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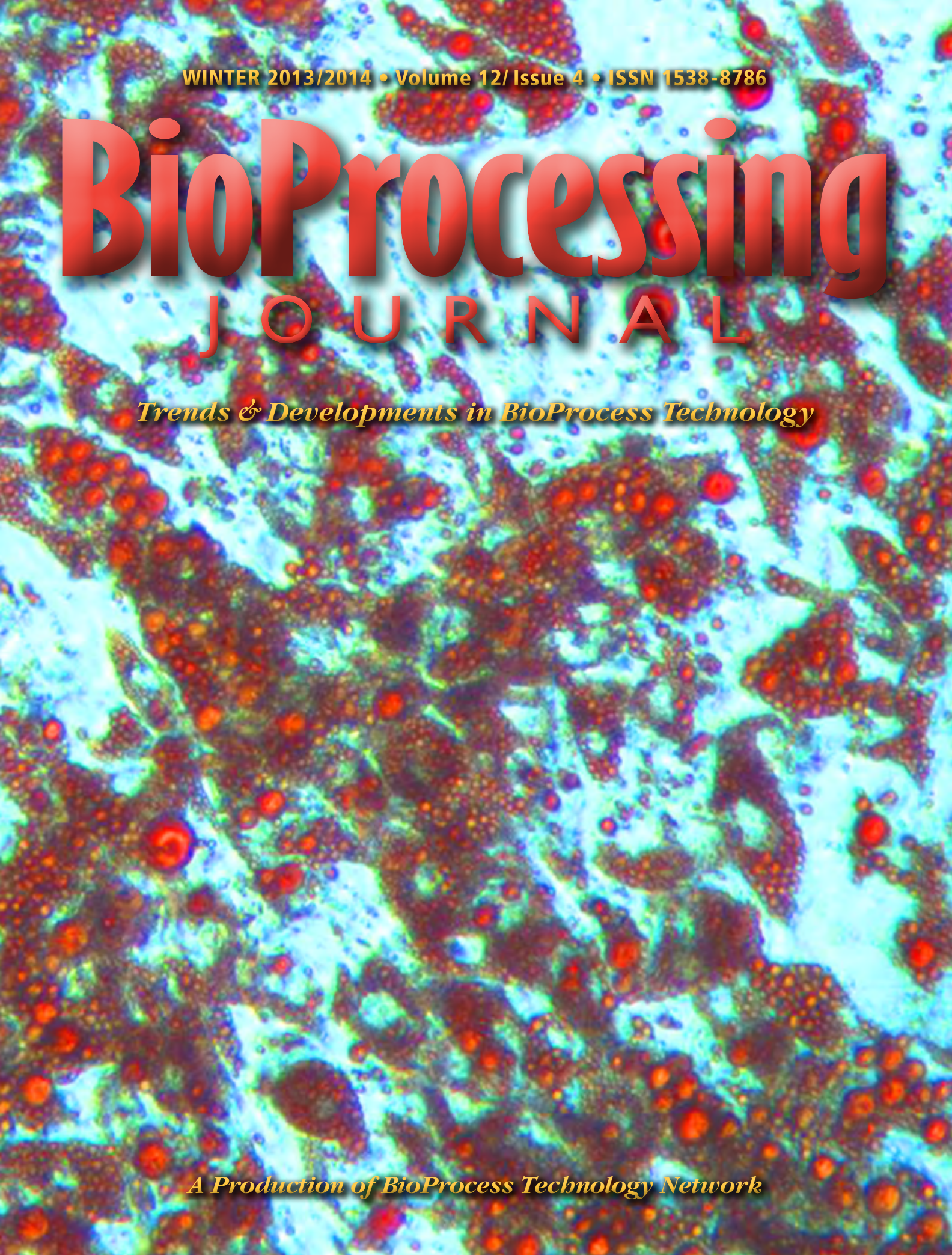

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TRENDS & DEVELOPMENTS IN BIOPROCESS TECHNOLOGY
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Log_{10} Reduction Factors in Viral Clearance Studies

By HORST RUPPACH

Introduction

Viral clearance studies are required for pharmaceuticals derived from human and/or animal sources such as recombinant proteins produced in eukaryotic cell lines, human blood products and vaccines, and even for some critical class III medical devices. It is mandatory to demonstrate that steps in the manufacturing process are capable of inactivating or removing potential viral contaminants. For this, a laboratory-scale (downscale) of the process step is developed and challenged with different model virus solutions. The viral concentrations are quantitatively determined in the feed material and the relevant product fraction. The ratio of both defines the reduction in virus and specifies the viral inactivation or viral removal capacity of the investigated process step.

