

Validating Virus Clearance in a Single-Use System

Part 1: Film Adsorption and Virus Inactivation Studies

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ABSTRACT

Single-use technology offers a number of benefits over traditional stainless-steel equipment, but manufacturers must be confident that single-use technology is appropriate for their most critical and aggressive processing steps. In this study, the use of a single-use mixing technology was evaluated and characterized for the performance of a detergent-based virus inactivation step during a monoclonal antibody production process.

Monoclonal antibodies (mAbs) are a leading class of biopharmaceuticals and can be used to treat many life-threatening diseases (1). A potential threat to patient safety is the presence of viruses in the finished biopharmaceutical product (2). Regulatory authorities require the process to be evaluated for the inactivation or removal of virus contaminants prior to Phase I studies, and where possible, for two orthogonal steps to be assessed as part of this validation (3). Various approaches can be used for the inactivation of viruses, such as low pH holds, solvent-detergent treatments, and UV inactivation (4).

Increasingly, the manufacture of biologics is being performed with single-use bioprocessing technology, which offers a number of benefits over traditional stainless-steel equipment. These advantages include greater flexibility, lower capital costs, and a reduced risk of product cross-contamination. Biomanufacturers must be confident that single-use technology is appropriate for their most critical and aggressive processing steps by being able to generate and review comprehensive data packages in partnership with their single-use equipment suppliers.

LFB Biomanufacturing is a contract manufacturing organization (CMO) specializing

in the manufacture of mAbs and recombinant proteins produced by mammalian cell culture. The company has recently invested in additional production capacity and is establishing a single-use production platform for the manufacture of mAbs including a detergent-based virus inactivation step. The company chose single-use mixing bags over stainless-steel tanks because they operate a multi-product facility using product-dedicated virus inactivation vessels. Using single-use mixers therefore decreases the investment required in having to purchase and validate a cleaning procedure for multiple stainless-steel mixers.

In this two-part article, the authors describe the testing performed to demonstrate the applicability of single-use mixing technology for a virus inactivation step performed using detergent (Triton X-100, MilliporeSigma).

The study was performed in a number of stages. Part one of this paper describes the study of the inactivation of enveloped viruses (xenotropic murine leukemia virus [X-MuLV], bovine viral diarrhea virus [BVDV], and pseudorabies virus [PRV]) by detergent (Triton X-100, MilliporeSigma) treatment within a single-use bag (Flexel, Sartorius Stedim Biotech). Part two of this paper



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will describe chemical compatibility and leachable studies performed on the single-use bags for magnetic mixers in the presence of detergent (Triton X-100, MilliporeSigma). To demonstrate the scalability of this approach, film adsorption and detergent homogeneity in a 50-L magnetic mixer were evaluated.

Experiments were performed by LFB Biomanufacturing in collaboration with Sartorius Stedim Biotech Confidence Validation Services.

CHEMICAL INACTIVATION BY DETERGENT

Solvent or detergent treatments for virus inactivation were originally developed for use in the manufacture of blood-derived medicinal products (5). They are an attractive option because they do not require expensive reagents or expensive equipment. The method is widely used and inactivates enveloped viruses by solubilizing the viral envelope's lipid membrane structure, thus preventing virus from binding to or infecting cells. The potency of recombinant proteins such as mAbs is typically unaffected because the detergent targets lipids and lipid derivatives rather than proteins (4). Virus inactivation treatments by detergents such as Triton X-100 (MilliporeSigma) are robust with respect to temperature, enabling these steps to be performed at room temperature without thermoregulation. The solvent that has been added must be removed from the final product, which is normally achieved by subsequent chromatography steps.

Low pH inactivation is also a commonly used method for virus inactivation during the production of mAbs. Neither low pH inactivation nor detergent treatments are effective against non-enveloped viruses such as parvo virus.

A previous study performed by LFB Biotechnologies, however, demonstrated that unlike low-pH inactivation, detergent (Triton X-100, MilliporeSigma) is effective for the inactivation of BVDV. An effective step is deemed one that provides a reduction factor of the order of 4 \log_{10} and is unaffected by small perturbations in process variables (6). The results are summarized in **Table I**.

