Received: 20 March 2026 | Revised: 3 July 2026 | Accepted: 7 July 2026

DOI: 10.1002/btpr.88547

RESEARCH ARTICLE

Bioseparations and Downstream Processing

BIOTECHNOLOGY PROGRESS

DNA concentration in harvest affects retrovirus clearance capabilities of protein A chromatography

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Abstract

Protein A chromatography is a key unit operation in biopharmaceutical purification, widely used to capture monoclonal antibodies (mAbs) from harvest cell culture fluid (HCCF). In addition to its primary function, protein A chromatography is also frequently tested for its ability to remove viruses. Although protein A specifically binds mAbs and should not interact with viruses, some studies have shown that logarithmic reduction values (LRVs) can vary between 1 and 4 $\log_{10}$ for retroviruses. This variance has been shown to depend on the mAb and feedstock compositions. However, it has not been evaluated so far which feedstock ingredients affect virus removal. To address this knowledge gap, a data set of virus spiking studies was analyzed and revealed a potential correlation between DNA content in HCCF and LRV of protein A chromatography. To evaluate this hypothesis, experiments with murine leukemia virus (MuLV) and retrovirus like particles (RVLP) spiked starting materials of three different mAbs were performed. In general, higher HCCF DNA levels were found to enhance LRV for both virus particles. A trend that was confirmed with RVLP spiked pure mAb. In addition, protein A chromatography washing buffers containing a detergent or a reducing agent were shown to improve LRV in RVLP spiked experiments. This study demonstrates the effect of DNA concentration on the virus removal capabilities of protein A chromatography. Findings that are especially useful when DNA is removed at early stages of the downstream process, for example by using functionalized depth filters or precipitation.

KEYWORDS
chromatography, DNA, endogenous retrovirus, monoclonal antibody, murine leukemia virus, protein a, virus

1 | INTRODUCTION

Protein A chromatography is a commonly used step in the downstream processing of biopharmaceuticals. This purification step leverages the ability of staphylococcal protein A to selectively bind to the Fc domains of antibodies, which is exploited for capture monoclonal antibodies (mAbs) from complex harvested cell culture fluid (HCCF).[1] While protein A chromatography generally yields mAbs with

high purity, residual process-related impurities (PRI) can remain in the eluate. Host cell DNA, chromatin and HCP are known to interact with mAbs leading to inefficient removal.[2,3] Adsorptive/functional depth filters are one way to shield the column from these impurities and reduce DNA and HCP content for intensified downstream processing.[4] Due to their optimized capacity, these filters also have the potential to simplify complex harvesting strategies and are therefore increasingly popular in biopharmaceutical processing.[5,6]

Biopharmaceutical products like mAbs are often produced in Chinese hamster ovary (CHO) cell cultures. Mammalian cell cultures like CHOs are vulnerable to virus infections and bear endogenous retrovirus like particle (RLVP) sequences within their genome. The virus safety of drug substances is therefore a regulatory requirement ensured by three pillars (i) virus testing, (ii) sourcing of virus free materials and (iii) virus clearance capabilities of downstream processes.[7,8] Substantial improvements in understanding virus clearance of downstream unit operations like viral inactivation,[9] removal by ion exchange chromatography[10–12] and virus filtration[13–15] have been made in recent years. Nevertheless, the mechanisms underlying virus removal by protein A chromatography are still not fully understood.[16–18] Due to its high specificity for the Fc part of mAbs and fusion proteins, it is assumed that viruses flow through the column while the protein of interest is retained. However, protein A chromatography shows a wide range of logarithmic reduction values (LRVs) between 1 to 4 $\log_{10}$ for retroviruses and 1 to 3 $\log_{10}$ parvoviruses.[17,18] Although LRV stays consistent for a specific mAb, it can largely vary between different mAbs. Further, it has been shown that virus removal of protein A chromatography is effective in the absence of mAbs. This suggests that nonspecific interactions between viruses and product components may interfere with effective virus removal.[19–21] The composition of the feedstock seems to play a major role as well.[19] Previous studies have shown that protein A chromatography spiking experiments yield lower LRVs when pure mAbs are loaded, relative to runs with HCCF as loading material. This led to the assumption that HCCF components are likely to disrupt the interactions between the mAb and retroviruses.[21]

