Establishing a surrogate model for inactivation of enveloped viruses to screen viral clearance conditions during biotherapeutics process development

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Abbreviations

A-MuLV	Amphotropic murine leukemia virus
BSL2	Biosafety level-2
CFU/mL	Colony forming units per mL
CMC	Critical micelle concentration
FDA	Food and drug administration
FTM	Fluid thioglycollate medium
HSV-1	Herpes simplex virus type-1
LBLP20	Lactose broth with LP20
LRV	Log reduction value
MVP	Mock virus particle
PFU/mL	Plaque forming units per mL
RT-qPCR	Reverse transriptase quantitative polymerase chain reaction
TSA	Tryptic soy agar
TSB	Tryptic soy broth
VC	Viral clearance
VI	Virus inactivation
X-MuLV	Xenotropic murine leukemia virus

Abstract:

Viral surrogates to screen for virus inactivation (VI) can be a faster, cheaper and safer alternative to third-party testing of pathogenic BSL2 (Biosafety Level 2) model viruses. Although the bacteriophage surrogate, Ø6, has been used to assess low pH BSL2 VI, it has not been used for evaluation of detergent-mediated VI. Furthermore, Ø6 is typically assayed through host cell infectivity which introduces the risk of cross-contaminating other cell lines in the facility. To circumvent contamination, we developed an in-house RT-qPCR (reverse transcriptase quantitative polymerase chain reaction) assay for selective detection of active Ø6 from a population of live and dead phage. The RT-qPCR assay was used to evaluate Ø6 inactivation in cell culture fluid of monoclonal antibody and fusion protein. Complementary Ø6 infectivity was also conducted at a third-party testing facility. The Ø6 RT-qPCR and infectivity data was modeled against VI of three BSL2 viruses, X- MuLV, A- MuLV and HSV-1 in corresponding therapeutics. Both Ø6 methods demonstrate that any VI agent showing Ø6 clearance of ≥ 2.5 logs would demonstrate complete BSL2 VI of ≥ 4.0 logs. Compared to BSL2 virus testing, this in-house Ø6 RT-qPCR tool can screen VI agents at 5% the cost and a turnaround time of 2-3 days versus 4-7 months.

Keywords: detergent inactivation, BSL2 enveloped virus, bacteriophage surrogate, RT-qPCR (reverse transcriptase quantitative polymerase chain reaction), monoclonal antibody, fusion protein

1. Introduction

Viral contaminants are a major concern in the biopharmaceutical industry in the manufacture of therapeutics of human, animal or microbial origin which include recombinant proteins, antibodies, plasma-derived immunoglobulins, hormones and vaccines [1, 2]. Most biologics processes therefore test to confirm the absence of viruses in cell lines, raw materials and intermediate products used in the production process [3]. Viral clearance (VC) validation studies are critical for FDA (Food and Drug Administration) approval of any IND (Investigational New Drug) or BLA (Biologics License Application) filings and are typically conducted at third party testing sites to demonstrate robust viral clearance for safe administration of therapeutics to patients. [4, 5] VC studies are conducted on dedicated downstream unit operations which either remove viruses by filtration or chromatography or inactivate viruses through the use of low pH or chemicals. Inactivation by low pH and chemicals / detergents such as Triton X-100 is commonly employed for enveloped viruses which will be the focus of this study. However, screening multiple virus inactivation (VI) candidates with a third party vendor is cost-intensive (up to hundreds of thousands of dollars) with long lead times (often 4 to 7 months), and requires shipping the therapeutic and / or inactivation agent out to the testing sites. The long turnaround time of 4 to 7 months and cost associated with the studies mean viral validation study results may not be available until later stages of development and scale-up of the manufacturing process. This delay in viral clearance raises the risk of validation failure thereby hindering commercialization and patient access to therapeutics. Circumventing the need to test unsuccessful VI conditions at a third party testing site, particularly at the development stages of the

process, leads to significant savings in cost and turnaround time. We propose a low-cost faster in-house VI screening alternative with a nonpathogenic viral surrogate. The surrogate inactivation data will enable the elimination of VI conditions that are less likely to meet FDA guidelines. As a result, only the most promising conditions would be carried forward for third party testing or validation studies with model viruses.

