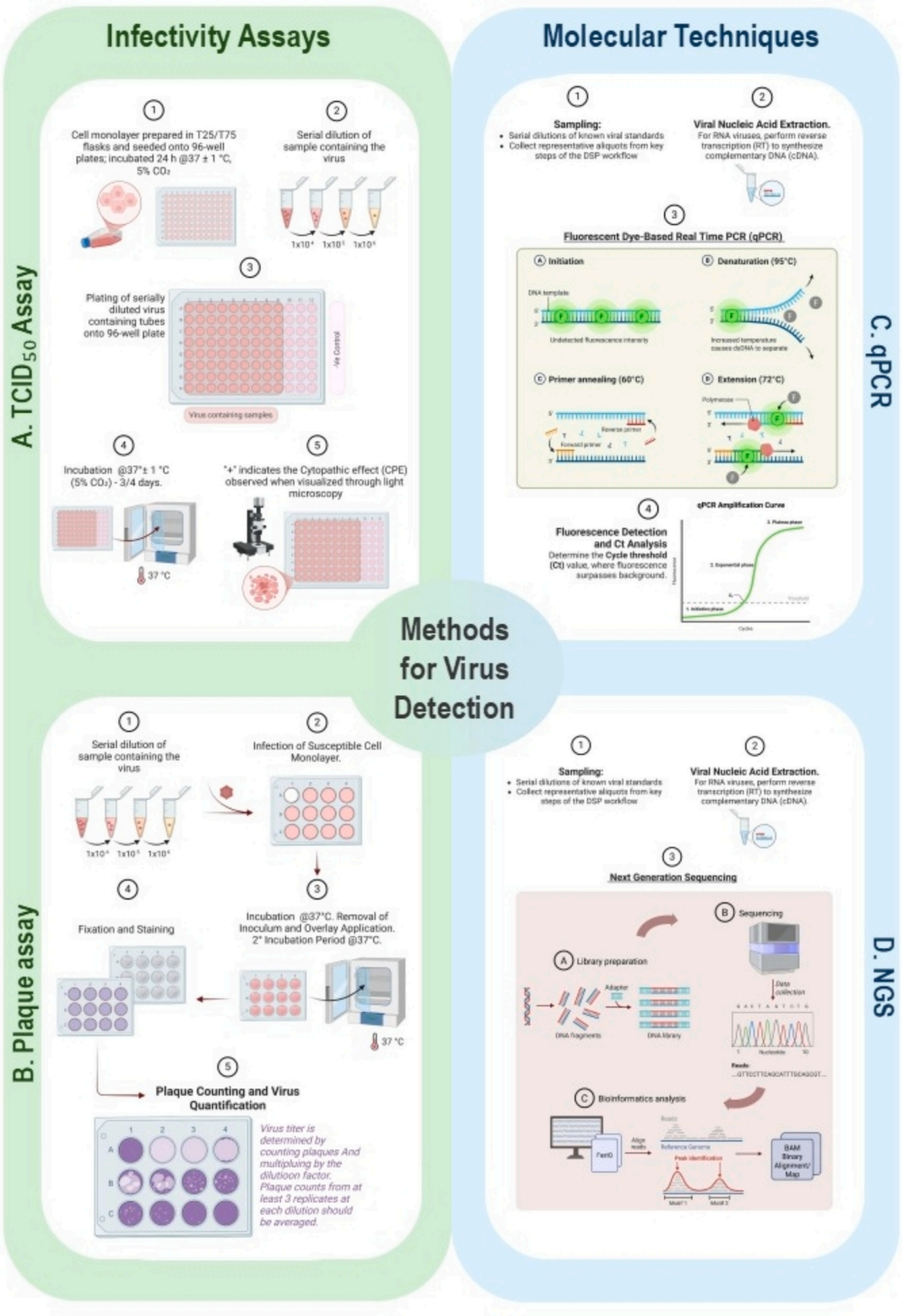

Fig. 2. Overview of the experimental workflows of the principal methods used for virus detection in downstream processing (DSP) during viral clearance studies. The figure illustrates the key analytical approaches: (A) $TCID_{50}$ assay, (B) plaque assay, (C) quantitative PCR (qPCR), and (D) next-generation sequencing (NGS).

evident at low viral loads, potentially leading to underestimation of virus presence. The subjective nature of CPE interpretation can also introduce variability and affect reproducibility. Finally, the applicability of the TCID50 assay is constrained by the inability to culture certain viruses in vitro, limiting its utility for comprehensive viral detection.

