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Quality by Design Approach for Viral Clearance by Protein A Chromatography

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ABSTRACT: Protein A chromatography is widely used as a capture step in monoclonal antibody (mAb) purification processes. Antibodies and Fc fusion proteins can be efficiently purified from the majority of other complex components in harvested cell culture fluid (HCCF). Protein A chromatography is also capable of removing modest levels of viruses and is often validated for viral clearance. Historical data mining of Genentech and FDA/CDER databases systematically evaluated the removal of model viruses by Protein A chromatography. First, we found that for each model virus, removal by Protein A chromatography varies significantly across mAbs, while remains consistent within a specific mAb product, even across the acceptable ranges of the process parameters. In addition, our analysis revealed a correlation between retrovirus and parvovirus removal, with retrovirus data generally possessing a greater clearance factor. Finally, we describe a multivariate approach used to evaluate process parameter impacts on viral clearance, based on the levels of retrovirus-like particles (RVLP) present among process characterization study samples. It was shown that RVLP removal by Protein A is robust, that is, parameter effects were not observed across the ranges tested. Robustness of RVLP removal by Protein A also correlates with that for other model viruses such as X-MuLV, MMV, and SV40. The data supports that evaluating RVLP removal using process characterization study samples can establish multivariate acceptable ranges for virus removal by the protein A step for QbD. By measuring RVLP instead of a model retrovirus, it may alleviate some of the technical and economic challenges associated with performing large, design-of-experiment (DoE)—type virus spiking studies. This approach could also serve to provide useful insight when designing strategies to ensure viral safety in the manufacturing of a biopharmaceutical product.

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KEYWORDS: protein A chromatography; virus removal; X-MuLV; SV40; MMV; retrovirus-like particles; risk ranking; QbD

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Abbreviations: CHO, Chinese hamster ovary; DoE, design-of-experiment; HCCF, harvested cell culture fluid; LRV, log reduction value; MMV, murine minute virus; QbD, quality by design; RRF, risk ranking and filtering; RVLP, retrovirus-like particles; SV40, simian virus type 40; X-MuLV, xenotropic murine leukemia virus.

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Introduction

Protein A chromatography is a bioprocess capture step that separates antibodies and Fc fusion proteins from harvested cell culture fluid (HCCF) via specific interactions with the antibody Fc region (Ghose et al., 2007a,b; Jungbauer and Hahn, 2004). Despite the heterogeneous nature of typical commercial antibody HCCF, it consistently provides efficient purification of antibodies from impurities such as host cell proteins and DNA (Fahrner et al., 1999; Hahn et al., 2003; Liu et al., 2010; Miesegaes et al., 2010a). This enables the implementation of platform process that hinges the use of Protein A capture step (Fahrner et al., 2001; Shukla et al., 2007). From a viral clearance standpoint, it is presumed that viruses largely flow through the column (Brorson et al., 2003a; Lute et al., 2008), along with most non-antibody cell culture components, during the loading phase and post-load wash. A small number of viruses do bind to the column however, via non-specific interaction to the media itself or through the mAb. Some viruses are presumably liberated by the elution pH transition, leading to low but variable levels of virus in the eluate. Because of this, and given that the non-specific binding of virus to the column does not involve well understood molecular interactions or controllable parameters, variable LRVs are often encountered (Miesegaes et al., 2010a).

In 2008–2009, the FDA conducted a data mining project where viral clearance information from over 200 regulatory

submissions spanning the past 20 years (IND, NDA, MF, and BLA document types) was extracted and assimilated into a searchable database (Miesegaes et al., 2010a,b). Protein A chromatography was included in this exercise and while it routinely afforded 1–4 $\log_{10}$ of clearance of many viruses, it appeared to be less consistent than other purification unit operations (e.g., flow-through mode anion exchange, virus filters). One could conjecture that part of this is due to protein A being operationally complex, that bind and elute mode columns in general tend to use more buffers with more phases, or since feedstock specific factors such as impurity composition often vary (Miesegaes et al., 2010a). Even though viral inactivation due to the use of low pH product elution buffers is relatively well-understood (Brorson et al., 2003b), the low pH effect on LRV is still not clear. To illustrate this, although the LRV distribution for MuLV using PCR-based assays (that only measures viral partitioning) was on average 1.7 $\log_{10}$ lower than that using infectivity assays (that measures the additional inactivation from low pH), there was no significant correlation between elution buffer pH and LRV (Miesegaes et al., 2010b). Finally, because the LRVs obtained from Protein A chromatography tend to vary from product to product, there has been a general agreement that more investigation is required to understand the mechanism and predict the outcomes of this operation as a virus removal step (Miesegaes et al., 2010a).

