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Understanding virus retention mechanisms on protein a chromatography based on using different wash buffers – Evaluating the possibility for a generic wash buffer toolbox to improve virus clearance capacity

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A B S T R A C T

During manufacturing of mammalian-cell derived monoclonal antibodies (mAbs) virus clearance capacity of the downstream process has to be demonstrated. The protein A chromatography step typically achieves less than 4 log_{10} and is not considered as a major contributing step. Having been successfully applied to host cell protein removal before, we used different wash buffers for three mAbs with two model viruses (Minute virus of mice and Murine leukemia virus) in series as well as separately to further understand major contributing interactions for virus retention and potentially design a generic toolbox of stringent wash buffers to be applied to various mAbs. Results indicate a major relevance of hydrophobic interaction for Murine leukemia virus (xMuLV) and mAb A, based on improved clearance for buffers additionally containing increased levels of hydrophobic compounds. This effect was less pronounced for Minute virus of mice (MVM), whereby hydrogen-bonds were expected to play a stronger role for this model virus. Additionally, electrostatic interactions presumably are more relevant for MVM retention compared to xMuLV under the conditions evaluated. A generic mAb and virus-independent stringent wash buffer toolbox could not be identified. However, based on our results a customized mAb and virus wash buffer design with improved virus clearance is possible, with here demonstrated log reduction increase by 1.3 log_{10} for MVM and 2.2 log_{10} for xMuLV for the protein A step compared to equilibration buffer alone.

1. Introduction

Downstream processing of biotherapeutic proteins typically consists of several chromatography steps, a final UFDF step as well as steps specifically incorporated to ensure virus safety.

Aim of downstream processing thereby is to deliver drug substance at high purity and high yield, while maintaining low manufacturing costs, ensuring robust removal of process and product-related impurities, also ensuring clearance of potentially endogenous and adventitious virus present during manufacturing.[1,2]

This is demonstrated in virus clearance studies, typically performed at CROs offsite, whereby the actual manufacturing process is performed in small-scale models. Model viruses, e.g. the retrovirus xMuLV or the parvovirus MVM are used during these studies to evaluate removal of retrovirus-like particle contamination as well as contamination by other viruses in the mammalian-cell derived production.[3–5] During these virus clearance studies, typically ion exchange or mixed-mode chromatography steps as well as inactivation and virus-filtration are evaluated as these are known to achieve high virus clearance capacity.[6,7] Affinity chromatography, such as protein A, despite being heavily used for capture of Fc-containing biotherapeutics,[8–10] is not regularly evaluated as it is rather achieving low virus removal capacity of 1–3 logs for MVM and slightly higher clearance of 1–4 logs for xMuLV[6,7,11,12] due to a combination of removal and inactivation during a protein A process.[7]

Despite the rather low clearance levels, protein A ensures significant removal of other impurities, e.g. host cell proteins, especially when using salt, detergent or solvent containing washes.[9] Thus, one approach to understand and improve virus clearance on the protein A step, thereby potentially assessing that step in future virus clearance studies, may be to evaluate buffer compounds for virus clearance based on their effect for host cell protein clearance. This follows the assumption that similar interactions are involved for host cell protein-mAb interactions as well as virus-mAb interactions, such as electrostatic, hydrogen bonding or hydrophobic interactions.[13,14] This assumption is also corroborated by results by Pan et al., showing similarities between virus co-elution and HCP co-purification.[12,14]

Based on previous publications, suitable buffer compounds for virus clearance may thus include e.g. arginine, urea as well as octanoate. The latter has been used for HCP removal and also for virus inactivation, however, not been tested for direct virus removal in a wash buffer.[15] Mpandi et al. evaluated octanoate for inactivation of viruses during a

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Abbreviations

CV	Column volume
HCP	Host Cell Proteins
WFI	Water for Injection
MVM	Minute virus of mice
xMuLV	Murine leukemia virus
UFDF	Ultra-/Diafiltration
CRO	Contract Research Organization
Logs	Logarithmic units
IPA	Isopropyl alcohol
LDAO	Lauryldimethylamine oxide
RVLPs	Retroviruslike particles
LRV	Leishmaniavirus

precipitation process, yet at slightly acidic pH conditions.[16] Arginine, NaCl as well as urea and propylene glycol have all been shown to facilitate HCP removal on the protein A step and have been investigated by various groups and companies.[17–19] This highlights these substances and similar compounds as potentially suitable for virus clearance as well.

Consequently, several groups have evaluated some of the HCP-removing compounds mentioned before to understand and improve virus clearance on the protein A step.[12,13,20] Studies also aimed at understanding the mechanism of virus retention depending on protein A backbone matrices.[12] Virus is not expected to interact strongly with the protein A resin, also based on results showing high virus recovery in wash and flow-through fractions.[21] Obtained log reduction for certain viruses was shown to be rather insensitive to process parameter changes and largely dependent on the mAb itself,[22] yet were shown to be also dependent on the type of resin.[12]

Yet, wash buffer pH has been shown to impact log reduction achieved, also use of 1 M NaCl as wash improved clearance significantly.[6,23] Furthermore, arginine has been known to interact with enveloped viruses, thereby inactivating them, both at low-pH and pH-neutral conditions as evaluated by several groups.[24–26]

Consequently, use of disruptive agents for on-column virus clearance has been evaluated, showing improvement of clearance to 3.1$\log_{10}$ by use of 1 M arginine by.[13] Likewise, Bolton et al., 2015 evaluated an arginine containing buffer at pH 4.7, with potentially pH-related contribution to clearance as well. Bach and Connell-Crowley further proposed electrostatic interaction being one mechanism of virus retention as well as co-elution on the protein A step, based on pH-modulating experiments.