Although feedstock composition has been described to play a major role in the virus removal capabilities of protein A chromatography, it has not been shown which components are involved in this effect. To address this knowledge gap, a data set of murine leukemia virus (MuLV) spiking studies was analyzed, revealing a correlation between DNA concentration in HCCF and LRV achieved with protein A chromatography. This observation was verified with three different mAbs by running spiking studies with low DNA HCCF generated using an anionically functionalized depth filter. Experiments with MuLV and RVLP showed that the LRV achieved with HCCF can be restored by spiking purified and fragmented CHO DNA to depth filtrate. An extended dataset with RVLP confirmed that addition of DNA to pure mAb also led to improved LRV. In addition, washing buffer additives were tested to address low LRV in protein A chromatography. The detergent Macrogol Lauryl Ether 9 distributed as Virodex™TXR-1 (TXR-1) and the reducing agent dithioerythritol (DTE) were shown to improve RVLP removal. These observations contribute to the mechanistic understanding of virus clearance capabilities of protein A chromatography and could facilitate the design of effective virus removal strategies during the development of affinity chromatography steps.

2 | MATERIALS AND METHODS

2.1 | Virus stocks and quantification

Ultracentrifuged X-MuLV virus stock solutions were provided by Charles River Laboratories, Biologics Testing Solutions, Cologne, Germany at titers of 11.1–11.2 $\log_{10}$ copies mL^{-1} and quantified using RT-qPCR by Charles River Laboratories, Biologics Testing Solutions, Erkrath, Germany. CHO retrovirus-like particle (RVLP) stock solutions were purchased from Cygnus Technologies, Southport, USA and quantified using the RT-qPCR protocol previously described by De Wit et al.[22]

2.2 | DNA purification

For preparation of host cell DNA spiking solution, CHO cells were removed from culture by centrifugation (4500 g/30 min). DNA was subsequently extracted using the Blood & Cell Culture DNA Maxi Kit (QIAGEN GmbH, Hilden, Germany) and fragmented with a Sonoplus HD 2070 ultrasonic homogenizer (BANDELIN electronics, Berlin, Germany), resulting in purified DNA with a concentration of 470 µg/mL.

2.3 | DNA quantification

CHO host cell DNA was extracted from samples using the PrepSEQ residual DNA sample preparation Kit (Applied Biosystems, Carlsbad, CA, USA). DNA quantification was performed on a Light Cycler® 480 II device (Roche) via real time quantitative polymerase chain reaction using a qualified in-house method.

2.4 | Protein a loading material

For this study, three mAbs were produced in genetically engineered CHO expression systems using standard methods. HCCF of mAb1 was obtained from 200 L pilot scale. mAb 2 and mAb 3 were expressed in DASGIP benchtop systems (Eppendorf, Hamburg, Germany) and harvested by centrifugation (4500 g/30 min) and sterile filtered using an 0.8/0.2 µm Sartopore 2 XLG filter (210 cm^2). Endogenous RVLP concentrations in HCCF ranged from 7.5 to 8.2 log10 RVLP/mL as determined by RT-qPCR.

HCCF with a low DNA concentration was generated by filtering HCCF using an Emphaze AEX functional depth filter (25 cm^2) equilibrated with 54 L m^{-2} phosphate buffered saline (PBS) at a flow rate

of 230 L m^{-2} h^{-1}. Pure mAb was generated by protein A chromatography using a MabSelect Prism A chromatography resin (Cytiva, Malborough, USA), titration of the eluate to pH 6.0 using buffer containing 1 M Tris, filtration using a Zeta Plus BC25S90ZB depth filter (25 cm^2) and dilution to the mAb titer of the HCCF. For control experiments, mAb-depleted HCCF was generated by collecting protein A flowthrough. A spiking mAb was prepared by concentrating pure mAb to the 10× concentration of the respective titer in the original harvest using Vivaspin15R (Sartorius, Goettingen, Germany) centrifugal concentrators with a 30 kDa molecular weight cut off. Subsequently, the concentrated mAb was spiked into the mAb-depleted HCCF to restore the original titer, thereby resulting in artificially generated HCCF.

For some experiments it was necessary to restore DNA content equivalent to HCCF. To do so the previously purified CHO host cell DNA was spiked to ~10^8 pg/mL into DNA depleted HCCF or pure mAb, respectively. All protein A starting materials were either spiked with 2% (v/v) of MuLV or 1% (v/v) of RVLP virus stock.