To gain additional insight, Genentech has performed a meta-analysis on its own viral clearance database. Incorporating Protein A chromatography validation studies from 22 mAbs and 30 processes over the past 15 years, it was observed that clearance of X-MuLV measured by QPCR ranged from 2 to 4 $\log_{10}$, while clearance of MMV trended lower, ranged from 1 to 3 $\log_{10}$. These ranges are consistent with observations reported by other companies (Miesegaes et al., 2010a,b). Given the limitations of post hoc meta-analyses, it was beyond the scope of this exercise to determine specific correlations between LRVs and process/feedstock conditions. Acquiring such information would benefit from statistically designed (e.g., design-of-experiment [DoE]) studies.

Implementation of quality by design (QbD)-based approaches within the biotech industry has become a recent means to assess complicating issues such as the impact of multiple operating conditions on product quality (FDA, 2006a; Rathore and Winkle, 2009, Rathore, 2010; Rathore and Devine, 2008; Rathore and Mhatre, 2009). One of the underlying principles behind QbD is that it is a risk and science-based approach to process validation. Another principle related to QbD is the identification of a multivariate "Design Space" in which process parameter impacts (including multivariate interactions) are explicitly related to critical quality attributes (such as viral clearance). In the context of viral clearance validation, the use of risk-based approaches can inform more efficient and rationale-driven strategies and study designs to establish Design Space, in contrast to traditional full characterization studies which typically include as many as 20–100 runs. Such traditional full studies can be extremely expensive and time-consuming to perform if using virus-spiking to determine viral clearance capacity. Previous publications report comparable clearance when using QPCR assays that quantify either CHO endogenous retrovirus-like particles (RVLP) or spiked X-MuLV, and that retrovirus removal by Protein A could be evaluated by RVLP removal using samples from a Protein A process characterization studies (Zhang et al., 2009). In this report, we propose an experimentally determined QbD-based approach for assessing viral clearance by Protein A chromatography.

Materials and Methods

Scale-Down Protein A Chromatography

Protein A chromatography was performed using a qualified scale-down model. All feedstocks that were loaded onto the columns were from large scale CHO cell cultures taken from routine production at Genentech, Inc. (South San Francisco, CA). Cells and debris were removed from the harvested cultures by centrifugation and depth filtration to yield HCCF.

Antibody purification from the HCCF samples was achieved by Protein A affinity chromatography (Prosep A or Prosep-vA High Capacity from Millipore, Billerica, MA or MabSelect SuRe from GE Healthcare, Pittsburgh, PA). The resin was packed in a 0.66 cm inner diameter glass column (Omnifit, Danbury, CT) with a 14–20 cm bed height resulting in a 4.8–6.8 mL final column volume.

Model viruses X-MuLV, MMV, and SV40 were purchased from BioReliance (Rockville, MD). Model viruses were spiked at 1/100th the volume for each virus into the feedstock and loaded onto the chromatography column. Chromatography was performed using an AKTA Explorer 100 chromatography system (GE Healthcare) at ambient temperature. Other than DoE studies, all model virus spiked studies used parameters, feedstocks, buffers, and resins equivalent to those used in the full-scale process of that particular product, except for protein load density and pooling criteria which were at maximum and widest, respectively. An acidic buffer (∼pH 3) was used for elution of product. Protein A elution pools were collected and pH adjusted to 6–8 prior to freezing. All virus-containing chromatography samples were frozen at −60°C prior to nucleic acid extraction. All samples were analyzed by Q-PCR assay for each model virus to determine the level of viral clearance. Product yield and chromatograms were evaluated after each run to be representative of the full-scale process.

In those studies, a control run without model virus spiking was performed. Samples from this run were spiked with known amount of model viruses to determine matrix interference and sample dilutions required for Q-PCR assays.

DoE Studies

For RVLP removal, the HCCF and Protein A pool samples were collected from process characterization studies. Protein A pool samples were adjusted to ∼pH 5 prior to freezing at −60°C. RVLP quantification by Q-PCR was performed by BioReliance.

For model virus spiked DoE studies, with the exception of tested parameters, all other parameters were run at target. The feedstocks, buffers, and resin were equivalent to those used in the full-scale process. All chromatography samples were frozen at −60°C. All samples were analyzed by Q-PCR assay for each model virus to determine the level of viral clearance.