They evaluated different wash buffers which would affect different virus-protein interactions and thereby understand the main interaction parameters during the protein A step responsible for virus retention and clearance.

They used 3 M urea, 10% IPA, also known to improve HCP clearance,[19] to impact hydrophobic or hydrogen-bonding interactions potentially present. A 0.1% Triton X-100 buffer was used to impact hydrophobic interactions, presumably by disrupting the lipid envelope of the xMuLV. Bach and Connell-Crowley identified improved clearance with these washes compared to a standard reference wash. Yet, clearance observed was lower than the one achieved with a control run where only virus was loaded, indicating additional interaction between virus and mAb and thereby corroborating results by Zhang et al., indicating virus interacting with mAb or impurities rather than with the resin. Bolton et al. achieved >5.5 log of clearance determined by PCR, employing an arginine containing wash buffer at low pH of 4.7 though with potential inactivation due to the pH, thereby disrupting electrostatic interactions and achieving complete removal of xMuLV.[20] The same group achieved a >5 log clearance of xMuLV by applying N, N-dimethyldodecylamine N-oxide (LDAO), a detergent, to a wash buffer, thereby presumably disrupting hydrophobic interactions.[20]

As these chemically different wash buffers disrupted different types of interactions and presumably virus-retention mechanisms, a combination of such wash buffers may even further improve virus clearance on the protein A step.

This hypothesis is corroborated by Bach and Connell-Crowley who concluded a multiple type of interactions between virus and mAb being present, with the potential of more stringent washes or a combination of stringent washes to improve clearance further.[13]

Pan et al. employed high-throughput screening to evaluate the impact of different wash buffer components on virus clearance by disrupting various interactions on a polymeric-based protein A matrix and agarose-based matrix.

They screened different excipients including salt, NaB as hydrophobic salt, a chaotropic agent, sorbitol aa protein stabilizing agent, as well as polysorbate 20 as detergent and their impact on removal of RVLPs. Among the substances screened, NaBenzoate achieved the highest log reduction, presumably due to its ability to interfere with hydrophobic and ionic interactions. Addition of polysorbate or sorbitol did not improve clearance.

Importantly, results discussed by several groups have shown a strong dependency of achieved log reduction based on mAb and feedstock, with rather low impact due to adaptations of process parameters,[12,21] thereby not necessarily allowing for generic filing of virus clearance study claims across different mAb products.

Thus, one option may be to use a toolbox of combination of several stringent wash buffers, also considering buffers known for improved HCP clearance, thereby disturbing various mAb-virus interaction as well as virus-resin interaction parameters at the same time. Such a combinatorial stringent wash toolbox may facilitate separation of virus from the mAb, irrespective of the type of mAb, feedstock and type of resin, also paving the way for generic filings in expectation of consistently high virus clearance across different mAb molecules.

Our aim was to evaluate the possibility for a combinatorial stringent wash toolbox, evaluating a series of different wash buffers applied to three different mAb-feedstock systems, with two model viruses, respectively on the protein A step. The selected mAbs were not specific to the viruses but were rather chosen due to company-internal projects. Additionally, to further understand the impact of different wash buffer components, we also evaluated different wash buffers separately with two mAb-virus combinations. These studies were performed in a research environment to gain further understanding of virus clearance solely.

2. materials and methods

2.1. Virus clearance studies with MVM and xMuLV

Combinatorial stringent wash toolbox - combination of stringent wash applied in series to three mAb-feedstock systems.

Three different mAbs in their respective feedstocks, provided by research colleagues were evaluated with a series of stringent wash buffers. For that purpose, each mAb in feedstock (clarified cell culture fluid of Chinese hamster ovary cells) was thawed prior to the study, filtered and then spiked with a certain amount of virus MVM or xMuLV, then being loaded onto the protein A resin (MabSelect SuRe, GE Healthcare, Uppsala, Sweden). The loaded protein A column was then exposed to a wash sequence with several different stringent wash buffers consecutively in series (20 mM Tris, 1 M NaCl, 75 mM Octanoate, 7.5% Isopropanol, pH 9.0; 20 mM Tris, 1 M NaCl, 10% propylene glycol, 75 mM Octanoate, pH 9.0; 500 mM Urea, 50 mM citrate, pH 9.0; 500 mM arginine, 10% propylene glycol, pH 9.0), followed by PBS wash and a mAb-specific reference wash. The use of different wash buffers in series aimed thereby at evaluating the overall potential for virus clearance improvement across different mAb-feedstock combinations together

with two model viruses, by using a set of wash buffers which would interfere with various interactions between virus and resin as well as mAb and feedstock impurities. The reference wash applied at the end of the sequence was also used for the respective mAb in previous studies to allow comparison of stringent wash results vs. a reference wash. Reference wash in previous studies was performed as duplicate experiment. Applying a PBS wash just before the reference wash at the end of the sequence ensured no carry-over of residual wash buffer components into the eluate fraction which would otherwise interfere with virus titer readouts.[13]

Besides wash buffer usage, process operating parameters were the ones also used in previous virus clearance studies. Additionally, with two mAbs, the impact of a prolonged wash exposure due to increased column volumes of wash applying the series of stringent washes consecutively vs. standard column volumes (CV) of reference wash has been evaluated. For that purpose, also the standard wash applied in the two projects has been prolonged to 18 CV to equal the CV used for evaluating the stringent wash buffers consecutively in series. Each chromatography run was performed in a single experiment when spiking either 1%, 2.5% or 5% MVM respectively, depending on the mAb. Same spike ratio was applied for the experiments using xMuLV as model virus.