3.2. Plaque assay

In the quantitative plaque assay (Fig. 2, panel B), a sample containing the virus undergoes serial dilution. Various dilutions are then used to infect susceptible, adherent cells typically arranged in a flat dish. After allowing time for the virus to attach to the cells and removing the inoculum, the cells are covered with a culture medium containing a semi-solid matrix, like agarose or methyl cellulose. As infected cells release new viral particles or virions, the semi-solid medium inhibits the virus movement, allowing infection only in the proximity of the original site. After some interval, cells surrounding the initially infected cell also become infected. If the infection leads to cell death, a visible clear zone or "plaque" is formed. Plaques can be observed without a microscope, though staining cells may enhance plaque visibility. For most viruses, there is a direct correlation between the number of plaques and the number of infectious particles in the initial sample. In this method results are expressed as plaque forming units (PFU) per millilitre (PFU/mL). It is important to note that not all viruses generate plaques, in such cases, an alternative assay like the $TCID_{50}$ must be used for logarithmic viral reduction. For the calculation of plaque assay following formula is used (Cen, 2019).

$$PFU \Big/ t = \sum \frac{c_1 + c_2 + \ldots c_n}{(n_1 + n_2 \times v_2 + \ldots n_n \times v_n) \times d}$$

Where,

t – the test volume that was added per dilution step to a plate;

c_1 - the PFU number of all the plates of the first dilution step (lowest dilution step) with nonconfluent plaques;

c_2 - the PFU number of all plates of the second dilution step with nonconfluent plaques;

c_n - the PFU of all plates of the last dilution (highest dilution step);

n_1 - the number of all plates of the first dilution step (lowest dilution step) with nonconfluent plaques to which c_1 corresponds;

n_2 - the number of all plates of the second dilution step with nonconfluent plaques (c_2);

v_2 - the dilution factor between n_1/n_2 (e.g. $n_1 = 10^{-3}$ and $n_2 = 10^{-4}$, then $v_2 = 0{,}1$);

n_n - the number of all plates of the last dilution for which PFUs were counted(c_n);

v_n - the dilution factor between n_1/ n_n (e.g. $n_1 = 10^{-3}$ and $n_n = 10^{-6}$, then $v_n = 0{,}001$);

d - the dilution step of c_1.

3.3. qPCR

It is also known as real-time quantitative PCR (Fig. 2, panel C); is a molecular biology technique used to amplify and simultaneously quantify a targeted DNA or RNA sequence in a sample. Unlike traditional PCR, which only provides the presence or absence of a target, qPCR allows for real-time monitoring of the amplification process by measuring the fluorescence emitted during each amplification cycle. This fluorescence correlates with the amount of DNA or RNA being amplified, enabling precise quantification of the target nucleic acid. In viral clearance studies, qPCR can be a valuable tool for quantifying viral genomes in samples, particularly when assessing the removal of enveloped viruses during purification processes like Protein A chromatography. While infectivity assays measure active viral particles, qPCR detects both infectious and inactivated viral genomes, making it useful for identifying viral presence after inactivation steps, such as low pH holds. For example, if enveloped viruses are spiked into a sample and subjected to Protein A chromatography, qPCR can quantify the viral genomes in both the chromatography load and eluate fractions. This allows for a clear distinction between viral removal by the chromatography process and viral inactivation by subsequent treatments, ensuring that both mechanisms of viral reduction are accurately accounted for.

qPCR offers several significant advantages, foremost among them its high sensitivity, which enables the detection of very low levels of viral nucleic acids often within a matter of hours—substantially faster than traditional infectivity assays. The technique's ability to quantify viral genome copies allows for precise measurement, while its broad applicability includes detection of viruses that are difficult or impossible to culture. Moreover, the widespread availability of standardized protocols and commercial kits facilitates reproducibility and simplifies implementation across different laboratories. Despite these strengths, qPCR also presents inherent limitations as it detects viral genetic material rather than infectious virus particles, the assay may overestimate viral contamination by amplifying nucleic acids from non-infectious or inactivated viruses. Consequently, qPCR does not provide direct information on virus viability and is insufficient as a standalone indicator of infectivity. Additionally, PCR is highly sensitive and more prone to false-positive results caused by contamination, so strict laboratory procedures are necessary to ensure the accuracy and reliability of the test data.