Real-Time Quantitative PCR (QPCR) Assays

The virus titer for each test sample was quantified by the Real-Time Quantitative PCR assay that provides automated quantification of input RNA or DNA copy number, associated with each virus. Viral genomic particle RNA (RVLP and X-MuLV), and genomic DNA molecules (MMV and SV40) were extracted from test samples using QiaAmp Viral RNA kit with the manual method, (Qiagen, Valencia, CA) or using Qiagen EZ1 virus mini kit v2.0 with EZ1/EZ1 advanced robotic system. For X-MuLV, SV40, and MMV, the manual extraction procedures were described previously (Shi et al., 1999, 2004; Zhan et al., 2002). Alternatively, automated multi-virus extraction procedure using robotic systems was also published (Lute et al., 2009). For RVLP, the viral RNA was extracted using Qiagen EZ1 virus mini kit v2.0 with the EZ1/EZ1 advanced robotic system, according to vendor instructions. Free viral nucleic acids not associated with intact virus particles were removed based on the digestion procedures described earlier (Lute et al., 2009; Zhang et al., 2009) with slight modifications (10 μL DNase I, 40 μL of DNase I buffer, and 350 μL sample volume).

Real time quantitative PCR assays were used to determine the copy numbers of viral DNA or RNA in test samples by the fluorescence produced from virus-specific primers and probe, purchased from Life Technologies (Carlsbad, CA). For X-MuLV, SV40, and MMV, the primers/probe sequences were described previously (Lute et al., 2009; Shi et al., 1999, 2004; Zhan et al., 2002). For RVLP, the primers were also described (de Wit et al., 2000), and the MGB probe sequence was 5′-TACAGGCGGAAAGCA-3′, labeled with FAM dye and a non-fluorescent quencher. The Q-PCR amplification reaction set-up and cycling conditions were described previously (Lute et al., 2009), except the reverse transcription step for X-MuLV and RVLP at 45°C for 30 min. In addition, the RT Q-PCR reagents used were purchased from Life Technologies.

X-MuLV and RVLP sRNA, MMV, and SV40 DNA used as quantification standards were prepared as described (de Wit et al., 2000; Lute et al., 2009).

Viral clearance by Protein A chromatography was expressed as $\log_{10}$ reduction value, or LRV, which is the difference of $\log_{10}$ (total virus) in load and elution pool. Total virus is obtained from virus titers (particles/mL) in samples and sample volumes (mL).

Results

Partitioning of Viruses in Protein A Chromatographic Fractions

In order to study virus removal mechanism of action (MOA) by Protein A, the partitioning of viruses in various fractions during the chromatography run procedures was evaluated. Mab 1 is a CHO-derived IgG1. Table I shows the amount of three model viruses, X-MuLV, MMV, and SV40 as well as RVLP in the fractions collected from mAb 1 Protein A process. The amount of each virus in the samples was determined by quantifying the virus RNA or DNA copy numbers using Q-PCR. For all four viruses, the amount detected in the flow through/wash fraction is comparable to those loaded onto the column, indicating that the majority of the viruses flow through, regardless of the type of virus. For each run, the level of viruses detected in elution pools was 1–2 $\log_{10}$ lower than that in load, thus reflecting the LRV. This data indicates that the flow through mechanism of virus removal by Protein A chromatography applies to all three model viruses as well as RVLP. As shown previously (Zhang et al., 2009), X-MuLV LRV is comparable to the RVLP LRV. In addition, even though endogenous RVLP levels in mAb 1 HCCF may be lower than spiked X-MuLV, the amount of both viruses in mAb 1 HCCF and Protein A pools are above the assay quantification level. Thus, LRVs reflect the actual separation power of the column, and are not limited by assay range. RVLP removal can be determined using the CHO RVLP QPCR assay.

Variable X-MuLV LRV Across mAbs

It has been observed that the removal of virus by protein A may vary across different mAb processes and feedstocks, although a consistent correlation with specific process parameters has yet to be identified (Miesegaes et al., 2010b). To determine whether the mAb/HCCF feedstock or specific aspects of the Protein A process (e.g., linear flow rate, buffer composition, etc.) is the main source of LRV variation across processes/products, X-MuLV LRVs were determined from 10 mAbs all using an identical Protein A process and resin (Fig. 1). The range of X-MuLV LRVs is from 0.8 to 2.9 $\log_{10}$ ($n = 14$). Meanwhile, LRV differences in duplicate runs by experimental variation are only between 0.2 (mAb 8 and mAb 11) and 0.5 (mAb 12) Log_{10}, resulting from the known technical variations from virus spiking, chromatography, sampling, and assay. Thus, these runs exhibited substantially less variation than the 10 mAbs, where all process parameters and buffer compositions were held constant; only the mAb/HCCF load material was different. This scatter, despite a uniform process, indicates that the

Table I. Model virus and RVLP partitioning and LRV by naïve ProSep vA for mAb 1 purification process.