The virus clearance study was split into two slots with some months for data analysis in between.

For the first slot, a combination of wash buffers, as shown in Fig. 1, was used with all buffers depicted in Fig. 1., used in series for a wash, i.e. in total up to 6 wash buffers in one single experiment, which would result in 18 CV in total. As reference, 18 CV standard wash was applied to exclude any impact on prolonged wash. This was done for 3 mAbs, with the 18 CV standard wash omitted for mAb C, as the impact of a prolonged wash of 18 CV would already be visible for mAb A and mAb B.

Thus, in total, for the three mAbs, eight experiments were performed, evaluating each mAb with two viruses and a series of stringent washes and in addition two of the mAbs with a prolonged wash based on the standard reference wash (see Fig. 1 as overview).

After data analysis, it was confirmed that the application of 6 wash buffers in series indeed had a positive impact on virus clearance. (Objective of that first slot was to identify if applying multiple wash buffers in series has an impact and whether to continue the study in more detail or not, due to relatively high costs associated with such a study, limited external resources (study performed externally with internal personnel travelling to the CRO) and thus limited number of experiments which can be performed within a given budget). Additionally, application of these wash buffers had no impact on yield, based on chromatogram overlays provided in Supplementary Fig. 1.

Thus, based on the positive outcome of the first slot, a 2nd slot was booked with a more detailed focus on wash buffers, also including slight variations into the buffer based on additional knowledge.

This 2nd slot is depicted in Fig. 2 and Fig. 3, which show a clearance per wash buffer to identify the buffers, most promising for virus clearance.

Virus clearance was calculated based on virus titer determination in load and eluate fractions, respectively. For MVM, titer was determined by large-volume plating and infectivity assays while for xMuLV PCR was applied to understand the impact on clearance and not inactivation.

2.2. *Detailed evaluation of several wash buffers*

For more detailed understanding on interacting forces, responsible for virus retention, a set of chemically-different wash buffers, with varying ability to interfere with those interactions, was designed and studied further, which are displayed in Table 1 (level of details on composition is restricted due to internal compliance regulations).