2.5 | Protein a chromatography

Protein A chromatography experiments were performed using a ÄKTA Pure 25 system equipped with an F9-C fraction collector and MabSelect Prism A chromatography resin (Cytiva, Malborough, USA). The chromatography system was operated with Unicorn 7.10 (Cytiva, Marlborough, USA). Each run was performed with a resin load of 30 g/L of the respective mAb or comparable volumetric load for runs with loads not containing mAb. Protein A chromatography was performed with a multi component buffer system (MCBS) for Equilibration (pH 7.1), Wash 2 (pH 5.9), and Elution (pH 3.4), and Wash 1 consists of a harsh washing buffer containing 2 M Urea, 1 M sodium chloride, and 5 mM EDTA (pH 7.0). All buffers and their exact preparation have been previously described by Trapp et al.[23] For wash buffer optimization, Wash 1 buffer was either prepared with additional 0.9 mM Cystein, 0.2% Virodex™ TXR-1, or 0.5 mM DTE. All buffers were prepared with purified water and filtered with a 0.45/0.22 µm Sartopore 2 (Sartorius, Göttingen, Germany) sterile filter.

3 | RESULTS

3.1 | Database evaluation

To identify potential impurities that could impact retrovirus removal during protein A chromatography, we investigated a data set of 47 projects run under similar conditions. As shown in Figure 1a, the data set indicated a potential correlation between the retrovirus MuLV and DNA concentration ($R^2 = 0.486$). Interestingly, lower DNA concentration in the protein A starting material seemed to be connected to lower LRVs. In contrast, when DNA concentration dropped toward <10^2 pg/mL, LRV below 1 was observed, which is considered insignificant for virus removal.[24] Our data did not show correlations between HCP concentration and LRV (Figure 1b). Further, evaluation of MVM, which represents a worst-case model for small, non-enveloped and physiochemically robust viruses, did not indicate an effect of DNA concentration on LRV under the investigated conditions (data not shown).

3.2 | Impact of DNA on MuLV removal

To verify the impact of DNA concentration on virus removal, spiking experiments with 2% (v/v) MuLV were conducted. Initial experiments were performed with HCCF of three different mAbs. To generate

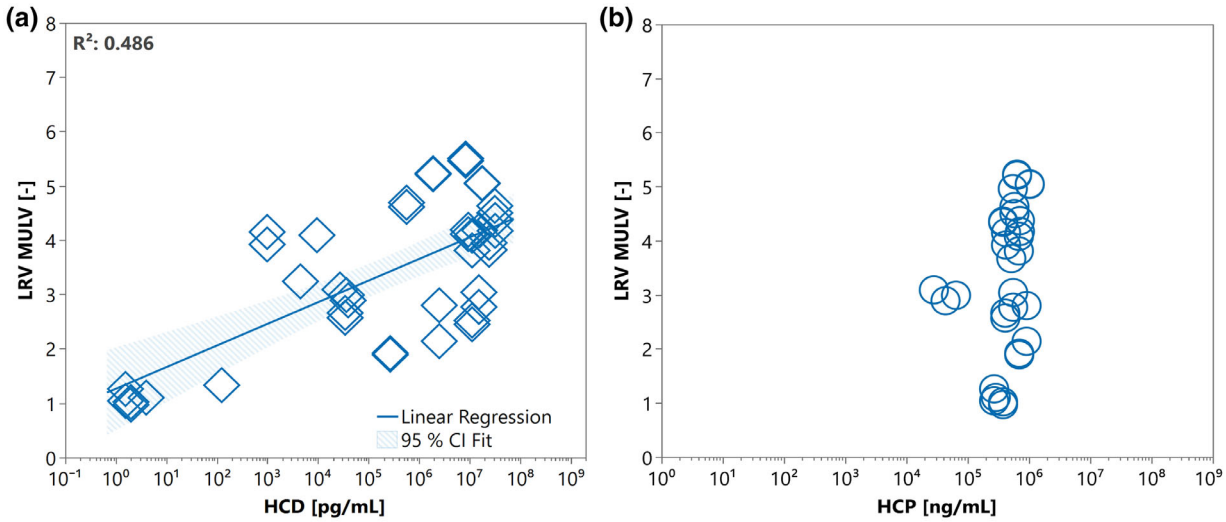


FIGURE 1 MuLV LRV of protein A chromatography virus clearance studies plotted against DNA concentration (a) or HCP concentration (b) in HCCF ($n = 47$). Solid line represents the linear regression. Shaded area represents the 95% Confidence Interval (ci) of Fit.