	Total virus RNA/DNA ($\log_{10}$ copies)			
Fractions	RVLP	X-MuLV	MMV	SV40
Load	9.95	10.65	9.92	10.06
Load flow through/wash	10.04	10.59	9.78	9.87
Elution	7.70	8.54	8.59	8.00
Viral clearance (LRV)	2.25	2.10	1.32	2.06

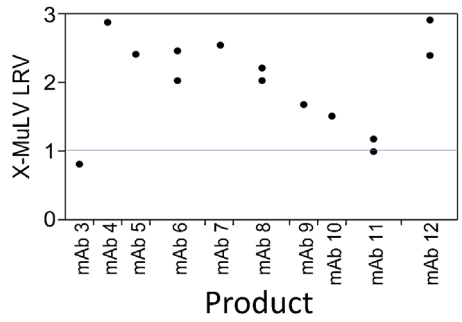

Figure 1. Removal of X-MuLV by protein A chromatography using an identical purification process. Data from mAbs 6, 8, 11, and 12 were from duplicate runs.

mAb/HCCF likely contributes to the variation in virus removal capacity by Protein A chromatography, as suggested previously using the FDA database (Miesegaes et al., 2010b).

X-MuLV and MMV LRV Correlation

It was noted that some viruses were removed better than others by protein A chromatography (Miesegaes et al., 2010b). To investigate this, historical virus clearance data from Genentech for X-MuLV and MMV from 22 mAbs in 30 processes ($n = 52$) were compared (Fig. 2a). Each data point represents X-MuLV LRV (x-axis) and the corresponding MMV LRV (y-axis) from a specific mAb and Protein A process. The data set includes virus removal by Protein A chromatography studies using Prosep A, Prosep vA, and MabSelect SuRe resins, both naöve and reuse resins. All X-MuLV and most MMV results were obtained using QPCR assays except a few MMV LRVs were from cell infectivity assays. This is a valid comparison because MMV is acid stable and there is predicted to be no inactivation component by protein A chromatography (Miesegaes et al., 2010b). Thus, measuring MMV, but not X-MuLV, clearance by TCID50 assay is functionally equivalent to measuring it by Q-PCR.

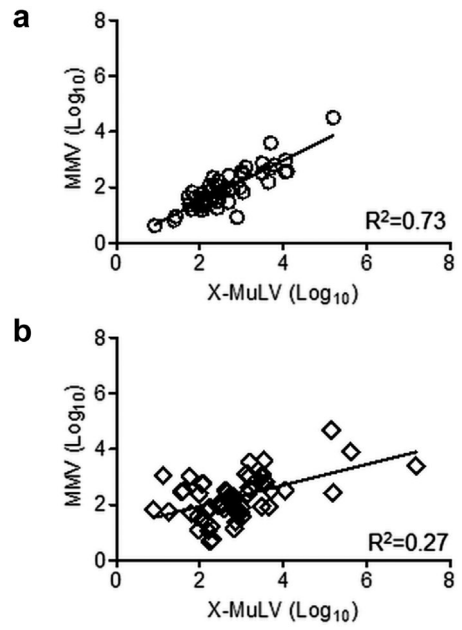

Figure 2. LRVs of X-MuLV and MMV from (**a**) Genentech validation studies over a period of 15 years ($n = 52$ data points); (**b**) viral clearance submissions across the industry from the CDER regulatory database ($n = 54$).

Results suggest that removal of both viruses follow the same general trend, that is, for a specific product/process, when X-MuLV LRV is high, MMV LRV is also high, and vice versa, with high correlation. In general, X-MuLV removal is higher than MMV removal ($n = 52$). On average, X-MuLV LRV is 0.67 $\log_{10}$ higher than MMV.

In order to see if the above observation could be generalized across companies, the LRVs of MuLV and parvoviruses from Protein A unit operations in CDER regulatory database (Miesegaes et al., 2010b) were correlated in a different scatter plot. Each data point represents a single product/study report where MuLV and MMV clearance were measured for the same Protein A unit operation. Studies were included if MuLV Log reduction values were measured by Q-PCR while MMV LRVs could be measured by either Q-PCR or infectivity. A subset analysis of Genentech-only records from the CDER regulatory database yielded a similar R^2 value (data not shown). In another comparison (Fig. 2b), the extent of a generalized trend was determined by incorporating MMV and MuLV data from viral clearance submissions across the industry. A lower R^2 value (0.27) for this analysis versus the Genentech-only analysis in (a) was observed. However, it should be noted that a lower correlation coefficient is not unexpected, given the ad hoc and retrospective nature of analyzing information from: (1) multiple companies; (2) a time period spanning more than two decades; and (3) varying or firm-specific-process platforms. Therefore, as the CDER database format cannot be appropriately controlled for, a fair amount of scatter was to be expected. Nonetheless, the correlation between the LRVs of the two viruses still existed, confirming the observation with the Genentech-only analysis.