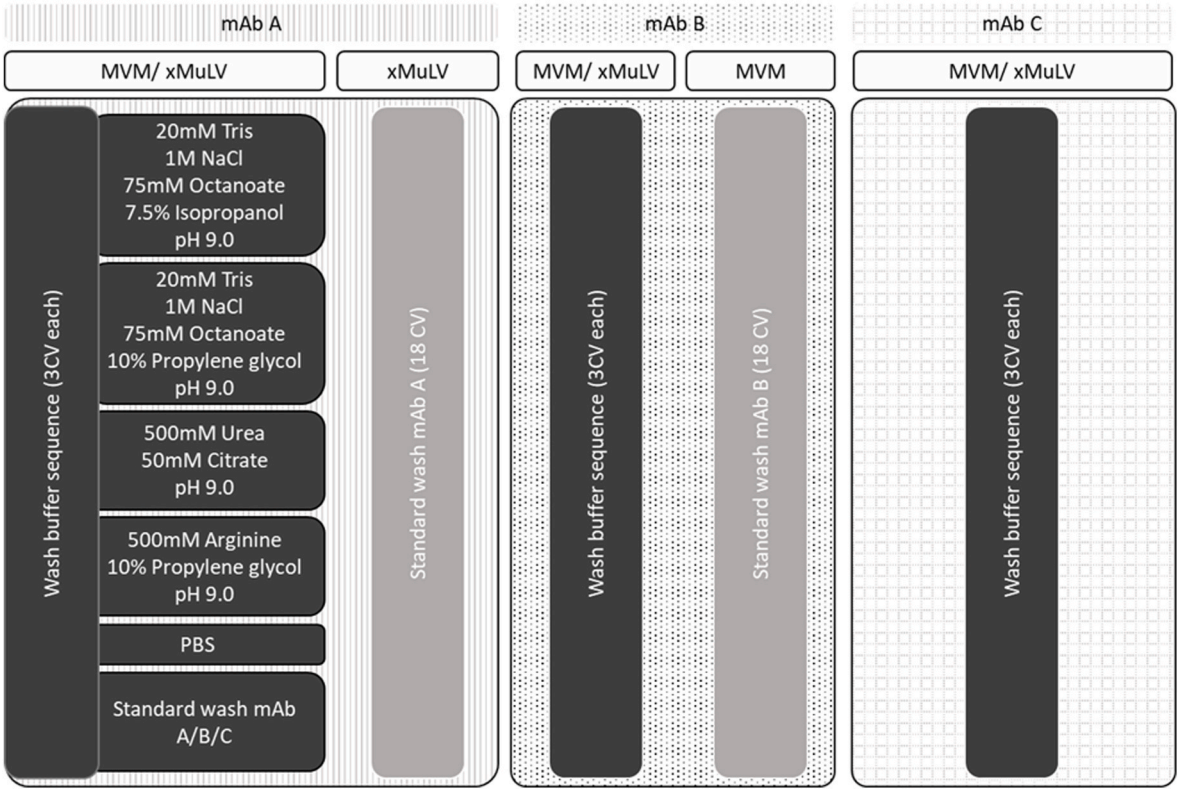

Fig. 1. Schematic overview of wash buffers evaluated for the three mAbs during first slot of virus clearance studies. The wash buffer sequence consisted of the six different buffers listed for mAb A. 3CV each were applied, while the respective wash buffer for each mAb was used as last buffer. This sequence was applied for both viruses and each mAb. As control, 18CV of the respective standard wash buffer was applied for xMuLV and mAb A as well as for MVM and mAb B. For budget reasons, this procedure was not carried out for mAb C.

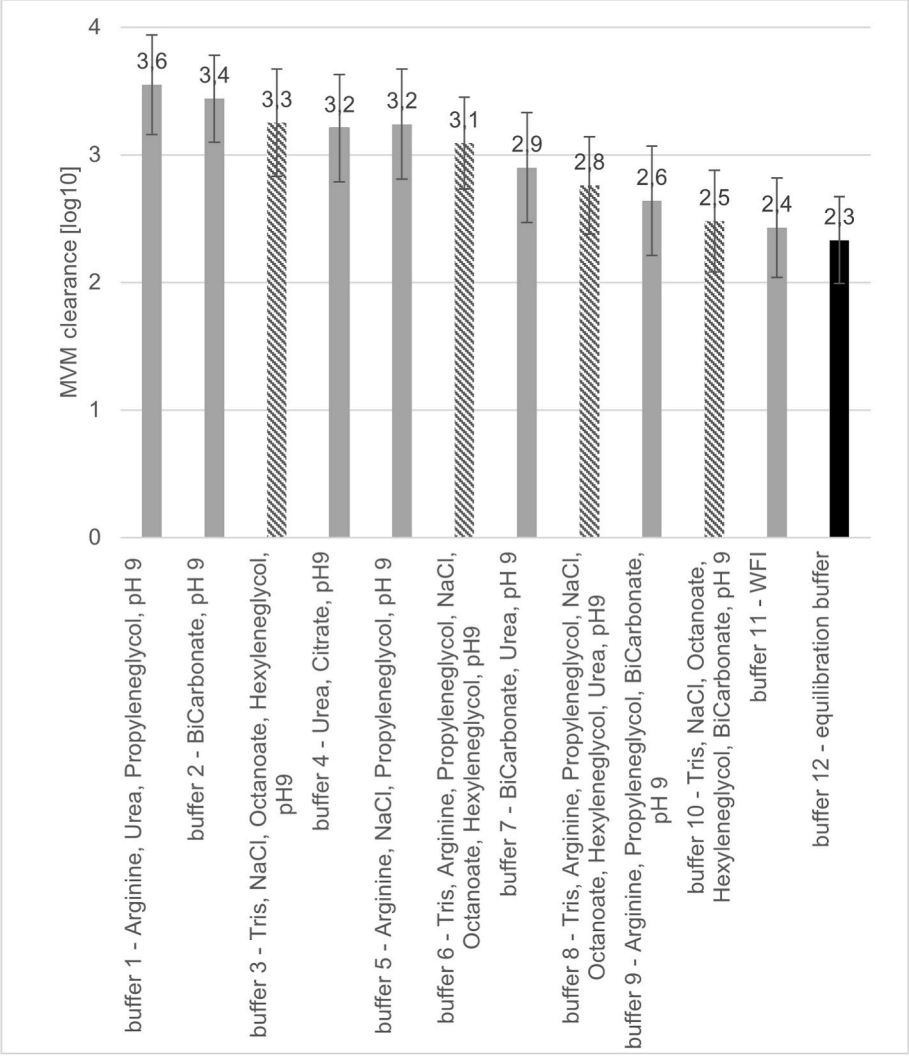

Fig. 2. MVM clearance on the protein A step in $\log_{10}$ with mAb B, depending on different wash buffers. The error bars represent a 95% confidence interval as provided by the CRO, based on a ± two-fold standard deviation obtained from the employed infectivity assay as performed by the CRO (Formula are given in the appendix). Black bar: reference wash with equilibration buffer. Bars with oblique stripes: buffer contains >15% hydrophobic compounds. Results are considered being significantly different to each other if obtained virus clearance values including confidence interval do not overlap, i.e., buffers 1–6 are considered resulting in significantly different clearance compared to buffer 12 used as reference.

Wash buffers were also designed in a way to ensure compatibility with manufacturing conditions, e.g. avoiding explosive hazards. Thus, a replacement of isopropanol by other substances, when compared to the wash composition employed during the stringent wash in series, can be explained.

Up to twelve different wash buffers were used, some of which would interfere with at least one or several of the following interactions, based on their compositions: electrostatic, hydrophobic, hydrogen-bonding. All reagents and chemicals used were employed as received by the vendors (all chemical raw materials provided by Merck Co. GmbH).