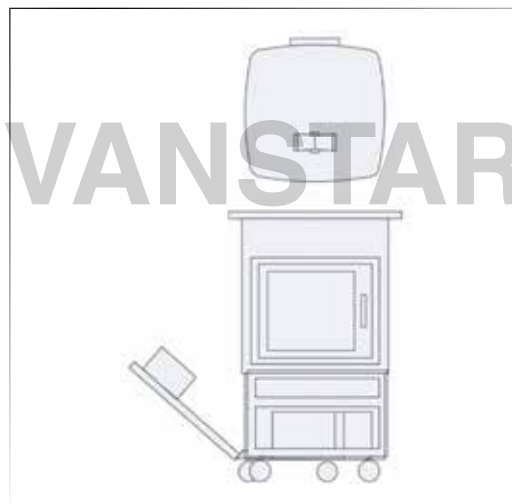
Manufacturers of biopharmaceuticals should consider the impact of selecting different processing techniques on product quality. LFB Biomanufacturing has internal data to show that their purification process is capable of producing a drug product with >99% antibody monomer and a detergent concentration that is below the level of detection.

Table I. Inactivation of bovine viral diarrhea virus by detergent treatment and low pH incubation.

	Detergent (Triton X-100, MilliporeSigma)		Low pH	
Initial viral load (\log_{10} TCID ₅₀ /mL*)	5.82	5.98	5.87	5.73
Virus titer after one hour (\log_{10} TCID ₅₀ /mL*)	< 2.23	< 2.23	4.95	5.31
Reduction factor (\log_{10})	> 3.59	> 3.75	< 1	< 1
Effectiveness	Effective		Not effective	

*TCID₅₀: 50% tissue culture infective dose

Figure 1. Schematic illustration of a single-use bag magnetic mixer (Sartorius Stedim Biotech). This product uses patented magnetic mixer technology (Pall).



STUDY DESIGN

At production-scale, the virus inactivation of a neutralized Protein A mAb-eluate is performed using detergent (Triton X-100, MilliporeSigma). The inactivation is performed at a final concentration of 1% (w/v) detergent for one hour at room temperature (20 ± 5 °C), in a magnetic mixer (Sartorius Stedim Biotech) (see **Figure 1**). A typical processing volume of 85 L of mAb is treated in a 200-L single-use bag (Flexel, Sartorius Stedim Biotech). To allow agitation, the bag includes a central magnetic impeller assembly, which is coupled to the magnetic mixer drive unit and rotates on a low-friction, inert bearing assembly designed to allow high mixing efficiency while avoiding particle shedding.

All figures courtesy of the authors.

Table II. Rational for the parameters selected during the studies.

Experiment	Process condition	Adsorption	Virus clearance
Bag size	200 L	75 mL	75 mL
Detergent (Triton X-100, MilliporeSigma) concentration (w/v %)	0.9 to 1.2	1	0.7
	Process condition	Process condition	Worst case
Surface volume ratio (cm ² /mL)	0.2	3.5	3.5
	Process condition	Worst case	Worst case
Contact time (hours)	1	2	1
	Process condition	Worst case	Process condition
Temperature (°C)	20 ± 5	20 ± 5	14±1
	Process condition	Process condition	Lower limit
Continuous mixing	Yes	Yes	Yes

Table III. Characteristics of commercial and scale-down single-use bags (Flexel, Sartorius Stedim Biotech).

Material description	200-L single-use bag for magnetic mixer	50-L single-use bag for magnetic mixer	75-mL scale-down bag
Study	In process	Detergent** homogeneity during mixing and adsorption	Detergent** film adsorption, virus inactivation studies
Film material	S40 film	S40 film	S40 film
Configuration	3D	3D	2D
Sterilization	Irradiation at 25-45 kGy	Irradiation at 25-45 kGy	Irradiation at 25-45 kGy
Agitation method	Magnetic mixer	Magnetic mixer	Orbital shaker
Working volume	130 L	24 L	Variable due to sampling
Agitation rate	55 rpm	50 rpm	20 oscillations/min*
Surface/volume ratio	0.2 cm ² /mL	0.2 cm ² /mL	3.5 cm ² /mL
Impeller material	Yes	Yes	Fragment

*The agitation rate has not been monitored during the viral inactivation study. It was set to 20 oscillations/min for the Triton X-100 film adsorption study.

**Triton X-100, MilliporeSigma

This bag is made from S40 film (Sartorius Stedim Biotech), which is a multi-layer film, including a gas barrier made of ethylene vinyl alcohol copolymer and a contact layer made of polyethylene.