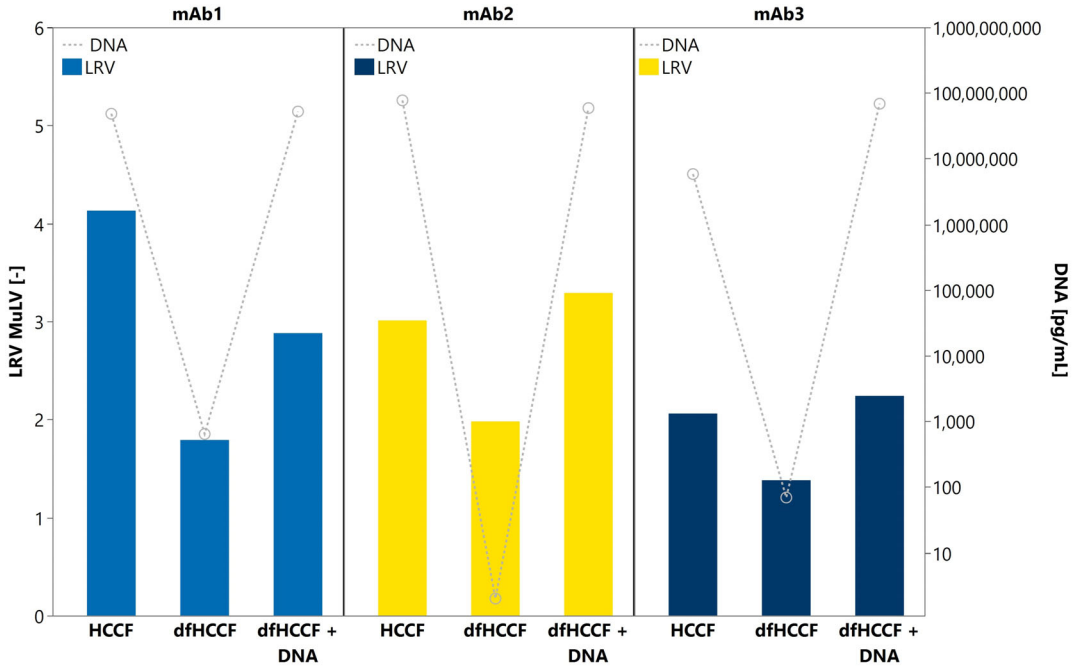


FIGURE 2 MuLV removal by protein A chromatography and corresponding DNA concentration of mAb1, mAb2, and mAb3 with harvest cell culture fluid (HCCF), HCCF filtered using an anionic functionalized depth filter (dfHCCF), and dfHCCF spiked with purified and fragmented CHO DNA (dfHCCF + DNA).

starting material with a low DNA content, HCCF was filtered with an anionic functionalized depth filter. The resulting starting material had a DNA concentration of 2–5000 pg/mL and showed lower LRVs compared to the untreated HCCF for all mAbs (Figure 2). To confirm that the removal of DNA led to the reduced LRV, purified and fragmented CHO DNA was spiked to restore the DNA concentration to $\sim 10^8$ pg/mL. This led to a remarkable improvement of MuLV removal and LRVs comparable to the initial HCCF for all mAbs.

3.3 | Impact of DNA on RVLP removal

For further investigation, an RVLP RT-qPCR assay was used to confirm whether the MuLV results were reproducible with RVLPs as spiking material.

For mAb1, the RVLP removal of HCCF resulted in a LRV of 2.8. When depth filtered HCCF was used, the LRV decreased to 1.5, but re-spiking DNA restored the LRV to 2.4 (Figure 3); this trend closely mirrors the observation from the MuLV experiments. In the case of mAb 2 and mAb 3, the differences in LRV between HCCF and depth filtered depleted HCCF showed comparable LRV. However, re-spiking DNA to depth filtered HCCF led to an improved LRV for mAb2 and a positive trend for mAb3 as well, indicating a positive effect of DNA concentration on RVLP clearance.

To better understand the impact of different protein A loading materials on RVLP removal, further spiking experiments were performed. The tested loading materials included cell culture medium (CCM) and mAb-depleted HCCF, as mAb-free matrices, pure mAb and pure mAb spiked with DNA, to evaluate the impact of DNA on RVLP removal in matrices with low impurity content, and an artificial HCCF consisting of mAb-depleted HCCF spiked with purified mAb, to examine whether short time exposure of mAb and RVLP leads to different LRVs than long-time exposure of both molecules during cultivation and harvesting.

As shown in Figure 4, the mAb free experiments based on CCM and mAb depleted HCCF showed higher RVLP removal capabilities (LRV >3.5) than HCCF. Pure mAbs showed contributable to moderate removal (LRV 1.5–2.6), which varied across the different mAbs. Adjusting DNA concentrations of pure mAbs to $\sim 10^8$ pg/mL (comparable to HCCF) improved the LRVs of mAb1, mAb2 and mAb3 toward moderate to effective removal (LRV 3.3–4.4), thereby confirming the positive effect of DNA on LRV observed for depth filtered HCCF. The artificially produced HCCF showed higher LRV (3.1–3.6) than the corresponding HCCFs. This effect was especially prominent for mAb3 where LRV was >2 $\log_{10}$ higher.