Two mAbs (mAb A and mAb B) with different isoelectric points and different feedstock were used for the experiments, spiking 5% MVM into mAb B containing feedstock and 2.5% xMuLV into mAb A containing feedstock. After loading the respective virus-spiked mAb-feedstock solution onto the protein A resin (MabSelect SuRe, GE Healthcare, Uppsala, Sweden), 3 column volumes of the respective stringent wash were applied (a different stringent wash for each experiment, in total evaluating twelve washes for mAb B and ten washes for mAb A, followed by 3 column volumes of equilibration buffer to avoid carry-over of wash compounds into the then followed elution. Virus clearance was calculated based on virus titer determination in load and eluate fractions, respectively. For MVM, titer was determined by large-volume plating and infectivity assays while for xMuLV PCR was performed.

Large volume plating was executed for improving the detection limit of samples that were analyzed. 200 µl of the diluted sample was added to a defined number of wells containing the indicator cells in 100 µl cell culture medium. The cells were cultivated for a specified incubation period before being inspected microscopically for virus-induced changes in cell morphology.

In general, virus reduction is analyzed by quantitative infectivity assays. The infectivity assay only detects viruses able to infect and replicate in cell cultures (infectious viruses). In the case of both virus removal and inactivation, the infectivity assay does not allow the distinction of removal and inactivation. Therefore, qPCR analysis is used to demonstrate reduction caused by removal. The qPCR analysis is a molecular biological method based on the quantitative detection of virus-specific nucleotide sequences. Compared to the infectivity assay qPCR analysis detects infectious as well as non-infectious viruses.

The RT-PCR method comprised RNA extraction of from test items, spiked with the internal control in-vitro transcript of xMuLV and with or without xMuLV virus spike using the QIAamp Viral RNA Mini Kit (Qiagen, Germany). Purified RNA is subsequently analyzed by real-time

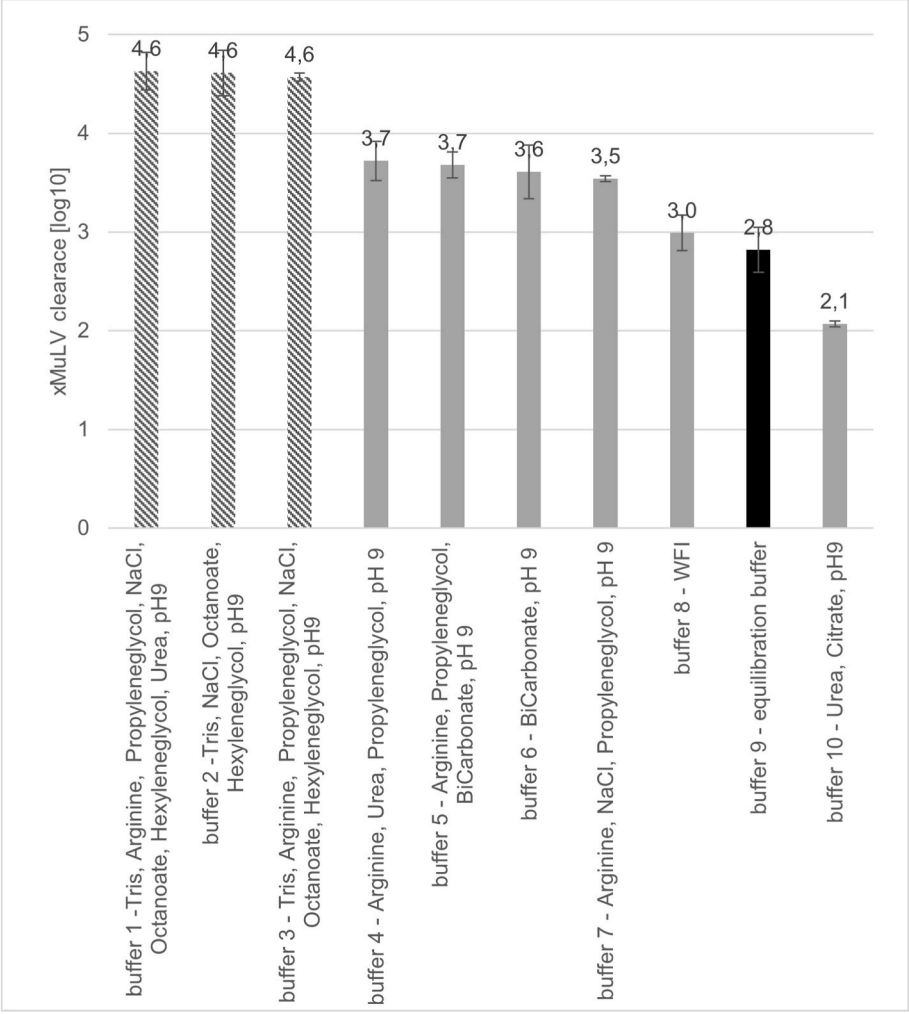

Fig. 3. xMuLV clearance on the protein A step in $\log_{10}$ with mAb A, depending on different wash buffers. The error bars represent a 95% confidence interval as provided by the CRO, based on a ± two-fold standard deviation obtained from the employed infectivity assay as performed by the CRO (Formula are given in the appendix). Black bar: reference wash with equilibration buffer. Bars with oblique stripes: buffer contains >15% hydrophobic compounds. Results are considered being significantly different to each other if obtained virus clearance values including confidence interval do not overlap, i.e., buffers 1–7 and buffer 12 are considered resulting in significantly different clearance compared to buffer 9 used as reference.