3.4. Next generation sequencing (NGS)

Conventional techniques for detecting adventitious viruses, such as in vitro and in vivo assays or qPCR-based methods, present several intrinsic limitations, including long turnaround times, restricted detection to predefined targets, and the inability to identify unknown viral agents. Moreover, these approaches fail to detect viruses that do not induce cytopathic effects (CPEs) or to capture the structural and molecular alterations triggered by viral invasion within host cells. To overcome these constraints, next-generation sequencing (NGS) (Fig. 2, panel D), also referred to as deep sequencing or massive parallel sequencing (MPS), has recently emerged as a powerful, unbiased tool for comprehensive viral detection (Fig. 2, panel D). By enabling the simultaneous sequencing of millions of nucleic acid fragments from a single sample, NGS provides a high-throughput platform capable of identifying a wide spectrum of viral genomes, encompassing both known and previously uncharacterized species. Its versatility allows for the sequencing of diverse nucleic acid types, making it a robust and sensitive technology suited to viral safety assessment. Recent studies have demonstrated that both short-read and long-read NGS platforms possess the sensitivity required to detect viral sequences at extremely low copy numbers, confirming their applicability in monitoring viral contamination in cell substrates and process intermediates (Hirai et al., 2024). Beyond its sensitivity, NGS offers the unique advantage of broad-spectrum detection without prior sequence knowledge, a feature that is particularly valuable for comprehensive virus surveillance and discovery in bioprocessing environments (Russell et al., 2025).

Within the analytical workflow (Fig. 2, panel D), viral nucleic acids extracted from the samples are processed through a defined NGS pipeline. In the initial phase, the extracted material is converted into sequencing-ready libraries. For RNA viruses, reverse transcription is performed to generate complementary DNA (cDNA), which is subsequently fragmented and ligated to platform-specific adapters, followed by amplification to produce a representative DNA library. The resulting libraries are then subjected to high-throughput sequencing (HTS), commonly on Illumina or Oxford Nanopore platforms, where nucleotide incorporation events are recorded, yielding millions of short reads that collectively represent the nucleic acid content of the sample. The raw data are then analysed through bioinformatic pipelines involving quality filtering, adapter trimming, and subtraction of host-derived sequences. The retained reads are aligned to reference genomes or assembled de novo to reconstruct viral genomes, enabling both the identification and

quantification of viral sequences. This data-rich analytical phase also supports the detection of specific sequence motifs or genomic regions of interest, typically visualized in alignment formats such as BAM files. Overall, the integration of NGS into viral clearance studies represents a paradigm shift toward sequence-based, information-dense monitoring of viral safety, providing an advanced and complementary layer of confidence alongside traditional assays.

4. Advances in chromatography-based viral clearance

4.1. Activated carbon (AC) for virus removal

Activated charcoal (activated carbon) is a highly porous, high-surface-area adsorbent that can bind a wide range of impurities through non-specific interactions. Its surface provides hydrophobic, ionic, and hydrogen-bonding sites that capture contaminants from bioprocess fluids (Arakawa et al., 2023). In the context of viral clearance, AC functions as an adsorptive depth filter in flow-through mode: the target protein (e.g. a monoclonal antibody) is formulated so that it does not bind strongly to the AC, while viruses and other impurities are retained on the charcoal. Viral particles (which often have hydrophobic or charged surface regions) can adsorb to the carbon matrix, becoming effectively removed from the product stream. AC filters have been shown to reduce viral titer significantly.