3.4 | Wash buffer evaluation

In addition to the examination of the impact of DNA concentration on LRV, we aimed on designing appropriate washing buffers which can increase the LRV of protein A chromatography. These experiments were performed in duplets with a 1% RVLP spike. mAb3 HCCF was chosen for the buffer testing since it showed the lowest LRV. To test whether reducing wash buffer additives have a positive effect on LRV, 0.2% TXR-1 was added to the high salt and urea containing wash

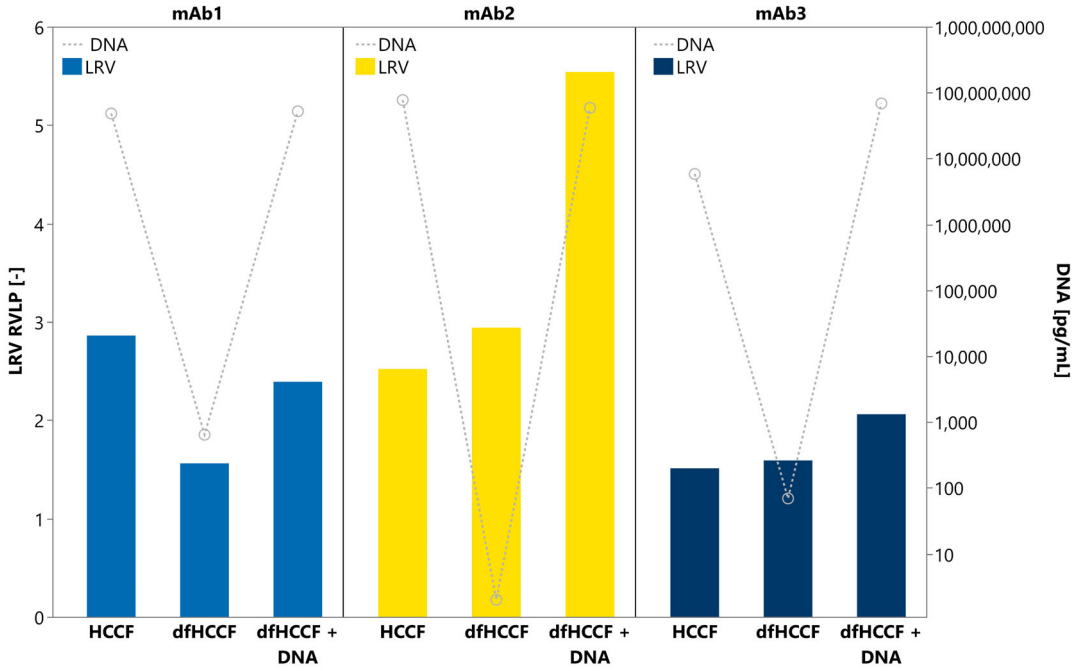
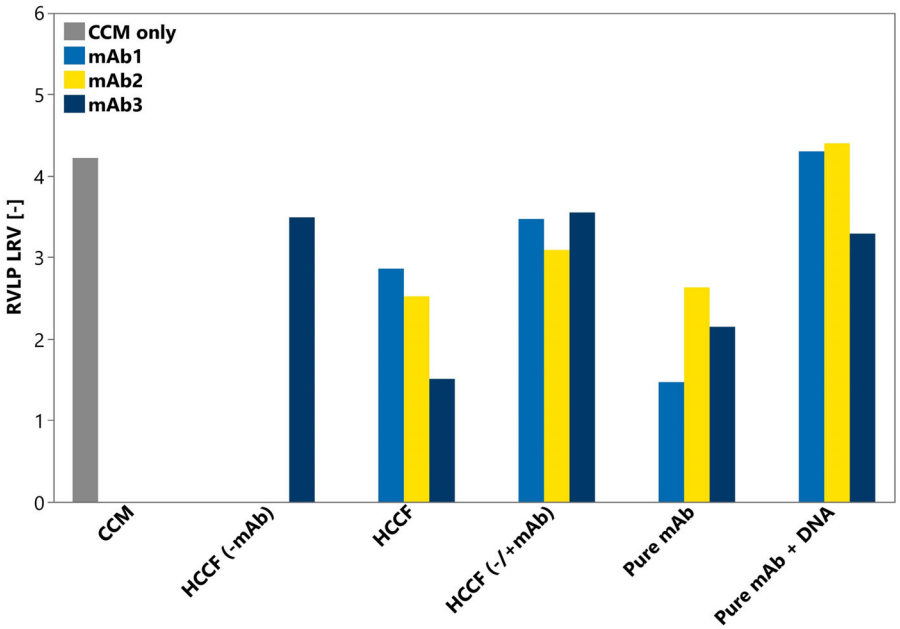