All vessels in the study were agitated at the maximum possible rate to ensure efficient mixing without causing vortex formation. The critical parameters for the inactivation step are the detergent concentration, product-detergent contact time, the film-surface area to product-volume ratio, and the temperature. The different studies were performed under worst-case conditions for contact time applied over adsorption, chemical compatibilities, and leachable studies (see **Table II**).

DESCRIPTION OF THE SCALE-DOWN MODELS

Virus clearance studies cannot be performed at production scale, as GMPs prevent the deliber-

ate introduction of viruses in the manufacturing area. Validation should be conducted in an adequate laboratory equipped for virology work using a scale-down model of the production process. Performance of the scale-down unit operation should represent, as closely as possible, the production procedure and reproduce critical process parameters that have significant effect on virus clearance (6). These studies are often performed in glass bottles that have the advantage of being stirred vessels and therefore somewhat similar to large-scale mixers. The disadvantages of using glass bottles are that the construction materials of the production-scale mixer are not mimicked, and possible interactions between the process and the container will not be identified. In this study, a 75-mL scale-down bag was used for the scale-down experiments. Adequate mixing that was representative of the large scale was ensured by visual inspection.

These scale-down models were representative of the single-use bags for the magnetic mixer. They were manufactured under equivalent process conditions, within the same class ISO 7 cleanroom environment, under the same ISO 9001 quality system, and were subjected to the same release testing.

An equivalent welding process was used to attach tubing to the scale-down bag chamber as is used for single-use bags (Flexel, Sartorius Stedim Biotech). Additional impeller parts, manufactured from representative construction materials, were added inside the scale-down bag to mimic the contact between the processing solution and the magnetic mixer impeller. The characteristics of the different bags used in the study are shown in **Table III**.

ADSORPTION AND INACTIVATION METHODS

An evaluation of detergent adsorption onto S40 film was conducted before virus-spiking studies could be performed. Significant binding could lead to a reduction in the concentration of detergent and limit the inactivation of viruses. Both film adsorption and virus-spiking studies were performed in 75-mL single-use bioprocess containers (Flexel, Sartorius Stedim Biotech).

Film adsorption study

To perform the film adsorption studies, a 75-mL single-use (Flexel, Sartorius Stedim Biotech) bag was filled with 63 mL of a 1% detergent (Triton X-100, MilliporeSigma) solution. Mixing generated by an orbital shaker was optimized to create a movement of the liquid and impeller without generating either a vortex or foam. Once effective mixing was established, the 1% detergent solution was incubated for 120 minutes at room temperature (20 ± 5 °C). Samples were periodically taken after 0, 30, 60, and 120 minutes incubation, and the concentration of detergent measured by high-performance liquid chromatography (HPLC).

A control experiment was performed in parallel by repeating the experiment in a 0.5-L glass bottle filled with 316 mL of 1% detergent solution. Mixing of the solution was performed using a magnetic mixer and a magnetic stirring bar. Samples were taken after 0 and 120 minutes.

Virus inactivation study

Virus inactivation studies were performed using samples from the manufacturing processes of two different mAbs: mAb A and mAb B. Samples of both processes were taken from the neutralized eluates of Protein A chromatography steps.

Table IV. Comparison of detergent (Triton X-100, MilliporeSigma) treatment conditions at production and small-scale of monoclonal antibody (mAb) A and mAb B.