In a pioneering study, Ishihara et al. (Ishihara et al., 2018) investigated the use of activated carbon (AC) as an alternative to Protein A chromatography for the capture of mAbs. The process employed a flow-through configuration, where the AC selectively adsorbed impurities such as high molecular weight (HMW) aggregates, low molecular weight (LMW) fragments, HCPs, residual DNA, and endotoxins, allowing the antibody to pass through unaffected. The results demonstrated product purity and recovery levels comparable to the conventional Protein A platform, with antibody yield around 80 % and no significant structural alterations. Subsequent work confirmed the viral clearance potential of AC, demonstrating >3 $\log_{10}$ reduction for both small non-enveloped (MVM) and large enveloped (MuLV) viruses in a single pass (Ishihara et al., 2018).

In addition, when AC is combined with an AEX membrane in series, the two steps provide orthogonal mechanisms to ensure a more robust viral clearance. As already anticipated, the AC step primarily scavenges a broad range of impurities (host cell proteins, DNA, endotoxin, virus, etc.) by adsorption, while the downstream AEX membrane adsorber captures any remaining negatively charged contaminants (such as virus particles and nucleic acids) via electrostatic binding. In a typical flow-through polishing configuration, the product antibody is adjusted to conditions where it carries little to positive net charge (e.g. operating below its pI) so that it does not bind the positively charged AEX membrane. Under those conditions, negatively charged viruses and DNA will bind to the AEX (quaternary amine) groups, whereas the antibody flows through unaffected(Thermo Fisher, 2021). This synergy means that any virus not adsorbed by the AC may be bound by the AEX membrane, resulting in overall high clearance. Moreover, using AC prior to AEX can protect the membrane from fouling: AC will remove many host cell proteins and lipids/endotoxins that might otherwise compete for binding sites or clog the AEX. The net effect is an efficient flow-through viral clearance train: AC adsorbs a spectrum of impurities (including a large portion of viral particles and endotoxin (Arakawa et al., 2025), and the AEX polishing step ensures log-order virus removal by capturing residual virions and DNA. Together, these flow-through steps can achieve viral clearance levels suitable for biopharmaceutical safety, while preserving the therapeutic protein yield.

Later, Kikuchi et al. (Kikuchi et al., 2022) reported LRVs of 3.0–5.8 for MVM and 3.5–3.8 for X-MuLV when using AC filters in single-pass mode with three different mAbs (mAb1, mAb2, mAb3). Fig. 3 illustrates viral clearance across different samples, including untreated hold controls, virus-spiked loads, and final product pools collected post-filtration.

Comparable LRVs were also observed when using a recirculation-based filtration system for 4, 8, and 23 h, suggesting that extended filtration time did not further improve viral clearance under those conditions (Kikuchi et al., 2022).

Interestingly, AC-based virus clearance outperformed traditional Protein A chromatography in some scenarios, achieving higher reduction levels (Zhang et al., 2014). One explanation for the lower LRVs sometimes reported with Protein A is the potential formation of virus–antibody complexes during capture, which may reduce viral removal efficiency (Li, 2022). Additionally, process parameters such as pH and flow rate were also shown to critically influence viral clearance. For instance, when filtering MVM at pH 7.0–7.1 and a flow rate of 41 L/m^2/h, LRVs were significantly lower compared to those obtained at pH 4.7–4.9 and 123 L/m^2/h. This enhanced performance at lower pH and higher flow may reflect hydrophobic interactions becoming more dominant when the pH approaches the virus's isoelectric point (Kikuchi et al., 2022).

Beyond performance, the use of AC also offers practical and economic advantages. Unlike Protein A resins, AC filters are disposable and relatively inexpensive, with scalability similar to depth filtration. This modularity makes AC attractive for next-generation purification workflows aiming to reduce cost and increase throughput. While further validation at scale is still needed, the accumulated evidence positions AC as a compelling alternative or complement to Protein A in next-generation biomanufacturing.