In general, cell line-based infectivity assays like the plaque forming units (PFU) assay or the tissue culture infective dose ($TCID_{50}$) assay are used to quantify the virus infectivity in the process solutions of a viral clearance study. In some cases, the quantitative polymerase chain reaction (qPCR) may be used. The viral titer derived from the viral infectivity assays is represented by log_{10}/mL values. For instance, viral stock solutions used to challenge a process step typically have a viral titer of $>10^7$ log_{10} PFU/mL or $TCID_{50}$/mL. The calculated ratio of the viral titer in the starting material and in the relevant product fraction defines the viral reduction, called log_{10} reduction factor (LRF), log_{10} reduction value (LRV), or sometimes simply log_{10} clearance.

The mode of the LRF calculation is outlined in the relevant guidelines for viral clearance studies.[1-3] If possible, the 95% confidence limit is calculated based on the 95% confidence limits of the single viral titers (the 95% confidence interval of the viral infectivity in: [A] the starting material; and [B] the final product fraction using the formula $\sqrt{a^2+b^2}$).

Reduction factors are the result of viral clearance studies. They quantitatively describe the potential or capacity of the investigated process steps to remove or inactivate different types of viruses. The single values determined for each process step are summed for an overall (or cumulative) reduction factor, which specifies the overall viral reduction capacity of a manufacturing process. This is required for model viruses that are likely viral contaminants of a product. For those, the potential contamination level in the final product can be calculated or estimated from analytical and/or literature data. An additional safety margin is added to define the overall log_{10} reduction, which should be addressed for this specific model virus in the manufacturing process.[4]

This article focuses on the aspects that should be considered when evaluating the significance of reduction factors calculated for single process steps and the whole manufacturing process.

Log Reduction Factor Calculation Effects

With Individual Process Steps

Viral titers are determined by cell-based assays that are known to be variable. The accuracy and precision depends not only on the experimental parameters such as the dilution steps and the number of replicates, but also on the training of the operators and the quality of the critical materials, particularly the model virus and the indicator cell line. Assays to determine the virus titers must be validated, and critical materials like viruses and cell lines must be qualified. The 95% confidence limit for assay results should be ± 0.5 log_{10} or less, as specified in the guidelines.[1-3] The precision of the calculated reduction factors depends on the precision of the titer determinations but can still differ significantly between two runs (experiments), even if

derived from assays with high precision.

Table 1 shows three examples of typical results from duplicate chromatography runs with moderate virus removal capacity.

The duplicate runs of each example are two independently spiked experiments. The virus titers were defined in the starting material and the product fraction and the reduction factors were calculated. The reduction factors in example 1 are not significantly different after considering the 95% confidence limits. Example 2 is also not significantly different, although the difference is greater. Example 3 has reduction factors that differ by 0.8 $\log_{10}$, and the 95% confidence limits do not overlap. There is a significant difference between the two runs, indicating a low robustness of the investigated process step or low precise titer determination. However, the titers of the two load materials in experiment 3 don't differ significantly (see confidence limits). Also, the titers defined in the two product fractions are comparable. Interestingly, the titer of the load determined in run 2 is on the higher end of the titer range, but the titer of the relevant product fraction is on the lower end of the titer range. The opposite is found in run 1 where the load titer is on the lower end and the product titer is on the upper end of the titer range and this causes the significant difference in virus reduction factors.

This example clearly demonstrates the dependency of the reduction factor on the variations of the biological assays. Therefore, differences of reduction factors by $<1 \log_{10}$ should be considered carefully. Frequently, differences come from assay variations and are not due to a difference in virus removal/inactivation capacity of the investigated process step. This is also acknowledged in the guidelines that consider reduction factors on the order of 1 $\log_{10}$ not significant.[1,2]

With the Overall Process

The overall reduction factor of a manufacturing process is the sum of the single reduction factors determined at each process step. There are different methods to calculate this sum. Table 2 shows the murine leukemia virus (MuLV) $\log_{10}$ reduction factors obtained from four different process steps, validated in each of the two runs, then summed by different methods.