Varying Process Parameters Does Not Affect RVLP Removal

It can be difficult to assess process parameter effects on LRV by evaluating the Genentech in-house database or the CDER database. It was noted that even though varying LRVs were observed across feedstocks and products for a given Protein A process (Fig. 1), when a single feedstock type was assessed with varying parameter settings (Zhang et al., 2009 and data not shown), consistent LRVs were observed. In order to see if process parameter effects on LRV could be determined, virus removal by a particular mAb (mAb 1) process was systematically analyzed using QbD principles.

As part of Genentech's implementation of the QbD initiative, risk ranking and filtering (RRF) exercises are performed on unit operations to identify parameters that have potential impact on certain product quality attributes (CMC Biotech Working Group, 2009; FDA, 2006b). The RRF assessment draws on scientific knowledge, platform information from other mAbs, and historical product-specific data. Once identified, these parameters are further evaluated in process characterization studies for parameter impact on product quality and process performance (e.g., purity and yield). The RRF assessment for viral clearance by mAb 1 Protein A chromatography identified a similar set of parameters as those identified from the process characterization study. All parameters that were considered to have a potential impact on virus removal were included in the process characterization study design. While the characterization studies are not specifically performed to evaluate the robustness of viral clearance, the same study design and existing retained samples can be used to evaluate virus removal based on RRF. It has been established that it is feasible to analyze RVLP removal using process characterization study samples, and that RVLP LRV can reflect the retrovirus removal capability of the Protein A process (Zhang et al., 2009). This approach is therefore used to support the multivariate acceptable ranges of the mAb 1 Protein A chromatography step for retroviral clearance.

The mAb 1 process characterization study used a total of three HCCF lots that were produced from the same cell culture process, feedstocks 1, 2, and 3. Within the characterization study, one set of experiments was a multivariate study consisting of a 16 run, ½ fractional factorial design (Resolution V), with five factors blocked by feedstock into two blocks of eight and augmented with four target runs (Table II). Factors tested were load density, load flow rate, wash buffer pH and molarity, and operating temperature. The RVLP LRVs are shown in Table II.

The average LRV for the 16 test runs was 2.0, comparable to the average LRV of 1.9 for the four target runs. Furthermore, the ranges of RVLP LRVs from this DoE were also comparable, 1.6–2.4 for the test runs and 1.7–2.1 for the target runs, as shown in Table II.

To determine if any of these parameters had any statistically significant effect on viral clearance, a regression analysis was performed (Fig. 3). The regression model consisted of five main effects, ten 2-way interactions and the block effect (feedstock or HCCF lot). The *P*-value of the model fit is 0.0135. The R^2 value is 0.99, while the root mean square error (RMSE) value is 0.0524. The estimates of LRV effect were rather small, when varying the parameter from the center point to the high or low end of the test range. The estimates were 0.20 $\log_{10}$ for feedstock, 0.13 $\log_{10}$ for load density, 0.12 $\log_{10}$ for load flow rate, and −0.05 $\log_{10}$ for operating

Table II. CHO RVLP removal by protein A chromatography for mAb 1 from multivariate study.

Run no.	Pattern[a]	Feedstock (Lot)	CHO RVLP LRV
1	00000	1	1.7
2	−++−+	1	1.9
3	−+−++	1	2.2
4	− − − −+	1	2.0
5	− −+++	1	2.2
6	++−+−	1	1.8
7	+++− −	1	1.6
8	+−++−	1	1.9
9	+− − − −	1	1.6
10	00000	1	1.8
11	00000	2	2.1
12	−+− − −	2	1.9
13	− −+− −	2	2.0
14	− − −+−	2	2.2
15	−+++−	2	2.2
16	+++++	2	2.3
17	++− −+	2	2.1
18	+− −++	2	2.4
19	+−+−+	2	2.1
20	00000	2	2.1

[a]Symbols in pattern indicate the run conditions at which the parameters are set. 0 is center point, + is high and − is low. The parameters are operating temperature (°C), wash buffer pH, wash buffer molarity (mM), load density (g/L), and load flow rate (CV/h) from left to right. Regression analysis indicated the following parameters to have statistically significant effects (*P*-values ≤ 0.05) on RVLP LRV: feedstock (0.005); load density (0.002); load flow rate (0.002), and operating temperature (0.038).