While low pH is typical for virus inactivation (VI), certain therapeutics such as fusion proteins often have low isoelectric points (pI) rendering them unstable at the low pH ranges employed during low pH-mediated VI. [6, 7] For such molecules, VI using a detergent such as Triton X-100 becomes a natural choice. However, current concerns over the harmful estrogenic effect of Triton X-100 have resulted in an industry-wide initiative to replace the detergent with environment-friendly VI agents. [8-10] Detergent-mediated inactivation also demonstrates an orthogonal means of VC particularly for next generation continuous manufacturing. [7, 11] Our study evaluates different detergents for VI using a viral surrogate.

VC studies employing pathogenic BSL2 (Biosafety level 2) or higher viruses are typically delegated to BSL2 approved-third party testing sites to ensure safe handling and operation during study execution. Over the years, different viral surrogates have been employed in the biotech industry as safe low-cost in-house alternatives for screening VC conditions. Viral surrogates include ØX174, T7, PRD1, and Ø6. [12-16] Viral surrogates, ØX174 and MVP (Mock Virus Particle) have been tested for chromatographic and filtration-based clearance of virus, MVM (Minute virus of mice), through qPCR and immuno-qPCR-based quantification, respectively. [17] Surrogate bacteriophage Ø6 has been employed for filtration-based clearance of virus, MuLV, through qPCR-based Ø6

quantification. Ø6 has also been evaluated for low pH VI technologies through infectivity assay-based quantification of Ø6. The comparable size of Ø6 of 75 nm diameter and its low propensity to aggregate make it an ideal MuLV surrogate for filtration-based VC. [16, 18] Ø6 belongs to the only known class of bacteriophage that has an outer lipid envelope thereby sharing morphological similarity with model enveloped viruses such as A-MuLV (Amphotropic murine leukemia virus), X-MuLV (Xenotropic murine leukemia virus), HSV-1 (Herpes simplex virus type-1) and SARS-CoV-2 virus. [12, 16, 19] Ø6 is thus an ideal candidate for assessing inactivation-based VC.

A critical step in the use of viral surrogates such as Ø6 is the method of quantification of the surrogate before and after inactivation. The two common methods of quantification include infectivity and RT-qPCR (reverse transcriptase quantitative polymerase chain reaction). The BSL1, i.e., non-pathogenic nature of Ø6 and its host, *Pseudomonas syringae,* make it the natural choice for infectivity-based assessment of VI conditions. In addition, the ease of propagation to obtain high titers of Ø6 make it ideal for spiking studies to determine VI- based clearance where a minimum of 4 or greater logs of clearance is required to demonstrate VC process robustness. [20-22] The other technique for Ø6 quantification is RT-qPCR. While infectivity assays can be employed for assessment of inactivation-based VC, RT-qPCR is an established quantification technique that has been used to quantify total virus particle count [23] and to quantify column or filtration-based VC [24]. RT-qPCR provides the advantage of shorter turnaround of day(s) compared to weeks. RT-qPCR is also advantageous as it removes the need for host cell-based phage propagation and thus avoids the toxicity and interference studies that often need to be conducted prior to infectivity-based quantification. Furthermore, the use of a host cell for surrogate propagation and quantification

means that the infectivity assay also pose added risk of cross-contaminating cell-lines dedicated for therapeutic production in shared facilities. [1] The phage RT-qPCR-based assay thus removes the bottlenecks associated with infectivity-based quantification of Ø6. The phage RT-qPCR method can expedite preliminary VC development, particularly for processes with limited prior knowledge on the extent of VI capability.