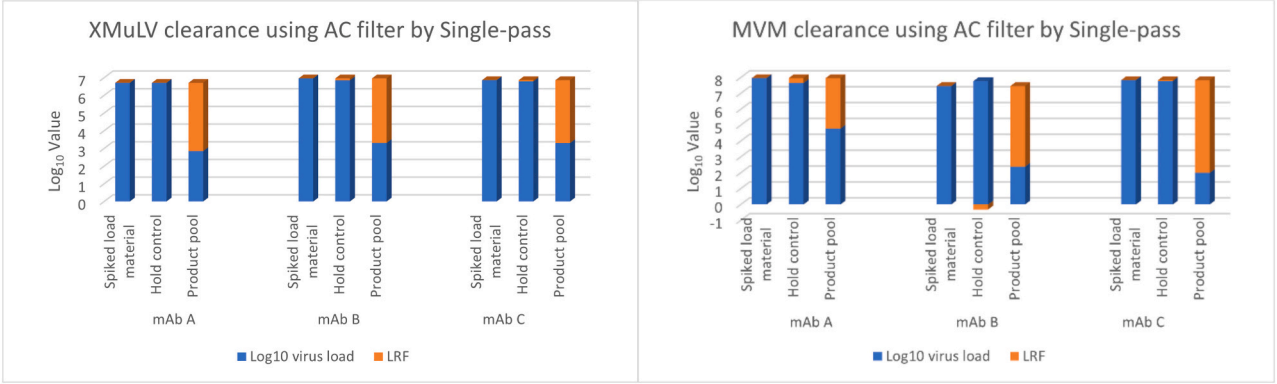

Fig. 3. Viral clearance achieved through a column-free single-pass filtration approach using activated carbon (AC) filters. The histograms report the LRVs for MVM and X-MuLV viruses when spiked into three different monoclonal antibody preparations (mAb1, mAb2, mAb3). The comparison includes: i. **Hold Control** – untreated spiked samples incubated under test conditions to assess virus stability; ii. **Spiked Load Material** – virus-spiked input prior to AC filtration; iii. **Product Pool** – filtrate collected after AC processing (Kikuchi et al., 2022).

4.2. Membrane chromatography vs. packed-bed resins

Membrane chromatography employs multilayered microporous membranes functionalized with ligands covalently attached throughout the internal pore surfaces, enabling efficient and convective interaction with target molecules across the entire membrane matrix (Liu et al., 2010). Majorly used functional ligands are described in Table S1.

4.2.1. Viral removal efficiency

Membrane adsorbers and packed-bed AEX resins operated in flow-through mode for polishing exhibit high viral clearance, quantitatively expressed by LRVs. Their effectiveness is mainly attributed to the net negative charge of most adventitious viruses at neutral pH, which favors adsorption to the positively charged matrix. In a head-to-head study, Miesegaes et al. compared multiple AEX membranes and resins under the same conditions and found comparable clearance of model viruses (including a small non-enveloped parvovirus, a medium-sized retrovirus, and a bacteriophage) for both membrane and column formats. In other words, membranes were just as capable as packed columns at achieving high LRVs for viruses when operated optimally (Miesegaes et al., 2014). This equivalence holds even for challenging small viruses; a recent study by Dolan et al. (Dolan et al., 2021) demonstrated that modern AEX membrane devices provide parvovirus (i.e. MVM) clearance equivalent to AEX resin columns. In practice, virus log reduction values of >5–6 logs are routinely obtained using quaternary amine (Q) membrane units for both large retroviruses and small parvoviruses (Shukla and Aranha, 2015), matching or exceeding typical resin performance. Notably, membrane adsorbers have achieved >6 LRVs even at very high load challenges (e.g. >1.7 kg of product per Liter membrane) and high flow rates (≥240 cm/h), highlighting that membranes can maintain robust virus removal under aggressive processing conditions.