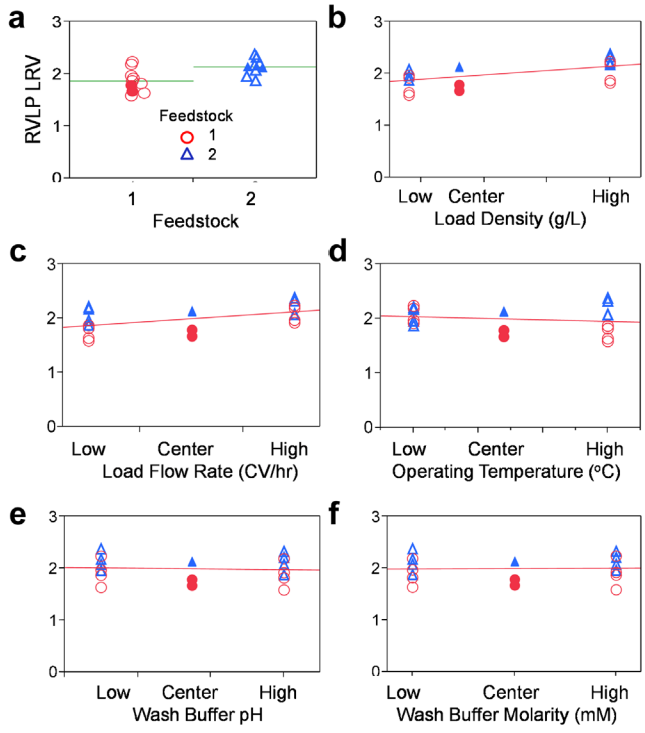

Figure 3. CHO RVLP removal by mAb1 protein A chromatography in response to (**a**) feedstock; (**b**) load density (g/L); (**c**) load flow rate (CV/h); (**d**) operating temperature (°C); (**e**) Wash buffer pH; (**f**) Wash buffer molarity (mM). Each symbol corresponds to two HCCF lots as feedstocks. Closed symbols are target runs and open symbols are test runs.

temperature, all well within the experimental variation, which includes at least the variations from chromatography, sampling and QPCR assays. In addition, LRV variation is dependent on the measurements of two samples (load and pool), and the estimates observed in this study were even lower than the assay variation for a single measurement, at 0.2–0.5 $\log_{10}$ (Brorson et al., 2002). Therefore, no practically significant effect on RVLP removal was observed within the tested ranges of the five parameters studied.

The RRF exercise identified similar sets of process parameters that had potential impact on virus and impurity removal by Protein A chromatography. Therefore, the worst case conditions for impurity removal were assessed for RVLP removal. Another set of experiments investigated worst case conditions for removal of impurities such as CHOP and leached Protein A. Parameters evaluated included load density, flow rate, wash and elution buffer molarity and duration, pooling, temperature, and various combinations of these parameters. Worst case studies, while they may exceed the final process multivariate acceptable ranges, are typically performed to better understand the process. When RVLP removal was assessed using these samples, the results were comparable from test runs and target runs, even under the most unfavorable conditions for certain impurity removal.

Taken together, RVLP LRVs shown in Tables II and III were combined together in Figure 4 and grouped into test and target runs. Figure 4 indicates that RVLP removal from these test runs is similar to those from target runs. The average LRVs were both 2.0 for the test runs and the target runs, and the ranges of LRVs were comparable as well, 1.6–2.4 for the test runs and 1.7–2.4 for target runs, supporting the conclusion that no practically significant parameter effect was observed in all tested runs within the tested ranges.

Therefore, removal of RVLP was not impacted by worst case parameter conditions as well as the combination of these conditions (Tables II and III). The Protein A chromatography step resulted in a similar reduction of RVLP for the test runs and the target runs (Fig. 4). No parameter tested was shown to have a significant impact on RVLP removal.

Table III. CHO RVLP removal by protein A chromatography for mAb 1 from worst case studies.

Run no.	Run[a]	Feedstock (Lot)	CHO RVLP LRV
21	Target	2	2.1
22	Test	2	1.9
23	Test	2	2.4
24	Test	2	1.9
25	Test	2	2.1
26	Test	2	1.9
27	Test	2	1.9
28	Test	2	1.8
29	Target	2	1.9
30	Target	3	2.1
31	Test	3	1.9
32	Test	3	2.1
33	Test	3	1.9
34	Target	3	2.4

[a]Target runs were run when all tested parameters were set at target conditions, while test runs were run when tested parameters were changed, either individually or in combination.

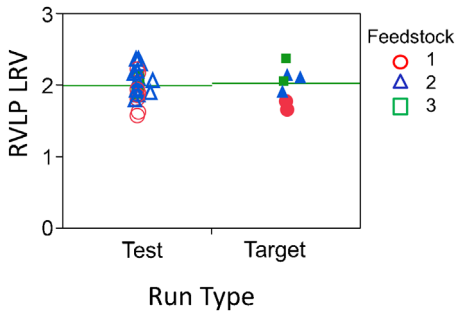

Figure 4. RVLP removal of test runs and target runs measured from mAb1 protein A characterization study samples.