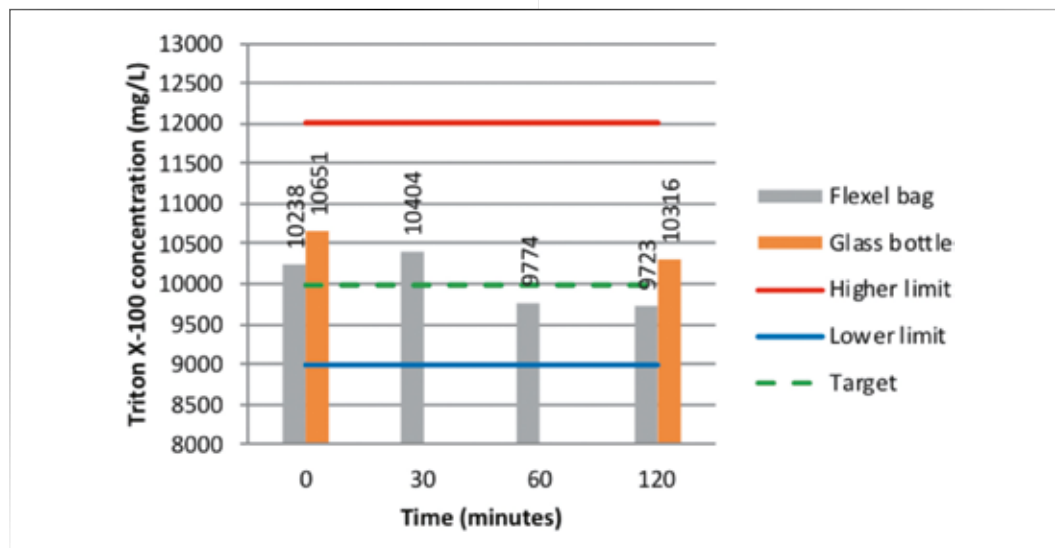
Parameter	Operating conditions at production-scale	Operation conditions at small-scale
Final detergent concentration (% w/v)	1	0.7
Duration (hours)	≥ 1	1
Temperature (°C)	20 ± 5	$14 \pm 1^*$
Incubation	Agitated	Agitated

*Except for mAb A; the experiment was performed under the worst-case condition of the process with respect to detergent concentration and duration (0.7% w/v detergent [Triton X-100, MilliporeSigma] for exactly one hour) and normal condition of the process for the temperature (20 ± 5 °C) for one of the two experiments.

The effectiveness of a detergent (Triton X-100, MilliporeSigma) treatment to inactivate viruses was evaluated according to the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) and European guidelines (3, 6). The detergent target concentration was 1% (w/v), and the specified limits were 0.9 and 1.2% (w/v). The protein concentrations of samples were less than 10 g/L. X-MuLV, BVDV, and PRV viruses (BioReliance) were used in the inactivation studies. All three viruses are enveloped and have either low or low-to-medium resistance to physical or chemical treatment. Viruses were quantified by end-point dilution in 96-well plates with the appropriate cell line (PG-4 [X-MuLV], MDBK [BVDV], Vero [PRV]). The mAb A sample was spiked with X-MuLV while the mAb B sample was spiked with all three viruses.

Inactivation experiments were performed in the 75 mL single-use bioprocess container. A summary of the operating conditions for the production-scale and small-scale detergent treatments is shown in **Table IV**. Small-scale experiments were performed below the specified limits for temperature, hold duration, and final detergent concentration. These were deemed “worst-case” conditions. Furthermore, although the specified lower concentration limit for detergent was 0.9% (w/v), an additional 20% security margin was applied during the virus-spiking study. This provides additional assurance by allowing data to be generated at a concentration as low as 0.7% (w/v), such that the design space studied exceeded specified limits.

Figure 2. Detergent (Triton X-100, Millipore Sigma) concentration in 75-mL single-use (Flexel, Sartorius Stedim Biotech) bag and a glass bottle over time.



A virus spike of 5% (v/v) was added to the mAb A and mAb B samples; an aliquot was removed to assess the virus titer entering the treatment. The spiked sample was then filtered. Spiked mAb samples were mixed with detergent to give a final concentration of 0.7% (w/v) and the mixed samples incubated in the bioprocess container.

Aliquots of the detergent-treated, mAb-containing solutions were taken at 0–1 min, 5 min, 30 min, and one hour. These were immediately diluted prior to virus titration. All assays were performed using the 50% tissue culture infective dose (TCID₅₀) infectivity method. The limit of detection was improved using the large-volume plating method in addition to the use of the standard TCID₅₀ titration.

Control samples were taken to which no detergent was added. No statistically significant change in virus titer was observed for the control.

RESULTS

Film adsorption study

Figure 2 summarizes the results from the detergent adsorption study. The detergent concentration decreased by 5% and 3.1% in the 75-mL single-use bag and glass bottle, respectively, over 120 minutes. In both cases, the final detergent concentration was within the acceptance range of 9.0 g/L to 12.0 g/L. The experiment showed no significant adsorption of detergent to the single-use bag. This result gave confidence that the single-use bag would be a suitable scale-down model for performing the detergent virus inactivation step.