In mode 1, the single values of run 1 of each step and the single values of run 2 of each step are summed. As a result, two similar overall reduction factors are defined. In mode 2, the minimal numbers and the maximal numbers obtained from each step are added. In this case, the two overall reduction factors vary by more than 1 $\log_{10}$, but the maximal range of overall virus reduction of the manufacturing

TABLE 1. Examples of $\log_{10}$ reduction factor calculations.

PROCESS FRACTION	EXAMPLE 1		EXAMPLE 2		EXAMPLE 3	
	Total Load ± 95% Confidence Limit $TCID_{50}$ [$\log_{10}$]					
	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2
Load	9.43 ± 0.21	9.72 ± 0.17	9.16 ± 0.27	9.50 ± 0.19	8.85 ± 0.22	9.22 ± 0.20
Eluate	7.12 ± 0.14	7.25 ± 0.19	7.14 ± 0.25	7.05 ± 0.20	7.32 ± 0.24	6.89 ± 0.25
Reduction Factor	**2.31 ± 0.25**	**2.47 ± 0.25**	**2.02 ± 0.37**	**2.45 ± 0.28**	**1.53 ± 0.33**	**2.33 ± 0.32**
Difference	**0.16**		**0.43**		**0.80**	

TABLE 2. Different modes of overall reduction calculation. Mode 1: summation of the single factors of run 1 and the single factors of run 2. Mode 2: summation of the lowest single factors and the highest single factors of each process step to determine the maximal and minimal overall reduction.

PROCESS STEP	MODE 1		MODE 2	
	Reduction Factor ± 95% Confidence Limit [$\log_{10}$]			
	Run 1	Run 2	Minimal	Maximal
Affinity Chromatography	2.01 ± 0.24	1.87 ± 0.23	1.87 ± 0.23	2.01 ± 0.24
pH Inactivation	5.02 ± 0.24	5.50 ± 0.26	5.02 ± 0.24	5.50 ± 0.26
AEX Chromatography	≥ 6.05 ± 0.21	≥ 5.77 ± 0.19	≥ 5.77 ± 0.19	≥ 6.05 ± 0.21
Virus Retentive Filtration	≥ 5.65 ± 0.17	≥ 5.81 ± 0.15	≥ 5.65 ± 0.17	≥ 5.81 ± 0.15
Cumulative Reduction Factors	**≥ 18.73 ± 0.43**	**≥ 18.95 ± 0.42**	**≥ 18.31 ± 0.42**	**≥ 19.37 ± 0.44**

process is better represented. The minimal number is the lowest overall reduction and the maximal number the highest overall reduction. Considering the 95% confidence limits of the reduction factors in mode 2, it covers a range of approximately 2 $\log_{10}$ (17.89–19.81) versus a range of 1 $\log_{10}$ (18.30–19.37) in mode 1. Mode 2 better reflects the biological assay variations and more clearly specifies the range of overall reduction. Mode 1 compensates for the variance of the single values. Therefore, the real range of overall reduction may be obscured. Calculating the mean values of each step before defining the overall reduction leads to the calculation shown in mode 3 (Table 3), which determines one value for the whole process. The overall mean value is comparable to the range in mode 1 and smaller than in mode 2, which, however, may better represent the real variance of overall reduction.

In summary, reduction factors in a single step can differ by up to 1 $\log_{10}$ and can be caused by the typical variations of biological assays. It does not automatically point to any significant difference or low robustness of the analyzed process step. This variation should also be considered when comparing data from two studies of the same process steps performed at different laboratories. Such differences can cumulate in the overall reduction factor of a manufacturing process. Presenting the maximal range of overall reduction provides the most transparency and reflects the assay variances typical for biological assays.

TABLE 3. Mode 3: summation of mean values of each process step leading to a mean overall reduction.