No Process Parameter Effects on X-MuLV, MMV, and SV40 Removal for mAb 1

Due to the correlation observed between X-MuLV and MMV LRVs, it is expected that the lack of parameter impact on RVLP removal within the parameter ranges studied can also be generalized to other model viruses. In order to confirm that there is no parameter impact on other model virus removal within the parameter ranges tested, a small DoE multi-virus (X-MuLV, SV40, and MMV) spiking study was performed.

The DoE experiment consisted of a 2-factor 4 run full factorial design augmented with two target runs (runs B and G). Load density and load flow rate were tested in ranges during the four test runs (runs C–F), combining with six selected parameters set to potential worst case conditions, including wash buffer molarity, flow rate, phase duration, elution buffer pH, flow rate and pooling. Runs B and G were performed with all process parameters set to target conditions. All three model viruses were spiked and analyzed for removal. In order to avoid the LRV results being confounded by a different load amount for virus when fixed spiked ratio is used, all spiked runs were performed with the same amount of virus loaded onto the column. The virus amount for each model virus was determined by what would have been spiked at 1% volume for the highest load density.

LRVs of each virus fell within a range less than 0.30 $\log_{10}$, well within experimental variation (Table IV). No parameter was observed to impact virus clearance within the ranges tested as comparable removal in all test runs and target runs for each virus was observed.

Discussion

Because multiple antibody processes have been validated for viral clearance, it is now possible to systematically evaluate industry's experience. This has been attempted by CDER by building a database from regulatory submissions by multiple

Table IV. Removal of X-MuLV, MMV, and SV40 by protein A chromatography for mAb1 from multivariate study.

Run no.	Load density (g/L)	Load flowrate (CV/h)	X-MuLV LRV	MMV LRV	SV40 LRV
B	Center point		1.88	1.30	1.76
C	Low	Low	1.68	1.30	1.81
D	High	Low	1.83	1.13	1.96
E	Low	High	1.70	1.24	1.73
F	High	High	1.87	1.43	1.85
G	Center point		1.94	1.31	1.89

Run A is a control run without virus spiking, while all other runs are spiked with X-MuLV, MMV, and SV40. Runs B and G are target runs. Runs C–F are multivariate runs with 2-factor (load density and load flowrate) full factorial design and six parameters at worst case conditions. Parameters included wash buffer molarity (mM), wash flowrate (CV/h), wash phase duration (CV), elution buffer pH, elution flowrate (CV/h), and end pooling (CV).

companies. Large companies with multiple products like Genentech also have the capacity to perform a retrospective analysis of their accumulated data. In this report, we examined overall clearance of model viruses by Protein A chromatography as contained within the CDER and Genentech databases. It is clear that when evaluating data from a broad range of firms, the removal of multiple types of virus varies significantly between mAbs. In contrast, when evaluating one antibody in DoE studies (i.e., mAb 1), consistent RVLP removal (Tables II and III) and model virus removal (Table IV) by Protein A, were observed. This trend was maintained even across runs with differing process parameters, indicating that viral clearance variation is likely not due to process parameter effects (or at least those assessed in this study). Further analysis of different Genentech mAbs with the same purification process however revealed that viral clearance still varies significantly between mAbs (Fig. 1). As also suggested by the CDER database, this is likely a result of feedstock effects, a phenomenon that was also suggested at the 2009 Viral Clearance Symposium (Miesegaes et al., 2010a). For example, Amgen reported at the symposium that in their experience, both X-MuLV and MMV removal varied across different mAbs yet clearance in replicate studies was reproducible. It has been reported that different mAbs may form different complexes with host cell proteins (Sisodiya et al., 2012), it is unknown whether viruses would form complexes with mAbs or host cell proteins or both. It requires further investigation to determine whether these LRV-variations are caused by the biochemistry of the antibody or other HCCF components like host cell proteins or DNA. For instance, one could separate the impact of mAbs from that of host cell proteins on virus removal by using feedstocks with or without mAbs. From a practical standpoint, the variability in LRVs obtained from different mAbs employing the same purification process makes the protein A unit operation not suitable for modular virus validation, where viral clearance validation data for a specific process step for one mAb can be applied to another mAb.