FIGURE 3 RVLP removal by protein A chromatography and corresponding DNA concentration of mAb1, mAb2, and mAb3 under different conditions. LRVs were determined for harvest cell culture fluid (HCCF), HCCF filtered using an anionic functionalized depth filter (dfHCCF), and dfHCCF spiked with purified and fragmented CHO DNA (dfHCCF + DNA).

FIGURE 4 RVLP removal by protein A chromatography in the presence and absence of mAb 1, mAb 2 and mAb 3. Tested matrices include Cell Culture Medium (CCM), mAb-depleted HCCF (HCCF (−mAb)), Harvest Cell Culture Fluid (HCCF), artificial harvest (HCCF (−/+ mAb)), pure mAb (mAb), and pure mAb spiked with DNA (mAb + DNA), consisting of mAb-depleted harvest spiked with concentrated pure mAb.



buffer. To address potential disulfide bonds either a cysteine or 0.5 mM DTE was added. As shown in Figure 5, the standard wash buffer resulted in an LRV of 1.23 ± 0.28. The addition of cysteine yielded an LRV of 1.77 ± 0.29. However, this increase is within the range of assay variability and therefore may not represent an improvement. In contrast, addition of the detergent TXR led to a substantial increase in LRV to 4.60 ± 0.15. Similarly, the addition of the reducing agent DTE markedly enhanced the LRV to 3.55 ± 0.01.

4 | DISCUSSION

4.1 | Correlation between DNA and virus removal

The present study was conducted to gain a better understanding of the impact of PRI on the removal of retroviruses. Based on data set and spiking experiments, DNA concentration was identified to have an impact on the virus removal capabilities of protein A

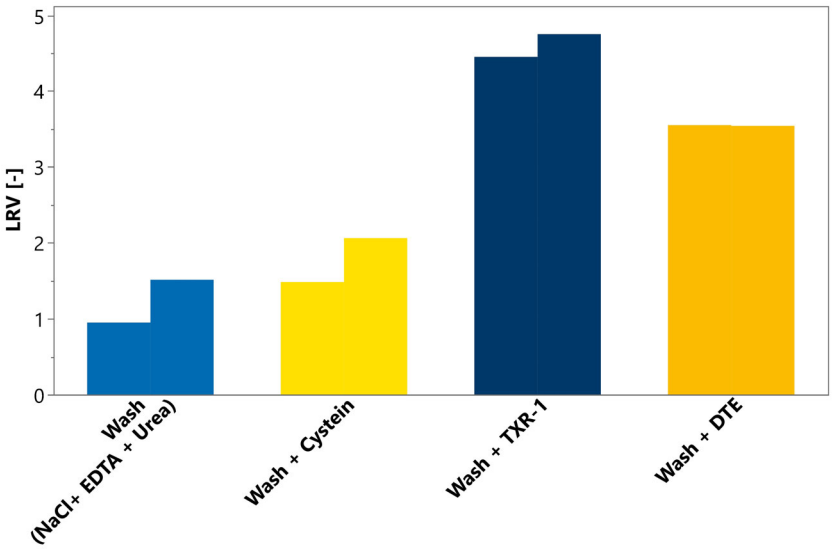


FIGURE 5 Evaluation of protein A wash buffer additives using RVLP spiked mAb3 HCCF ($n = 2$). Standard washing buffer containing urea, sodium chloride and EDTA was tested to benchmark. 0.9 mM cysteine was added to the standard washing buffer to generate a mildly reducing environment. 0.5 mM DTE was added as a strongly reducing agent. Additionally, 0.2% of the detergent Virodex™TXR-1 was tested.

chromatography. We showed that LRVs were lower when DNA concentration was reduced and restored again when fragmented CHO DNA was spiked. Previous studies indicated that the removal of MuLV or RVLP is highly dependent on feedstock, with variations in type and composition of feedstock having a higher impact than typically tested process parameters like load density, flow rate, or temperature.[19,21] A study digging deeper into the impact of impurities by Pan et al. observed that pure mAb shows lower MuLV removal than mAb containing HCCF. An observation that was suspected to be caused by mAb-virus interactions that may be bridged by HCP.[20] However, our data did not show a major role of HCP concentration. Although we cannot exclude the effects of specific HCP since HCP ELISAs capture a broad and heterogenous range of HCP rather than concentrations of specific proteins. Further, potential HCP-mediated effects may be masked at higher DNA levels, where DNA-mediated interactions dominate.