PROCESS STEP	MODE 3
	Reduction Factor ± 95% Confidence Limit [$\log_{10}$]
	Mean
Affinity Chromatography	1.95 ± 0.33
pH Inactivation	5.32 ± 0.35
AEX Chromatography	≥ 5.93 ± 0.28
Virus Retentive Filtration	≥ 5.74 ± 0.23
Cumulative Reduction Factors	**≥ 18.94 ± 0.60**

Dependency of Reduction Factors on Viral Load and Assay Sensitivity

The reduction factor is a suitable parameter to describe the viral reduction potential or capacity of a process step. A high number represents a high potential, and a low number represents a reduced potential. A reduction to the limit of detection indicates a higher potential than is demonstrated. In the latter case, and for all process steps with high viral reduction capacities, the reduction factor depends on the experimental conditions. A viral clearance study should be designed so that a high reduction potential can be demonstrated in principle. The dynamic range, the principle reduction factor, which can experimentally be demonstrated, depends on two factors: the viral load in the starting material and the sensitivity of the assay applied for the relevant product fraction.

Viral Load

The viral load is determined by the titer of the viral stock solution used to spike the intermediate process material and the spike ratio. Both should not significantly change the starting material composition and subsequent process parameters. Highly purified viral stock preparations may allow for high spike ratios (up to 10%) without any impact to the downscale. The same can be achieved with high-titer viral stocks at a low spike ratio. In principle, the combination of both would allow for extremely high loads.

The ICH Q5A and EMA guidelines[1,2] recommend a viral load "as high as possible"[5] or "sufficient."[6] The EMA guideline also indicates risk for a load that is too low.

The EMA guideline gives examples of "effective" virus reduction combined with reduction factors.[7] It is important to understand that effectiveness is not reduced to the reduction factors—only this was and still is misinterpreted. The virus reduction potential or capacity (not effectiveness) expressed by reduction factors can be ranged as shown:

≤1 $\log_{10}$	*Not significant*
1–2 $\log_{10}$	*Indicative/contributable*
2–4 $\log_{10}$	*Moderate*
>4 $\log_{10}$	*High*

This may lead to an overestimation of the viral removal capacity in adsorptive process steps.[8] What is the significance of a sufficient load? Should extremely high viral loads such as 10^8–10^9 $\log_{10}$/mL, $TCID_{50}$, or PFU be adjusted in viral clearance studies? There is a significant risk that extremely high viral loads will impact the downscale, particularly at high spike ratios. Another consideration is the relevance of extremely high loads in viral clearance studies in relation to contamination situations that may arise in the manufacturing process. It can be calculated for some potential contaminants but not for unknown contaminants. For most recombinant products and mAbs produced in an eukaryotic cell line, categorized case A and B according to ICH Q5A,[1] the risk of extremely high loads in intermediates of the purification process will be low. A very high load of a viral contaminant in the bulk harvest would probably be detected upstream, as reported for most of the published contamination cases. Rather, a low to medium contamination level

may enter the purification process undetected. Particularly for the small virus retentive filtration step frequently placed at the end of the downstream process, an extremely high viral contamination load is unlikely. Applying extremely high loads for this specific step may not adequately reflect a real contamination case. In addition, very high viral particle concentrations may impact the downscale even if highly purified virus stocks are used.

In summary, there is always a risk that extremely high viral loads may impact the downscale of the investigated process and may not simulate a real contamination situation. Loads in the range of 10^5–10^7 $\log_{10}$/mL, $TCID_{50}$, or PFU in the starting material are generally high enough to challenge a process step and ensure a sufficient dynamic range, particularly when combined with high-sensitivity assays.

Assay Sensitivity

The use of high sensitivity assays with the final product fraction is another method to ensure a high dynamic range of reduction, even at limited viral loads. The sensitivity of the infectivity assay depends on the volume of the original process fraction, which can be screened for viruses in a cell culture. In large volume plating (LVP), or bulk assays, a high volume of the non-cytotoxic and non-interfering dilution of the process sample is given to the detector cell line and the cultures are monitored for virus-specific effects. The minimal dilution defines the volume of the process solution that can be reasonably screened for virus. If the product fraction sample requires only a low dilution, a considerable amount of the original process solution may be assayed. The larger the volume screened, the higher the sensitivity and the higher the reduction factor that may be demonstrated. The benefit of this method is that it has no impact on the process itself.