To the best of our knowledge, Ø6 has not been employed for comprehensive assessment of detergent-mediated BSL2 VI in downstream processing of biotherapeutics using either infectivity or RT-qPCR. In spite of the advantage of RT-qPCR over infectivity, the primary challenge of the RT-qPCR assay is its inherent inability to differentiate between live versus dead phage. RT-qPCR studies using RNase and viability dye have been conducted to differentiate live from dead or heat-inactivated viruses, such as rotavirus and norovirus [25-27]. Infective Ø6 detection was achieved through the use of enzymes, protease and RNase [28]. We propose to develop and utilize a combination of protease, RNase and viability dye-mediated RT-qPCR to assess the extent of inactivation of Ø6 in response to different VI agents in presence of biotherapeutics. Due to the RNA-based genome of the phage, the quantification of Ø6 involves the use of the enzyme reverse transcriptase to construct DNA from genomic RNA to prior to its qPCR-based quantification. This modified qPCR technique is termed as RT-qPCR (reverse transcriptase quantitative polymerase chain reaction). The quantification of Ø6 was conducted using both infectivity and the developed RT-qPCR assay to assess VI agent-mediated inactivation. We tested the inactivation of Ø6 with seven different detergents and low pH in the cell culture harvest of two modalities of therapeutics. The detergent-mediated phageinactivation results from both assays were compared against

inactivation data for three model BSL2 viruses in the corresponding detergent and therapeutic conditions. The BSL2 VI and quantification was conducted by infectivity assays in respective host cells at a third party testing site for comparison. The choice of BSL2 virus encompassed enveloped viruses of differing size and genomic content. [3, 29, 30] The viruses include X-MuLV and A-MuLV (the MuLVs have RNA genome, 80-120 nm diameter) and HSV-1 (DNA genome, 120-200 nm diameter). [2] Statistical models were developed to assess the correlation in the inactivation data sets for Ø6 against those of the BSL2 viruses. The choice of a range of BSL2 virus ensured the robustness of Ø6 VI model and its broader applicability as a VI surrogate in downstream purification. Statistical models generated show strong correlation between phage inactivation with the BSL2 virus inactivation thus demonstrating proof-of-concept of the proposed surrogate model.

2. Material and Methods

This section details the methods of phage and virus inactivation, and assays to evaluate subsequent clearance which include virus infectivity and the developed phage infectivity and phage RT-qPCR assay. Detergents tested include OG (Octyl glucoside), DDM (Dodecyl-β-D-maltopyranoside), NaTC (Sodium taurocholate) and Trehalose-6-dodecanoate [31-36]. Known VI agents Triton X-100, Ecosurf [8] and LDAO (Lauryldimethylamine-oxide) [9] have been tested as positive controls. A comprehensive list of detergent can be found in the supplementary information (SI), **Table S1** and a schematic of the phage inactivation and quantification methods are shown in **Figure 1**.

2.1 Detergent-induced virus inactivation and infectivity assay

Virus, X-MuLV was spiked into clarified cell culture harvest fluid of mAb and viruses, A-MuLV and HSV-1 were spiked into clarified cell culture harvest fluid of Fusion protein 1. The extent of inactivation after 1 hour of incubation at 2-8 °C was evaluated using infectivity assay in Vero indicator cells for HSV-1 and PG4 indicator cells for X-MuLV and A-MuLV. Toxicity and interference studies were conducted prior to the detergent-mediated inactivation studies. Toxicity studies were conducted with different dilutions of detergent-spiked therapeutic to determine the dilutions necessary to have less than 20% indicator cells showing cytotoxic effects. Interference studies were used to determine the dilution needed to avoid interference on growth of indicator cells. The interference studies involved adding different dilutions of virus into different dilutions of detergent-spiked therapeutic followed by addition to the indicator cell line for virus quantification by the plaque assay. Samples that altered the virus stock by more than 0.5 log10 were considered to be interfering. [8, 37] Following incubation with detergent and virus, the therapeutic samples were diluted to non-toxic / non-interfering concentrations before addition to indicator cells for quantification. Corresponding virus-spiked therapeutic samples with no detergent were also quantified by the plaque assay. The sample was quantified by adding it at non-toxic concentrations to the cell line at the beginning and end of hold time of the experiment. The difference in virus concentration before (VI load) and after (VI pool) the detergent addition over the duration of the experimental hold time of 1 hour gives the extent of inactivation or LRV (Log reduction value).