Table 1
Detailed composition of the different wash buffers used for evaluating interacting forces responsible for virus retention.

	Composition	pH
Buffer 1	Arginine, 500 mM Urea, Propyleneglycol	9.0
Buffer 2	BiCarbonate	9.0
Buffer 3	50 mM Tris, 1 M NaCl, Octanoate, Hexylenglycol	9.0
Buffer 4	500 mM Urea, Citrate	9.0
Buffer 5	Arginine, 750 mM NaCl, Propylenglycol	9.0
Buffer 6	50 mM Tris, Arginine, Propylenglycol, 1 M NaCl, Octanoate, Hexylenglycol	9.0
Buffer 7	BiCarbonate, 500 mM Urea	9.0
Buffer 8	50 mM Tris, Arginine, Propylenglycol, 1 M NaCl, Octanoate, Hexylenglycol, 500 mM Urea	9.0
Buffer 9	Arginine, Propylenglycol, BiCarbonate	9.0
Buffer 10	50 mM Tris, 1 M NaCl, Octanoate, Hexylenglycol, BiCarbonate	9.0
Buffer 11	WFI	9.0
Buffer 12	Standard Wash buffer for mAb	9.0

PCR of xMuLV target and internal control sequences on a LightCycler 480 System (Roche Diagnostics, Germany) equipped with LightCycler Software version 1.5. The assay utilizes in vitro transcript copy number standards to generate standard curves, which are used to determine the xMuLV genome copy number in test items and controls.

All experiments were carried out in a virus clearance lab at a CRO.

3. Results and discussion

3.1. Combinatorial stringent wash toolbox - combination of stringent wash applied in series to three mAb-feedstock systems

To confirm results published by previous authors, indicating a mAb and feedstock-dependent log reduction as well as to evaluate the overall potential log reduction improvement, we used a combination of several washes in series. Three mAbs, each with a different isoelectric point and being present in different feedstock, were subjected to a series of four stringent wash buffers, followed by a PBS as well as mAb-specific wash and evaluated for MVM and xMuLV clearance, respectively.

In total, 18 column volumes of wash buffer were applied to each of the mAbs.

Results, depicted in Table 2, confirm the aforementioned mAb and

Table 2
Log_{10} reduction values obtained for series of stringent wash buffers in comparison to reference buffer, for different mAb-feedstock systems. A confidence interval of 95% provided by the CRO is represented by ± and based on the employed infectivity assay and calculated two-fold standard deviation. Reference runs with equilibration buffer are derived from project specific studies and were performed as duplicate experiments, thus two values are given for reference runs. For some results, no additional standard deviation could be calculated, thus no additional confidence interval given, indicated by ± 0.00. In case max clearance could not be calculated due to none virus being present in the eluate, values are indicated with a ">".

mAb	wash	Log_{10} reduction MVM *pI* 6.2	Log_{10} reduction xMuLV *pI* 5.8
mAb A	Stringent wash in series	2.99 ± 0.34	>4.73±0.09[2)]
	Reference with equilibration buffer (duplicate experiments for MVM)	2.38 ± 0.33 2.48 ± 0.36	2.68 ± 0.00 2.82 ± 0.00
	Reference with prolonged wash with equilibration buffer	Not done	2.36±0.00[1)]
mAb B	Stringent wash in series	3.74 ± 0.36	>4.07 ± 0.04
	Reference with equilibration buffer (duplicate experiments for MVM)	2.33 ± 0.42 1.92 ± 0.37	3.01 ± 0.05
	Reference with prolonged wash with equilibration buffer	2.74±0.00[1)]	Not done
mAb C	Stringent wash in series	3.58 ± 0.38	>4.09±0.06[2]
	Reference with equilibration buffer (duplicate experiments for MVM)	2.27 ± 0.40 1.68 ± 0.35	3.87 ± 0.00
	Reference with prolonged wash with equilibration buffer	Not done	Not done

[1)] Clearance obtained when prolonging the reference wash to 18 CV to understand the impact of prolonged wash duration.
[2)] Non symmetric confidence interval provided by CRO, for simplicity only more narrow confidence interval displayed.

feedstock-dependent capability of virus clearance on the protein A step[12,21]

Reference runs in Table 2 refer to results when applying only a standard reference wash to the given mAb molecule. Additionally, when prolonging that standard wash to 18 column volumes, performed for two experiments, obtained clearance was within the 95% confidence interval of those results when not prolonging the wash, also with no tendency of either increased or decreased clearance due to wash length. Thus, prolongation of the standard wash did not result in improved clearance, based on Table 2 and wash duration therefore had no impact on our results, at least for a window of 3–18 CV and the given wash buffer composition.

Generally, for Table 2, a higher clearance is observed for xMuLV, compared to MVM, also reported by previous groups.[1,7,12] Importantly, as some of our buffers contained arginine, in addition to removal inactivation is expected for xMuLV, adding 1 log_{10} in addition based on results by Bolton et al.[20] Yet, this effect is excluded from our results as xMuLV was evaluated by PCR, thereby only considering the removal effect and not any virus inactivation.