Improving the sensitivity may be helpful in determining the virus reduction capacity. Figures 1 and 2 show how improvement of the detection limit led to a higher reduction factor and the identification of the real viral reduction capacity. The low pH inactivation of MuLV, in an antibody solution derived from a Protein A purification step, was analyzed in four different runs. The kinetics of inactivation were the same in all of the runs. Two different volumes of the final two time points were analyzed in the LVP assays, leading to two different sensitivities: 0.49 $\log_{10}$/mL (Figure 1) and –0.81 $\log_{10}$/mL (Figure 2). In Figure 1, the infectivity reached detection limits and the reduction factors ranged from ≥ 5.29 to ≥ 5.73 (Table 4). When a larger volume was analyzed, residual infectivity was found in the final two samples of all runs. A larger reduction by almost 1 $\log_{10}$ (6.29–6.69) could

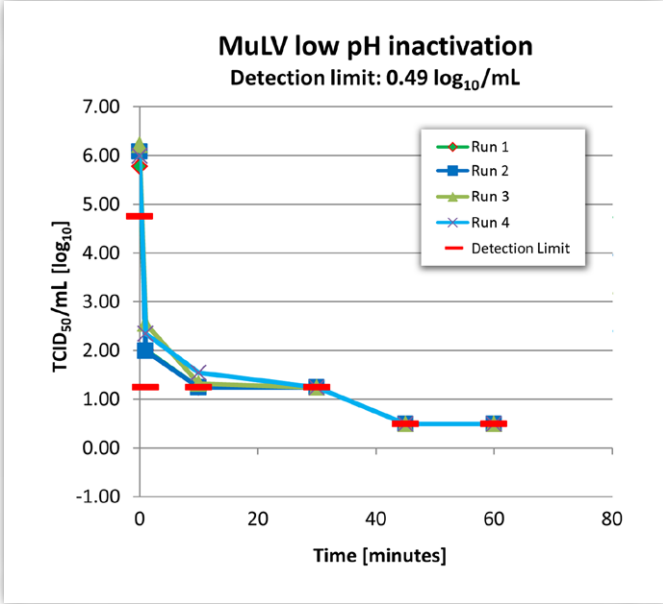

FIGURE 1. Kinetics of MuLV low pH inactivation (detection limit at 0.49 $\log_{10}$/mL).

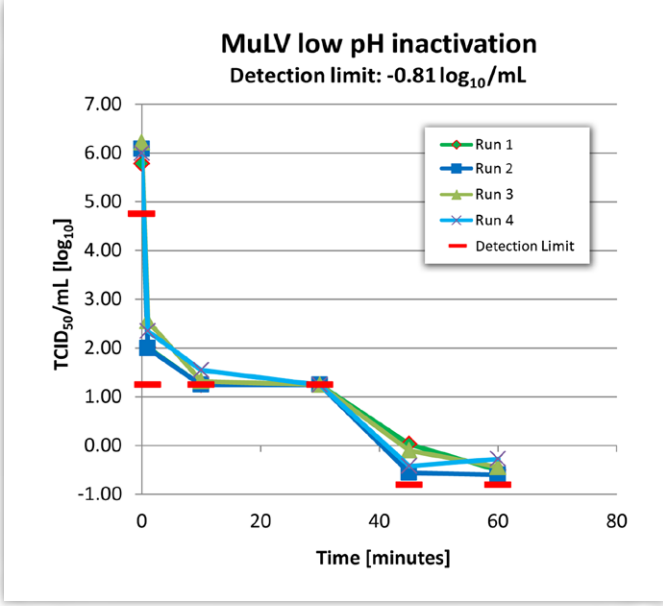

FIGURE 2. Kinetics of MuLV low pH inactivation (detection limit at –0.81 $\log_{10}$/mL).

TABLE 4. Reduction factors calculated for MuLV at two different detection limits after 60 minutes of low pH inactivation.