$$LRV_{\text{Virus infectivity}} = -\log_{10}\frac{[Virus]_{\text{VI pool with detergent}}}{[Virus]_{\text{VI load without detergent}}}$$

2.2 Detergent-induced phage inactivation

Clarified cell culture harvest was spiked with phage followed by adding known final concentrations of detergent to induce phage inactivation. The harvest was held at room temperature for one hour from time of addition of detergent followed by quenching the detergent-induced phage inactivation by 16.7 fold dilution with water and PEG (for RT-qPCR assay) or Glycerol (for infectivity assay). The dilution leads to a final detergent concentration that is greater than 4 times below the critical micelle concentration (CMC) of the detergent thereby ensuring the quenching of the reaction. A control sample of harvest material spiked with phage but with no detergent was treated similarly to detergent- and phage- spiked harvest sample. The extent of inactivation of phage (DSMZ, *Pseudomonas* phage Ø6, Catalog number: 21518) using different detergents was evaluated using both RT-qPCR and infectivity-based assay based on the method suggested by Gendron et al [22].

The phage concentration in both detergent-treated (VI pool) and control (VI load) samples were quantified using RT-qPCR and infectivity and the relative quantities was used to determine extent of inactivation, LRV (Log Reduction Value) as follows

$$LRV_{Phage,qPCR \vee infectivity} = -\log_{10} \frac{[Phage]_{VI\ pool}}{[Phage]_{VI\ load}}$$

2.3 Phage RT-qPCR assay

For each test condition, 300 μL of 0.5 M EDTA, pH 8.0 (Invitrogen™, Catalog number: AM9260G) and 300 μL of phage, Ø6 at 8.9 x 10^9 PFU/mL (DSMZ, Catalog number: 21482) were added to 2.23 mL of clarified cell culture harvest. The final concentrations of EDTA and phage were 5 mM EDTA and 8.9 x 10^8 PFU/mL phage, respectively. Respective detergent stock and water was also added to the mixture to reach target detergent

concentrations as given in Supplementary Information (SI), **Table S1**. The samples were incubated at room temperature for 1 hour before quenching reaction with a 16.7 fold dilution with a mixture of 40.1 mL water and 10 mL PEG (Abcam, PEG Virus Precipitation Kit, Catalog number: ab102538). A harvest sample with phage but without detergent was used as control. Aside from the addition of PEG to facilitate the precipitation of phage, 4 μL Glycogen (Wako, DNA extraction kit, Catalog number: 295-50201) was added to help with visualization of pellet. For harvest matrix at pH of 8.0 or greater and the protein pI at~5.0 or less, the pH was titrated down to ~ 6.0 ± 0.2 with 60 to 80 µL of 1 N HCl. The lowering of pH from 8.0 to 6.0 ± 0.2 was used to optimize precipitation and recovery of phage by adjusting the pH closer to the isoelectric point (pI) of the protein (pI of 5.0) in question. For samples subjected to low pH VI, 600 to 800 µL of 1 N HCl was added to harvest at pH 8.0 to target final pH of 3.6 ± 0.2. At the end of 1 hour, the low pH VI reaction was quenched with 200 to 300 µL of 0.5 M Tris to raise pH to 6.0 ± 0.2. The sample was subsequently treated with water and PEG in a similar manner to the detergent samples. All samples were refrigerated overnight to maximize the precipitation of phage. The precipitated phage was recovered the next day by centrifugation at 3,200 x g for 30 minutes at 4 °C and the pellet resuspended with 60 μL water for Fusion protein 1 and Fusion protein 2 (smaller pellets) and 120 μL water for mAb (larger pellet). The resuspended pellets were treated with 46 μL of 20 mg/mL RNase A to final concentration of 8.6 mg/mL (Invitrogen™ PureLink™ RNase A, Catalog number: 12091021). 1.28 μL 20 mM aqueous PMA or propidium monoazide (Biotium, Catalog number: 40019) was added to the mixture to final concentration of 0.24 mM. The solution was incubated for 1 hour followed by addition of 10 µL proteinase K at ~ 600 mAU/ mL to final concentration of 55.9 mAU/mL (Qiagen, Proteinase K , Catalog no: 19131) and 10 μL protease at 1070 mAU/mL to final concentration of 99.7 mAU/mL (Qiagen, Protease Solvent, Catalog no: 1021055) for another 30 minutes. At this point, 125.7 μL RNase A was added to reach final concentration of 32 mg/mL for additional 30 minutes followed by LED illumination for 30 minutes to activate PMA to intercalate into solvent-exposed RNA. Following treatment, the RNA from the