To generate HCCF with low DNA concentrations, the material was pretreated using anionic functionalized depth filters. In addition to DNA removal, such filters are known to reduce host cell proteins (HCP)[4] and may also affect nucleic acid–associated complexes, cell debris, and membrane fragments, which could contribute to changes in virus clearance behavior.

Although the positive impact of DNA was confirmed by spiking experiments using purified and fragmented DNA, a contribution from additional co-removed impurities cannot be excluded and may also influence virus clearance.

To reveal the exact mechanisms of DNA-mediated enhancement of viral clearance by Protein A chromatography, further experiments on molecular interactions are required which were beyond the scope of this manuscript. However, our results allow for the formulation of several hypotheses. Based on the DNA LRV correlation, the first assumption to be made is that there might be electrostatic interactions between DNA and mAbs. Such interactions could shield the mAb's surface and thereby reduce interactions with MuLV or RVLP. However, protein A chromatography is usually run with at least one washing buffer containing high salt concentrations, which addresses electrostatic interactions.[2] Taking this into consideration, the presence of DNA might disrupt other mechanisms of co-purification, like low affinity interactions between retroviruses and mAbs (such interactions have been reported for lipases[25]), or formation of inter-molecule disulfide bonds between mAb and virus.

4.2 | Impact of different mAbs

The general trends after addition and removal of DNA were similar. However, the LRV values varied between the three mAbs used. A highly product dependent variance of retrovirus clearance between 1 and 3 $\log_{10}$ has been shown previously in literature and database evaluations.[16,17] This co-elution of retrovirus is most probably caused by interactions between mAb and retrovirus and has been shown to be reproducible for the individual mAbs.[19] This is in line with previous studies, which showed the importance of product-specific virus–mAb interactions as a main driver of limitations in virus removal using protein A chromatography. Bach and Connell-Crowley (2015) and Pan et al. (2019) reported that retroviruses can bind to mAbs and co-elute with the product, resulting in reduced LRVs.[20,21]

4.3 | Differences between RVLP and MuLV

We compared protein A chromatography runs of MuLV and RVLP-spiked runs. Most of the results, including those from depth-filtered and DNA-spiked starting material, showed comparable LRV values. However, the results of RVLP runs with HCCF showed lower virus clearance compared to MuLV runs. The concentration of intrinsic RVLP co-expressed with the mAbs was within 7.5–8.2 log10 RVLP/mL, which approximately corresponds with the target spiking concentration of 8 log10 RVLP/mL. Interestingly, when an artificial HCCF was generated by combining mAb-depleted HCCF, pure mAb and

spiked RVLPs, the resulting LRVs were comparable or even higher than those of the MuLV spiked HCCF experiments. Thereby suggesting that RVLPs co-expressed with mAbs might behave differently than RVLP spiked to the starting material under certain conditions.

These differences may be attributed to the interaction history of the particles. RVLPs co-expressed with mAbs are exposed to the HCCF environment over extended periods; during this period, interactions with mAbs, DNA, or other impurities might form and stabilize. Such long-term incubation could promote co-elution behavior during protein A chromatography. In contrast, RVLP spiked into the starting material are only exposed to the matrix for a limited time, potentially resulting in fewer interactions.

This exposure history might also be connected to the lower RVLP LRVs in HCCF compared to the MuLV data since spiked MuLV also has a limited exposure time.

4.4 | Effectiveness of wash buffer ingredients

After increasing the understanding of virus clearance and protein A chromatography, we aimed to examine strategies to improve retrovirus removal by testing two hypotheses. First, we hypothesized that affinities between the lipid bilayer of retroviruses or membrane proteins might lead to co-elution. We assumed that disintegration of the retroviral membrane could increase virus removal. To disintegrate the virus, the environmentally friendly detergent TXR was added to a high salt, urea and EDTA containing Wash 1 buffer. This led to reproducible effective removal of RVLP, with an LRV of 4.60 ± 0.15 compared to 1.23 ± 0.28 with Wash 1 buffer only. This is in line with previous findings that focused on the on-column inactivation of MuLV using detergents in washing buffer.[26–28] Although incorporating detergents into protein A washing buffers has advantages from an operational point of view, it comes with the drawback that it is hard to discriminate between physical virus removal and chemical inactivation since both are based on disintegration of the viral membrane. Therefore, the cumulative LRV achieved by protein A chromatography and a separate detergent treatment might be higher than that of a wash step containing detergent. An issue that can be addressed by clever experimental designs of the viral clearance study.[29]