MuLV Sensitivity	Log_{10} Reduction Factors After 60 Minutes			
	Run 1	Run 2	Run 3	Run 4
Low 0.49 $\log_{10}$/mL	≥ 5.29	≥ 5.59	≥ 5.73	≥ 5.50
High –0.81 $\log_{10}$/mL	6.29	6.69	6.66	6.27

be demonstrated (Table 4) but it also revealed the actual potential of the analyzed low pH treatment—a reduction in a range of 6.5 $\log_{10}$. In addition, at a higher sensitivity, the biphasic inactivation mode was better demonstrated.

Instead of applying extremely high loads, with the risk of impact to the downscale or creating an artificial load that may not reflect the real situation, the improvement of the sensitivity level may provide both higher reduction factors and demonstrate the inactivation or removal capacity of the investigated process step. Of course, cytotoxicity or interference effects can limit the volume that may be reasonably screened for virus and reduce the sensitivity of the detection assay. In such cases, a high viral load in the starting material is required to ensure a dynamic range suitable to demonstrate the viral reduction capacity.

Reduction Factors and Viral Reduction Effectiveness

Reduction factors are numbers that describe the potential or capacity of a process step to remove or inactivate viruses. High numbers indicate a high potential and low numbers indicate a low potential. However, reduction factors should not be equal to viral reduction effectiveness. The viral reduction effectiveness depends on many additional factors, and reduction factors are only one aspect. This is demonstrated by the following two examples.

Table 5 shows the reduction factors determined for the two enveloped viruses, MuLV and bovine viral diarrhea virus (BVDV), after 30 and 60 minutes of low pH treatment.

TABLE 5. Reduction factors determined for the two enveloped model viruses MuLV and BVDV after 30 minutes and 60 minutes of low pH treatment.

Time of Treatment	Log_{10} Reduction Factors			
	MuLV		BVDV	
	Run 1	Run 2	Run 1	Run 2
30 minutes	≥ 4.28	≥ 4.58	2.56	3.11
60 minutes	≥ 4.28	≥ 4.58	≥ 4.42	≥ 4.82

The reduction factors are the same for both viruses and no residual infectivity was determined after 60 minutes of treatment. The viral reduction capacity is the same for both MuLV and BVDV. Thirty minutes earlier, significant infectivity was determined for BVDV but not for MuLV. Though the capacity of viral inactivation was the same after 60 minutes, MuLV was more effectively inactivated than BVDV. It demonstrates that the kinetics of inactivation, in addition to the reduction factor, is an important parameter that defines the effectiveness of a viral inactivation step. A large reduction factor does not automatically make a process step highly effective, and it shows why the demonstration of the viral inactivation kinetics is important and specified in the relevant guidelines.[1,2] For note, in order to demonstrate robust viral inactivation, high sensitivity assays should be applied to the two final time points (see also Figures 1 and 2).

Another example is the viral reduction validation of two model viruses, MuLV and minute virus of mice (MVM), on a chromatography step performed in the binding mode (Figure 3). In addition to the elution product of the peak fraction, small fractions immediately before and after peak collection were analyzed for