Compared to the respective reference runs (not considering the runs with prolonged reference wash duration), log reduction values for MVM clearance could be improved by 0.5–1.4 LRV, depending on the mAb. For xMuLV, clearance could be improved by 0.2–1.9 LRV, also depending on the mAb. Interestingly, there is no correlation between general virus clearance log reduction, clearance improvement and specific mAb, meaning for mAb A lowest MVM improvement was observed, whereas this was the case for mAb C with regards to lowest observed xMuLV clearance improvement. mAb A in contrast, showed strongest xMuLV clearance improvement. Interaction between virus, e.g. xMuLV and mAb target protein follows similar principles as interaction between HCP and target protein,[22,27] also being dependent on the virus itself as well as the Fab part of the mAb molecule.[28] The observed difference may also be related to different pIs of the mAbs used, with improved clearance for MVM in case the mAb displayed a less positive charge based on the pI. For xMuLV this effect has not been observed, despite, pI of xMuLV is ~5.8 and thus slightly lower than for MVM with pI ~6.2.[29]

While mAb B and C had slightly lower pIs compared to mAb A, they also allowed for better log reduction improvement, compared to mAb A. However, log reduction to be achieved seems also largely being dependent on feedstock parameters, with mAb B and C displaying a 5.8 and 6.2-fold increased HCP level expressed as per ng/mL compared to mAb A, respectively. Therefore, one assumption is that the higher abundance of HCPs in the load solution of the protein A step may compete with the virus when binding to resin and/or mAb, thereby resulting in a significant improvement in removal when applying the series of stringent wash buffers. However, while this seems to be the case for MVM, it is not for the stringent washes employed for xMuLV clearance, potentially related to the higher pI of xMuLV and thus reduced electrostatic interaction.

3.2. Detailed wash buffer evaluation with mAb-virus combinations

Based on the data of the stringent wash buffers applied in series, we designed a combination of suitable stringent wash buffers to further understand the clearance improvement for the different mAbs, evaluating wash buffers in a more detailed manner with two mAb-virus combinations and thereby elucidate the type of interactions most strongly involved in virus retention.

3.3. mAb B and MVM

For MVM and mAb B, 12 different buffers were evaluated, each with a different composition to further understand the importance of chaotropic, electrostatic-interfering, hydrophobic-interfering and H-bond breaking buffer compounds on virus retention and thus clearance (Fig. 2). Clearance in the reference run, employing an equilibration buffer as wash, was 2.3 log_{10} (buffer 12). Results are considered being significantly different to each other if obtained virus clearance values including confidence intervals do not overlap, i.e., buffers 1–6 are considered resulting in significantly different clearance results compared to buffer 12 serving as reference (Fig. 2). Among the stringent wash buffers, buffer 1, containing arginine, urea and propylene glycol was the one achieving the highest log reduction of 3.6 log_{10}. This may be explained by its composition: arginine interrupting electrostatic and hydrophobic interactions, urea as chaotropic agent breaking H-bonds and propylene glycol interfering with hydrophobic interactions. For buffer 2, bicarbonate, due to its ability of building H-bonds via its C-and O-atoms,[30] may act as H-bond breaker and also achieved a high clearance of 3.4 log_{10} whereas urea alone achieved 3.2 log_{10} (buffer 4). Interestingly, a combination of urea and bicarbonate (buffer 7) resulted in minor clearance improvement, compared to the reference run only, considering assay variation, however, may also obtain good clearance. Buffers, containing bicarbonate together with salts (NaCl or Arginine) and propylene glycol, hexylene glycol as well as octanoate did not result in improved clearance, the reason to be elucidated further.

Compared to the reference run with equilibration buffer, overall clearance could be improved by up to 1.3 log_{10}, using buffer 1.

3.4. Interactions responsible for virus retention

Increasing buffer components, known to interfere with hydrophobic interactions (octanoate, hexylene glycol and propylene glycol) above 10–15% did not result in improved clearance (compare buffers 3-6-8-10, highlighted with oblique stripes vs. the other buffers, highlighted as solid bars). These results indicate that for MVM retention with mAb B, hydrogen bonding plays a dominant role, based on results with urea or bicarbonate alone, as well as electrostatic interactions, based on buffers containing NaCl or arginine. These results also confirm results from the stringent washes applied in series, indicating electrostatic interactions

playing an important role on virus retention.

3.5. mAb a and xMuLV

For xMuLV and mAb A, ten buffers were evaluated in total (Fig. 3). Results in Fig. 3 are considered being significantly different to each other if obtained virus clearance values including confidence intervals do not overlap, i.e., buffers 1–7 and buffer 12 are considered resulting in significantly different clearance compared to buffer 9 serving as reference. Three buffers achieved a log reduction >4.6 $\log_{10}$, compared to 2.4 $\log_{10}$ for the reference run with equilibration buffer only. Of those three buffers, two buffers (1 and 3), based on their composition, presumably are interfering with hydrophobic interactions, electrostatic interactions as well as acting as H-bond breakers. This has been the case e.g. when the buffer contained arginine, known to act as hydrogen-bond breaker as well as interfering with electrostatic interactions as well as containing octanoate and hexylene glycol. A similar effect has been discussed by previous authors, indicating a virus-mAb interaction being based on e.g. electrostatic interaction, hydrophobic interaction as well as hydrogen-bonding.[12,13] Thus, using buffers designed specifically to fulfill these requirements, e.g. replacing octanoate and/or hexylene glycol by similar compounds may be a way to improve virus clearance.