4.2.2. Binding capacity for viruses and large impurities

Membrane chromatography offers fundamental advantages in binding large particles like viruses due to convective mass transport and macroporous structures. Unlike porous resin beads (which have diffusive pores often <100 nm), membrane adsorbers have pore sizes on the order of microns (e.g. >3 μm for Sartobind Q) and ligands accessible throughout those open channels. This means viruses are not excluded from the binding surface, they can freely diffuse or convect into the membrane's internal structure and attach to charged ligands. Consequently, membranes can exhibit much higher effective binding capacities for viruses compared to conventional resins, which only bind viruses on external or shallow bead surfaces. For example, Sartorius reports that its Q membrane adsorbers have about one order of magnitude higher dynamic binding capacity for viruses than an equivalent Q resin, since size-exclusion effects are negligible. This trend is corroborated by independent studies: convective-flow media (membranes or monoliths) consistently show higher binding capacity for large biomolecules (viruses, DNA, high–MW aggregates) than do diffusive particle columns (Boi et al., 2020). Liu et al. (Liu et al., 2010) had noted that implementing AEX membranes in polishing leverages this ability to capture trace impurities (including viral particles) very effectively. In summary, membrane adsorbers can bind a greater quantity of viral particles per unit volume than packed beds in many cases, on the order of 5–10× more for large viral vectors has been reported, because resin pores impose steric and diffusive limitations that membranes avoid. This high capacity for viruses directly contributes to strong clearance performance even at high contaminant loads.

4.2.3. Flow-rate Independence and productivity

One of the clearest advantages of membrane chromatography is its performance at high flow rates. In packed-bed columns, binding of large species is diffusion-limited, so increasing flow (decreasing residence time) often causes a sharp drop in dynamic binding capacity and virus removal. Membranes, by contrast, rely on convective flow through large pores, making their performance relatively independent of flow rate within a broad range. Boi et al. (Boi et al., 2020) conducted a direct side-by-side comparison of a Q membrane vs. a Q resin (both 3 mL bed volume) using a model protein and found that while the packed resin had higher equilibrium capacity, the membrane's usable capacity held up far better at short residence times. At a superficial velocity of ~100 cm/h, the resin's dynamic binding capacity for BSA was ~62.8 mg/mL, versus 20.7 mg/mL for the membrane (Boi et al., 2022), a gap expected due to the resin's greater surface area. However, because the membrane could be operated at much higher flow (with minimal loss in efficiency), its throughput productivity (mass processed per volume per hour) reached 111 mg/mL·h, which was 3.3-fold higher than the resin column's productivity under its optimal flow conditions (Boi et al., 2022). This illustrates that membranes excel in high-throughput polishing: even if a resin offers more capacity at slow flows, a membrane can compensate with dramatically faster flow rates (often 10–30× faster linear velocities). In practical terms, membrane units can be run at 5–30 column-volumes per minute (ultra-short residence times) without sacrificing viral clearance. The result is shorter processing times and smaller device volumes for the same amount of material. In fact, convective membrane systems have been highlighted as enabling much higher load processing in polishing steps while still achieving full viral clearance. The superior productivity and flow-rate resilience of membrane adsorbers at scale has been widely recognized in the bioprocessing community (Boi et al., 2020).

4.2.4. Robustness to pH and conductivity variations

Virus removal by AEX systems is governed by electrostatic interactions between the negatively charged viral particles and the positively charged surface of the adsorbent. Therefore, pH and conductivity are critical process parameters, as they directly influence charge interactions and, consequently, viral binding efficiency. Both membranes and resins require conditions in which viruses (and other impurities such as DNA) remain negatively charged—typically at neutral to mildly acidic pH—and have shown comparable virus clearance within this range (Miesegaes et al., 2014; Boi et al., 2022). However, conductivity exerts a stronger influence on performance: elevated salt concentrations can impair virus binding by competing for active sites and shielding electrostatic interactions. For example, standard Q resins show a marked decline in viral clearance when feed conductivity approaches ~1 M NaCl, with LRVs dropping from ~5 to as low as 1–2(Shukla and Aranha, 2015). To address this limitation, salt-tolerant membrane adsorbers such as Sartobind® STIC PA (bearing primary amine and guanidinium ligands) have been developed. These devices maintained >5 LRVs clearance for both retrovirus and parvovirus even at conductivities up to 150 mM NaCl, outperforming conventional Q membranes that require low-salt conditions for optimal virus binding(Shukla and Aranha, 2015). Membranes have also demonstrated resilience under high protein loads and moderately elevated conductivity. Boi et al.(Boi et al., 2022)showed that although extremely high product densities may reduce viral retention to some extent, such effects are manageable through proper sizing and buffer optimization. Moreover, Miesegaes et al. (Miesegaes et al., 2014) demonstrated that AEX membranes retained viral clearance performance even when challenged with elevated levels of CHO-HCPs, fish genomic DNA, or mouse DNA, conditions under which resin-based systems showed greater sensitivity and performance variability. Additional evidence from comparative studies supports the superior robustness of membranes under challenging process conditions. The Natrix® Q membrane (Millipore Sigma), for example, achieved up to 7.5 LRVs for MVM at high conductivity (10 mS/cm) and pH 7.5 under a high protein load (10 kg/L), significantly outperforming modern Q resins by ~3 LRVs under identical conditions. Under similar conditions, this membrane also demonstrated high removal efficiency for MuLV (≥4.9 LRVs), PRV (≥5.9 LRVs), and Reo-3 (≥6.3 LRVs), confirming its broad-spectrum virus clearance capacity. In addition to biochemical robustness, the single-use format of membrane devices contributes to operational