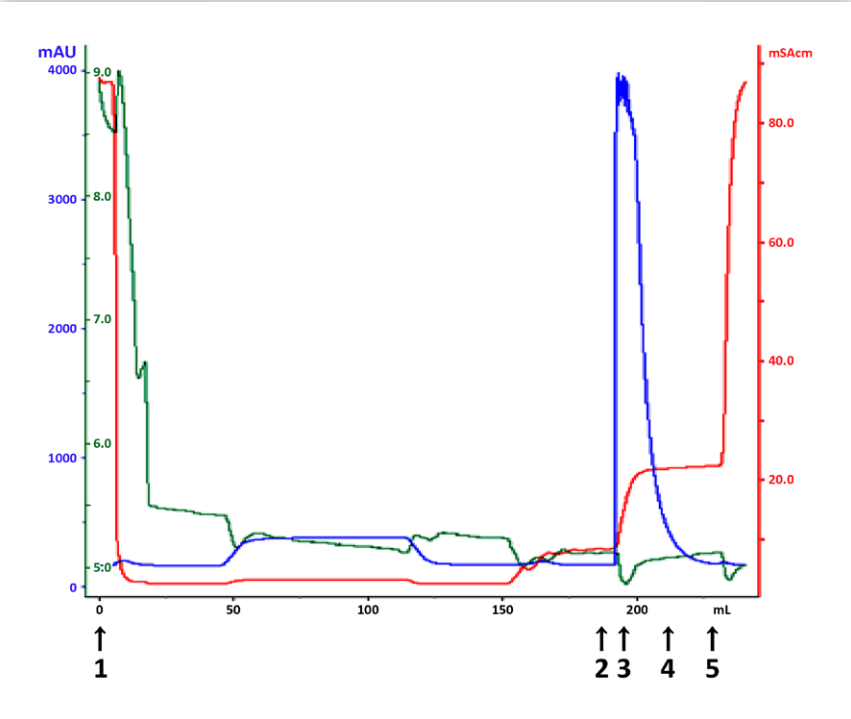

FIGURE 3. A chromatography step performed in a binding mode. The numbers indicate the starting point for the different fractions collected during the chromatography step.

virus content to determine the robustness of viral removal.

For both viruses, large reduction factors and no residual infectivity were demonstrated in the product peak fraction (see Table 6). Also, no residual infectivity was measured in the pre-elute and post-elute fraction of the MVM run, indicating a robust removal that will not be affected by slight changes in the product peak collection. No residual infectivity was determined in the pre-elute fraction of MuLV, but significant infectivity was found in the post-elute sample. The virus that elutes shortly after peak product collection indicates a risk that residual infectivity may enter the peak product fraction. Both viruses had high reduction levels, to the limit of detection, in the product fraction. However, the effectiveness of virus removal was different—MVM was more effectively removed than MuLV (Table 6).

In summary, reduction factors describe the viral reduction capacity of a process step, but high reduction factors do not automatically characterize a process step highly effective.

TABLE 6. Viral loads and reduction factors determined for MVM and MuLV model viruses.

PROCESS FRACTIONS	MVM		MuLV	
	Total Viral Load Per Fraction	Reduction Factor	Total Viral Load Per Fraction	Reduction Factor
	$TCID_{50}$ [log_{10}]	[log_{10}]	$TCID_{50}$ [log_{10}]	[log_{10}]
Load	8.81	—	7.98	—
(1) Flow-Through/Wash	≤ 4.01	≥ 4.80	≤ 4.01	≥ 3.97
(2) Pre-Elute	≤ 1.19	≥ 7.62	≤ 1.29	≥ 6.69
(3) Product Peak	**≤ 2.29**	**≥ 6.52**	**≤ 2.40**	**≥ 5.58**
(4) Post-Elute	≤ 1.09	≥ 7.72	3.67	4.31
(5) Strip	9.22	−0.41	6.87	1.11

Overall Reduction Factors and Viral Reduction Effectiveness

The viral reduction capacity of some process steps are very high, based on the nature of clearance and the reduction factor, which can be demonstrated, only depends on experimental conditions. For instance, the main mechanism of viral removal by small virus retentive filters is based on small pore size (approximately 20 nm). Accordingly, the capacity to remove medium- to large-sized enveloped viruses, like MuLV (90–100 nm), is very high. A breakthrough is usually not observed, and the possible reduction factor depends only on the load in the starting material and the sensitivity of the assay used on the filtrate. In principle, very high reduction factors may be calculated by applying high viral loads and using high-sensitivity assays. This would be attractive when virus, virus-like particles, or retrovirus-like particles (risk case B-E in ICH Q5A[1]) must be addressed in the viral clearance study and a specific overall reduction factor must be achieved, based on the risk assessment. Extremely high reduction factors would make it easier to meet the specified cumulative reduction. A required overall reduction of 15 log_{10} for an enveloped virus, for instance, can be achieved by two clearance steps, each showing a clearance of 7.5 log_{10}, or by three steps, each showing a clearance of 5.0 log_{10}. Reduction factors >7 log_{10}/mL will require high viral loads in the starting material and a high sensitivity assay for the product sample. The drawbacks of very high loads were outlined earlier, and the sensitivity of assays is sometimes limited by cytotoxicity and interference of the filtrate. But more importantly, the two-step approach would focus the entire viral reduction efficacy too much on the reduction factors. As previously outlined, reduction factors are only one aspect in the qualification of effective virus reduction. For an entire manufacturing process, the effectiveness of overall clearance depends on additional factors, including:

- The number of process steps analyzed
- How many steps showed reduction to the limit of detection
- The different principles of virus reduction applied
- The nature of the process steps analyzed

Though the reduction factors in the three-step approach are significantly lower (5.0 log_{10} versus 7.5 log_{10}), the effectiveness of viral reduction is better demonstrated. Instead of only two, three principles of viral reduction are analyzed. All three steps show a high removal/inactivating potential—even the numbers are smaller than in the two-step approach. One step in both approaches should include small virus retentive filtration. Small virus retentive filtration

is generally recognized to be a robust viral removal step based on its size exclusion principle. Large enveloped viruses are always removed to the limit of detection. The reduction factor that can be demonstrated depends on the experimental conditions only. Demonstration of ≥ 5.0 $\log_{10}$ or ≥ 7.5 $\log_{10}$ will not make an important difference in the efficacy of viral reduction through this step.

In summary, virus safety through viral reduction in the manufacturing process is better demonstrated by a documented three-step approach and high reduction factors, rather than a two-step approach and extremely high reduction factors. The same will be true for four-step or even five-step approaches. High reduction factors will probably not be demonstrated for all steps, but required overall reduction will usually be achieved. Identifying the reduction potential of many different viral reduction principles in the manufacturing process provides a better understanding of virus safety through viral clearance and increases the safety of the product. The more comprehensive information can be valuable to assess the impact to the viral risk in case of changes or unexpected occurrences in the production process of a biologic.

Summary

The reduction factor is an important parameter to define for the capacity of a single process step to remove or inactivate viruses. The overall reduction factor is the sum of the single reduction factors. It is calculated to describe the overall removal and inactivation capacity of the manufacturing process and address a risk assessment made for viruses, virus-like particles, and retrovirus-like particles that are identified or relevant for the product. Reduction factors are calculated from virus titers with typical variances for infectivity assays. Accordingly, reduction factors may vary significantly, and this should be considered when comparing results and calculating the overall clearance capacity of an entire process.

The extent of the single $\log_{10}$ reduction factors depends first on the process step capacity and secondly on experimental conditions, such as the viral load in the spiked starting material and the sensitivity of the product-relevant virus quantification assays. It is important to understand that different results in $\log_{10}$ reduction factor numbers derived from different laboratories for the same process step and process can be caused by assay variations, the different experimental conditions applied, and the statistics used to calculate titer, particularly titers at low infectivity. The last aspect can significantly contribute to differences in the results between laboratories. This aspect was not discussed in this article due to its complexity and should be examined in a future article.

Although reduction factors are suitable to quantitatively describe viral removal or the inactivating capacity of an investigated process step, they do not sufficiently define the viral reduction effectiveness. The definition of effectiveness requires the consideration of many parameters such as:

- The appropriateness of the test viruses used
- The design of the clearance studies (orthogonal principles of reduction)
- The $\log_{10}$ reduction achieved
- The time-dependence of inactivation
- The potential effects of variation in process parameters on virus inactivation/removal (robustness)
- The limits of assay sensitivities, and
- The possible selectivity of inactivation/removal procedure(s) for certain classes of viruses

These are detailed and explained in the EMA and ICH guidelines.[1,2]

The $\log_{10}$ reduction factor is an important parameter to quantify the viral reduction capacity of both single process steps and the entire production process. These should, however, not be overstated in the evaluation of the viral reduction effectiveness. Extremely high loads, apart from their relevance, and extremely high reduction factors may increase the overall reduction but do not provide significantly higher virus safety.

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[1] ICH Harmonized Tripartite Guideline Q5A (R1), 1999: *Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin*.

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[3] FDA (CBER), 1997: *Points to consider in the manufacturing and testing of monoclonal antibody products for human use*.

[4] See reference [1], specifically appendix 5, p 29.

[5] See reference [2], specifically chapter 5.9, p 7.

[6] See reference [1], specifically chapter 6.2.3, p 12.

[7] See reference [2], specifically chapter 6.2, p 8.

[8] See reference [2], specifically chapter 7.2, p 10.

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