Virus Inactivation Study

A minimum 4.74 log reduction of X-MuLV virus titer was observed when mAb A was treated for one hour with detergent (Triton X-100, MilliporeSigma). A reduction factor (RF) of 3.84 log₁₀ was observed in a single sample of mAb B that was treated with the detergent for one hour. This was the only sample in which virus was detected after one hour of treatment. A replicate experiment showed a RF of ≥4.12 log₁₀. The minimum observed RF for BVDV and PRV were ≥5.18 log₁₀ and ≥5.01 log₁₀, respectively.

These data demonstrate that this step is very effective for the inactivation of enveloped viruses. These results are satisfactory, considering that an effective viral inactivation/elimination step is generally assumed to have a RF ≥4 log₁₀. The results were consistent with LFB's expectations. The full virus inactivation results are shown in **Table V**.

CONCLUSION

The use of single-use technologies for biomanufacturing offers a number of advantages over stainless-steel equipment, notably increased manufacturing flexibility and lower capital costs. The successful implementation of single-use technology, however, requires comprehensive analysis of how the equipment is likely to perform under process conditions. Performing this analysis is facilitated when the consumable and associated hardware have an existing, comprehensive data package to support its intended use. Representative scale-down models of the large-scale consumable are also useful.

Table V. Log reduction factors of viruses in the neutralized Protein A eluates from two monoclonal antibody (mAb) processes treated with detergent (Triton X-100, Millipore Sigma). TCID is tissue culture infective dose. X-MuLV is xenotropic murine leukemia virus. BVDV is bovine viral diarrhea virus. PRV is pseudorabies virus.

	Virus	Load virus titer (\log_{10} TCID ₅₀ /mL)	Timepoint virus titer (\log_{10} TCID ₅₀ /mL)				Reduction factor
			T = 0-1 min	T = 5min	T = 30min	T = 1hr	
mAb A	X-MuLV	6.63	3.83*	NT	3.83*	1.45*	$\geq 5.18 \pm 0.36$
	X-MuLV	6.19	3.83*	3.83*	3.83*	1.45*	$\geq 4.74 \pm 0.32$
mAb B	X-MuLV	5.76	3.83*	3.83*	3.83*	1.92	3.84 ± 0.47
	X-MuLV	5.67	3.83*	3.83*	3.83*	1.55*	$\geq 4.12 \pm 0.22$
	BVDV	6.63	3.83*	3.83*	3.83*	1.45*	$\geq 5.18 \pm 0.28$
	BVDV	6.81	3.83*	3.83*	3.83*	1.45*	$\geq 5.36 \pm 0.29$
	PRV	6.72	3.83*	3.83*	3.83*	1.45*	$\geq 5.27 \pm 0.33$
	PRV	6.46	3.83*	3.83*	3.83*	1.45*	$\geq 5.01 \pm 0.34$

*The Poisson distribution was used to calculate the virus titer when no virus was detected in the sample (minimum detection limits).

In this study, the use of a single-use mixing technology was evaluated and characterized for the performance of a detergent-based virus inactivation step during a mAb production process. Testing showed that no significant adsorption of detergent to the bioprocess container occurred. Enveloped virus-spiking studies showed that a minimum reduction factor of $3.84 \log_{10}$ could be expected in this single-use step and that, in most cases, the reduction factor would be greater than $\geq 5.01 \log_{10}$.

In the second part of this paper the authors will demonstrate how further tests showed that single-use (Flexel, Sartorius Stedim Biotech) bags for magnetic mixers had excellent chemical compatibility with a 20% solution of the detergent (Triton X-100, MilliporeSigma) and that leachables were negligible when extractions were performed with 1% detergent. Finally, the authors will describe a scale-up study showing that the magnetic mixer provided sufficient agitation to ensure detergent homogeneity within the single-use bag.

Note: A recent amendment of Annex XIV in the European Union (EU) REACH regulation (7) will lead to the substance 4-(1,1,3,3-tetramethylbutyl)phenol, ethoxylated being prohibited from January 4, 2021 unless an authorization is granted. The Triton X-100 family is included in this new amendment based on the fact that it degrades to the substance mentioned above. This is due to concerns over the environmental impact of the compound's degradation products. For this reason,

EU biomanufacturers must seek alternatives to Triton X-100 with equivalent efficacy and safety. We believe our research is valuable for biomanufacturers not affected by the EU REACH regulation but also that the approach we have adopted is informative for biopharmaceutical companies inside of the EU considering using other detergents for virus inactivation steps.

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