Second, we added Cysteine or DTE as reducing agent to washing buffers. While the cysteine buffer only led to a slightly improved LRV of 1.77 ± 0.29, no residual RVLP was detected in the protein A eluate after washing with DTE. The lower performance of the Cysteine buffer might be attributed to unfavorable pH and reaction time, both of which can have a significant impact on the functionality of Cysteine buffers.[30] The DTE showed good performance and increased the LRV to 3.55 ± 0.01. However, introducing reducing agents into a washing buffer of course also comes with the risk of reducing the product itself (e.g., fragmentation of heavy and light chains for mAbs or general disulfide bond shuffling), which might compromise their use in manufacturing processes. In addition, the potential impact of these additives on product quality as well as their clearance downstream of affinity chromatography should be considered when adding the proposed additives. Although being rare inter-protein disulfide bonds between mAb and retroviral surface proteins are a potential explanation for the positive effect of the Cysteine and DTE in the washing buffer. Retroviruses such as MuLV have envelope proteins (env) consisting of a surface (SU) and transmembrane (TM) subunit, which are linked by a labile disulfide bond. The isomerization of the SU-TM subunit disulfide bond leaves the TM cysteine as free thiol, which is crucial for rearrangement and finally cell entry.[31] Interestingly, isomerization or reduction of the disulfide bridge has been shown to also occur in presence of reducing agents, physical stress or urea.[32] Further, the interchain disulfide bonds of the mAb are known to be solvent exposed.[33,34] Considering this and the reducing environment in freshly harvested material caused by reducing enzymes from the host cell cytosol,[35,36] scrambled disulfide bridges between mAb and virus could occur during reformation of disulfide bonds. However, it cannot be excluded that reducing environments lead to destabilization or partial unfolding of mAbs, thereby leading to conformations that are not favorable for mAb interactions and vice versa improve the LRV.

It should be noted that the interpretation of washing buffer effects is based on overall LRV values without fraction-resolved virus quantification. While such data would provide additional mechanistic insight, this was beyond the scope of this work. Furthermore, experiments under native conditions without urea could help to differentiate between potential disulfide-mediated interactions and denaturation effects but were also beyond the scope of this work.

5 | CONCLUSION

In our present study, we showed that DNA content in HCCF can impact the removal of retroviruses. We showed that low DNA concentration in the starting material correlates with lower LRV. This finding is especially relevant when it is intended to use filters, precipitation, or other methodologies that are known to reduce DNA before protein A chromatography. We also showed that this correlation can be seen with MuLV and RVLP. Although it was out of the scope of this study to reveal the mode of action of the correlation between DNA content and retrovirus removal, our results might lead to a better understanding of virus removal by protein A in general and support virus clearance design of downstream processes.

AUTHOR CONTRIBUTIONS

Lukas Döring: Conceptualization; methodology; investigation; formal analysis; writing – original draft; visualization; data curation. **Sven Schubert:** Conceptualization; methodology; writing – review and editing; project administration; validation. **Nora Roos:** Project administration; writing – review and editing; methodology; conceptualization. **Johannes Winderl:** Supervision; writing – review and editing. **Annika Müller:** Investigation; formal analysis; writing – review and editing. **Anna Ratermann:** Investigation; data curation. **Matthias W. Kron:** Conceptualization; supervision; writing – review and editing. **Jürgen Hubbuch:** Conceptualization; writing – review and editing; supervision.

ACKNOWLEDGMENTS

The authors would like to thank Thilo Grob, Head of Process and Analytical Science at Rentschler Biopharma for making this study possible, Simon Baukmann, Sarah Thalhofer, Verena Kössler, and the whole Process and Analytical Science for their support and the always constructive discussions.

FUNDING INFORMATION

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

PEER REVIEW

For transparency, the peer review documents associated with this article are available at https://doi.org/10.1002/btpr.88547.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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How to cite this article: Döring L, Schubert S, Roos N, et al. DNA concentration in harvest affects retrovirus clearance capabilities of protein A chromatography. *Biotechnol. Prog*. 2026;e88547. doi:10.1002/btpr.88547