reliability by eliminating cleaning and regeneration steps, thus avoiding performance drift over repeated cycles. This format also minimizes cross-contamination risks and ensures consistent batch-to-batch performance.

Taken together, these findings reinforce the positioning of anion-exchange membrane chromatography as a superior alternative to packed-bed resins—not only achieving equal or greater log reduction values (≥5–6 LRVs), but also offering enhanced productivity, higher throughput, and greater virus-binding capacity. When combined with lower buffer consumption, modular scalability, and process simplification through disposable formats, membranes represent a robust, efficient, and scalable platform for virus removal in modern downstream bioprocessing.

5. Implementation in continuous processing

Continuous bioprocessing offers notable advantages in product consistency, productivity, and cost reduction (Chiang et al., 2019). However, transitioning from traditional batch production to a continuous platform entails significant business and regulatory hurdles. A particularly critical challenge is viral safety, as even in continuous mode the process must ensure robust viral clearance through dedicated inactivation and filtration steps (Chiang et al., 2019). Johnson et al. (2017) emphasized that meeting regulatory requirements will require seamless integration of viral testing and clearance/inactivation technologies into continuous downstream processes (Johnson et al., 2017). Accordingly, recent development efforts have focused on adapting major downstream steps – capture chromatography, low-pH viral inactivation, and virus filtration – to operate in a continuous manner (FDA, U. S, 2023).

For the capture step, studies indicate that multi-column continuous chromatography can achieve virus removal comparable to batch processes. Chiang et al. (2019) performed a design-of-experiments comparing continuous dual-column Protein A capture (using 1 + 1 and 1 + 2 column configurations) against equivalent batch capture for two monoclonal antibodies spiked with model bacteriophages φX174 and PR772 (Chiang et al., 2019). They observed no reduction in viral clearance efficacy in continuous mode, LRVs were similar to those in batch operation under both best-case and worst-case conditions (Chiang et al., 2019). Importantly, parameters known to influence viral removal (e.g. wash volumes, additives, elution pH, and pool cut criteria) had similar effects in batch and continuous modes (Chiang et al., 2019). This suggests that a well-designed batch-scale experiment can predict viral clearance performance for full-scale continuous capture chromatography (Chiang et al., 2019).

In the case of viral inactivation, continuous processing has also proven effective. David et al. (2019) demonstrated that a continuous low-pH inactivation step (using a coiled-flow inverter reactor) could inactivate an enveloped retrovirus (X-MuLV virus) as effectively as the conventional batch hold (David et al., 2019). In their study, >4 $\log_{10}$ virus reduction was achieved within the first ~15 min of acid exposure in both continuous and batch formats (David et al., 2019). Two distinct Protein A elution pools at different pH levels were tested in continuous mode, and no residual infectivity was detected – findings that mirrored batch results and affirmed that batch-derived inactivation kinetics can translate to continuous operation (David et al., 2019).