3.6. Interactions responsible for virus retention

Additionally, a certain concentration of buffer substances, capable of disturbing hydrophobic interactions, seems to be required. Of the ten buffers evaluated, a log reduction >4.6 was only achieved if the buffer contained >10–15% compounds capable of disturbing hydrophobic interactions (buffers 1,2,3, bars with oblique stripes). For buffers containing less than 10–15% of these compounds (bars colored in blue), achieved log reduction was only 3.6 ± 0.1 $\log_{10}$, indicating stronger importance of hydrophobic interactions being responsible for xMuLV retention. These observations have also been corroborated by Bach and Connell-Crowley as well as,[6] who showed a significant improvement of 3–4 $\log_{10}$ applying a wash containing Triton X-100 as well as urea/IPA, compared to an improvement of only 2 $\log_{10}$ when applying washes which disrupt electrostatic interactions only, all in relation to a reference run.

Johnson et al., also pointed out a stronger hydrophobicity of xMuLV compared to MVM, when evaluating the retention time after subjected to a hydrophobic interaction chromatography,[31] being a possible explanation for these observations and the increased clearance based on increased hydrophobic buffer compounds.

As visible from Fig. 2, ability of H-bond breaking alone was not sufficient in contrast to mAb B and MVM clearance, as buffer 10, containing a chaotropic agent (urea), achieved a slightly lower log reduction compared to the one observed in the reference experiment with equilibration buffer. This has also been reported by Pan et al., evaluating RVLP clearance with different buffers.[12] While they saw, depending on the mAb, only a minor improvement when applying a chaotropic compound-containing buffer, improvement in clearance could be further enhanced when also adding additional substances, e.g. NaB as hydrophobic salt, thereby interfering with both hydrophobic and electrostatic interactions.

Generally, for mAb A and xMuLV, a combination of electrostatic, hydrophobic and hydrogen-bonding interactions seems to be responsible for virus retention. Additionally, improvement compared to a reference run with equilibration buffer was 0.7 $\log_{10}$ for a wash containing arginine, NaCl as well as propylene glycol (buffer 7), compared to 1 $\log_{10}$ for the same buffer when applied to MVM and mAb B (Fig. 1, buffer 5). This indicates that electrostatic interactions may be more relevant for MVM retention compared to xMuLV retention. This has already been concluded based on the results from the stringent wash buffer series experiments.

4. Conclusion

We were able to corroborate results by previous groups, indicating the mechanism of virus retention on the protein A step being governed by hydrogen bonds as well as electrostatic and hydrophobic interactions. Our results further confirm the previously observed mAb and feedstock dependent virus retention and thereby limited ability to improve virus clearance when using a generic wash approach. Additionally, we further evaluated the impact of different interaction mechanisms on virus retention.

For MVM, interaction and retention seem primarily governed by electrostatic interaction and formation of hydrogen bonds. This has been corroborated by our results, showing improved clearance when applying chaotropic substances as well as salts, e.g. arginine or NaCl.

While addition of hydrophobic compounds further improves clearance, this effect is not as pronounced as with xMuLV, which showed a significant log reduction improvement, when applying a wash buffer combination of compounds capable of interfering with electrostatic, hydrophobic and hydrogen-bond interactions. For xMuLV especially, we observed an improved clearance in case the percentage of hydrophobic compounds in the buffer exceeded a certain level, indicating hydrophobic interactions playing an important role in xMuLV retention.

Bach and Connell-Crowley indicated the potential for generic filings for virus clearance claims, based on applying stringent wash buffers.[13]

Yet, based on our results when applying a series of combination of stringent wash buffers to three different mAb-feedstock systems, a generic wash buffer recipe, consistently achieving significantly improved virus clearance, is likely so far not possible.

However, we were able to at least show the possibility, to improve clearance by applying certain wash buffer compositions to the mAb and virus, thereby yet with the need to adapt these buffers to specific model viruses and likely also mAb-feedstock systems. Furthermore, knowing the interactions responsible for improved virus clearance as disclosed in this manuscript may be a way to design wash buffers in certain way, e.g. selecting ones previously successfully used for HCP clearance to, based on their component's interactions, improve virus clearance. None of the potential buffers decreased product yield significantly (compare Supplementary Fig. 1). An impact on product quality is not expected but should clearly be part of further studies and evaluations.

Yet, interaction parameters for virus retention differ for the different viruses, thereby impeding a generic approach for improving virus clearance. Yet, based on a careful design of wash buffers, virus clearance can be improved, also on the protein A step, with here reported improvement of 1.3 $\log_{10}$ for MVM and 2.2 $\log_{10}$ for xMuLV, compared to reference buffers, thereby making this step more attractive to be considered in virus clearance studies or to be claimed for additional log reduction achievements.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: the authors are currently employed by Sanofi-Aventis Deutschland GmbH.

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Appendix

$TCID_{50}$ calculation:

$$\log_{10} TCID_{50}/ml = -\left(-Y_0 + \frac{d}{2} - d \bullet \sum P_i - v\right)$$

Standard Deviation:

$$s_e = d\sqrt{\sum \frac{p_i(1-P_i)}{(n_i-1)}}$$

95% Confidence limit

$$c = \pm 2\, s_e$$

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biotno.2024.03.001.

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