The historical data mining of Genentech viral clearance results showed a correlation between the removal of the large retrovirus, X-MuLV, and the small parvovirus, MMV. These viruses are both routinely studied in virus removal validation studies. In general, X-MuLV LRV trends higher than MMV LRV; by 0.7 $\log_{10}$ on average. The range of the difference is 0–1.5 $\log_{10}$ (Fig. 2). Interestingly, the CDER database also showed that the mean Q-PCR-measured MuLV LRV was 0.7 $\log_{10}$ greater than MMV LRV based on 3.0 $\log_{10}$ versus 2.3 $\log_{10}$ in Figure 2 of the published article (Miesegaes et al., 2010a), largely corroborating the Genentech data analysis, although with more scatter.

The mechanism behind the observation of higher X-MuLV LRV compared to MMV LRV remains unclear. LRV is determined by the amount of virus that co-elutes with the antibody. This is determined by the sum of non-specific binding to the Protein A resin and interactions among the virus, the antibody and the Protein A resin. One of the possible explanations to different LRVs using different viruses relates to their size relative to the average pore-size of the chromatography beads. On the other hand, one must consider recent evidence arguing that binding of at least some viruses to the Protein A column is usually very weak (Bach et al., 2012), except in the presence of HCCF, arguing against the size-related pore-trapping hypothesis. This, and additional complexities due to HCCF components have made it difficult to predict or model virus behavior in a Protein A chromatography process. For instance, different viruses possess different physical and chemical properties, including shape, rigidity, net charge, charge distribution, hydrophobicity, binding affinity and kinetics to the Protein A ligand and the antibody. All of this is presumably influenced by the presence of mAb or HCCF components. Similar to host cell protein interactions with mAb demonstrated in Sisodiya et al., one could separate the impact of mAb from other HCCF components on the removal of each virus type by using purified mAb, or using HCCF with high levels of host cell proteins or DNA for example. Despite the complexity of the process, almost all the viruses evaluated here share the same partitioning pattern during a typical Protein A process (Table I), where the majority of the viruses flow through the Protein A column. The one counter-example appears to be Reo-3, where it has been reported that there is little virus in the flow through fraction (Miesegaes et al., 2010a), possibly related to its non-specific interaction to the column resins that associated with virus prep due to heterogeneity in the Reo-3 virus preparation. While efforts to improve virus spike preparation and QC procedures have been initiated due to known variations in prep quality and resulting impact on LRV (Bolton et al., 2005; PDA, 2010), standardization of Reo-3 preparations is likely to lag those of less complex viruses (e.g., parvovirus), and/or those more relevant at early-phase of development (e.g., retrovirus; Miesegaes et al., 2010a). Nevertheless, there is a correlation between the LRVs from X-MuLV and MMV in the case of the Genentech mAbs and to a lesser extent, in the CDER regulatory database. Therefore, for a specific mAb process, the removals of these two viruses

correlate with X-MuLV LRV likely to be higher than MMV LRV.

The QbD approach to bioprocessing (ICH Q8) can include the establishment of multivariate Design Space(s) for manufacturing processes, when feasible and warranted. To accomplish this, DoE process characterization studies are performed to evaluate process parameter effects, individually and in combination, on product quality and on process performance. The RVLP approach proposed in this article, for the first time to attempt QbD for protein A, presents a practical option to evaluate multiple process parameter effects on virus removal. Using the mAb 1 process as an example, RVLP clearance can be measured using samples generated during DoE-based process characterization studies. This is possible because the process space where consistent RVLP LRV occurs is within what was tested in a typical characterization study, while other tested process outputs were also within the acceptable range (data not shown). Furthermore, it can be argued that the lack of parameter effect demonstrated for RVLP clearance is applicable to all model viruses (X-MuLV, MMV and SV40). The evidence supporting this argument is (1) comparable removal measured by RVLP and X-MuLV shown in a previous publication (Zhang et al., 2009); (2) a similar partitioning pattern among all three virus types (Table I), indicating similar MOA of virus removal by the Protein A chromatography; and (3) the overall correlation of MuLV and MMV removal (Fig. 2), in conjunction with lack of parameter effects on RVLP removal (Tables II and III, Figs. 3 and 4). The lack of parameter effect on the removal of all model viruses was experimentally confirmed in a small DoE virus spiking study with consistent LRVs observed from each of the model viruses (Table IV).

There are many advantages of using this RVLP approach. First, it is certainly more desirable to study RVLP, a CHO endogenous component present during the actual production, and the source of the drug safety concern (ICH Q5A). Moreover, the DoE studies for viral clearance by a chromatography step become much more manageable and economic, since it uses the existing samples from characterization studies without the need for additional chromatography runs or virus-spiked runs. By doing so, this approach efficiently probes the MOA of viral clearance by affinity chromatography. It is particularly advantageous for this unit operation as the complexity and lack of understanding mechanisms makes modular virus removal claims for protein A unit operations untenable at the present time.

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