Finally, for viral filtration, small-scale models have been developed to emulate extended continuous operation. Lute et al. (2020) evaluated an older-generation parvovirus filter (Planova 20 N) and a newer Planova BioEX filter under continuous flow conditions (Lute et al., 2020). Their data showed that both membrane filters could reliably remove >4 $\log_{10}$ of a spiked parvovirus surrogate (bacteriophage PP7) when run continuously for up to 4 days(Lute et al., 2020). Moreover, both filters handled a simulated high–loading "elution peak" (with elevated protein, salt, and virus levels) with only a modest increase in filtration pressure and without any loss of virus retention performance (Lute et al., 2020).

These findings underscore that key viral clearance steps, from multicolumn capture to low-pH inactivation and long-duration filtration, can be successfully implemented in continuous bioprocessing without compromising viral safety.

6. Conclusions and future directions

DSP represents a critical phase in mAb manufacturing, where viral safety is ensured through a multilayered combination of capture, inactivation, and removal strategies. This review has summarized the main unit operations including Protein A chromatography, low-pH inactivation, virus filtration, and ion-exchange steps, and their respective contributions to viral clearance.

Chromatographic techniques not only purify the product but also contribute to partial virus reduction. Among them, Protein A remains the cornerstone of the capture step, while AEX in flow-through mode offers robust removal of a broad viral spectrum, including non-enveloped viruses. Virus filtration using size-exclusion membranes provides a highly effective physical barrier, achieving >4–6 $\log_{10}$ reduction for even the most resistant species like MVM. Viral inactivation, whether via low-pH or solvent/detergent treatment, remains essential, though associated risks such as protein aggregation and environmental toxicity have prompted the adoption of stabilizers and alternative surfactants. Innovative solutions, such as membrane adsorbers, activated carbon filtration, and mixed-mode chromatography, are enhancing the viral safety toolkit while supporting high-throughput and modular DSP configurations.

The future of viral clearance in monoclonal antibody manufacturing is being redefined by the pursuit of efficiency, resilience, and seamless integration into continuous downstream processing (CDSP). What was once a series of isolated safeguards is rapidly evolving into a cohesive, high-performance system embedded within the broader architecture of next-generation biomanufacturing.

Continuous processing is no longer a distant goal: multicolumn Protein A capture, uninterrupted low-pH inactivation, and extended-duration virus filtration have already demonstrated viral clearance levels on par with, or superior to, traditional batch methods. The reproducibility of critical parameters, including pH, viral load, flow rate, and wash volumes, across scales confirms the scalability and regulatory viability of these innovations.

Membrane adsorbers are leading the charge toward resin-free purification, offering exceptional virus-binding capacity, tolerance to high conductivity, and compatibility with fast, high-throughput operations. In parallel, activated carbon filtration, when strategically combined with AEX membranes, is enabling orthogonal virus removal strategies that are both efficient and gentle on the product.

On the molecular front, stabilizing excipients such as arginine and uncharged extremolytes are emerging as key allies in mitigating aggregation during stress-prone inactivation steps, preserving structural integrity without compromising viral safety.

Together, these advances are more than incremental, they represent a shift toward a new paradigm of viral clearance: one that is not only compliant and effective, but also agile, scalable, and environmentally conscious. As membrane-based platforms, biodegradable surfactants, and CDSP technologies continue to mature, the field moves closer to a future where viral safety is no longer a constraint, but a built-in advantage, fueling the rise of flexible, high-efficiency, and sustainable biomanufacturing ecosystems.

Declaration of competing interest

The authors declare no conflicts of interest related to the publication of this review. This work was developed independently and is not influenced by any commercial or financial interests. The analysis of viral clearance strategies in downstream processing aims to provide a critical overview of current technologies and regulatory perspectives, with relevance for the biopharmaceutical manufacturing field. At the time of

writing, the authors have no affiliations or financial relationships with companies or organizations that could benefit from the content of this review.

Acknowledgments

The author sincerely thanks the colleagues at the Eurofins Virus Laboratory (Eurofins Biolab Srl, Eurofins Biopharma Product Testing Italy) for their valuable support and collaboration throughout the study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biotechadv.2025.108784.

Data availability

No data was used for the research described in the article.

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