phage samples was extracted from the phage and eluted in 20 μL water using RNA extraction kit (QIAamp MinElute Virus Spin Kit, Catalog no: 57704). The extracted RNA was annealed with forward and reverse primer (TaqMan™ Universal PCR Master Mix, Catalog number: 4318157). 14 µL of extracted RNA was incubated with 62.5 nM forward and reverse primer in 16 μL volume for 5 minutes at 99.9 °C followed by cooling to 4 °C at 0.8 °C/s and holding the plate at 4 °C for 3 minutes. 4 μL of Superscript Mastermix (Thermofisher, SuperScript IV Mastermix) was added to samples for reverse transcription to DNA as per protocol. 1 μL of forward and reverse primer each (1 μM final concentration) and 0.3 μL (1 μM final concentration) of probe along with 50 μL TaqMan master mix, 16 μL cDNA and 31.7μL water were mixed to run RT-qPCR in quadruplicates of 25μL reaction volume each. The primer and probe information for Ø6 were obtained from Gendron et al. [22] The amplification was run at 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min with a plate read after the elongation step.

2.4 Phage infectivity assay

One mL of *P. syringae* culture suspension (DSMZ, *Pseudomonas* sp., Catalog number: 21482) measured at 8.6×10^7 CFU/mL was transferred to 90 mL LBLP20 (Lactose Broth with LP20) and incubated at 20-25 °C for 72 hours. The 72-hour LBLP20 culture of *P. syringae* was swirled to thoroughly mix before transferring 1 mL to duplicate set of 9 sterile test tubes labeled from 10^{-1} dilution to 10^{-9} (total of 18 sterile test tubes). The phage-spiked samples were thawed completely from -70 °C to room temperature on day 1 of infectivity assay and vortexed three times for one second each time before transferring 1 mL to a 9 mL TSB (Tryptic Soy Broth) for a 1:10 dilution (10^{-1}). The 10^{-1} dilution was vortexed three times for one second each time, and then 1 mL of the 10^{-1} dilution was transferred into a separate 9 mL TSB for the 10^{-2} dilution. This serial dilution was continued to a final dilution of 1:1,000,000,000 (10^{-9}). Each serial dilution of the phage culture

suspension was vortexed three times for one second each before transferring 1 mL of each serial dilution of phage culture suspension to the host culture in their corresponding labeled test tube. Each test tube was vortexed three times for one second each before the addition of 10 mL molten TSA (tempered to < 45 °C) into the test tubes. 9 TSA plates were labeled as 10^{-1} dilution to 10^{-9} in duplicate (total of 18 TSA plates). Each serial dilution test tube was vortexed three times for one second each before transferring TSA within tube onto its corresponding labeled TSA plate with adequate mixing by swirling. The addition of molten TSA and overlaying onto their corresponding labeled TSA plate was done by sets of every 1 dilution to prevent the molten TSA from solidifying. Controls were prepared for all media and diluents used. All phage titer TSA plates were incubated at 20-25 °C and observed on day 5 of incubation.

An average of the Plaque Forming Units per mL (PFU/mL) at different dilutions was taken for each sample as long as the values were within an order of magnitude of each other. For specific VI agent-Fusion protein 2 samples, the PFU/mL was observed to vary more than 2 orders of magnitude over the range of dilutions tested. The lower PFU/mL observed at lower dilutions could be attributed to cytotoxicity in the reporter cell line and may overestimate LRV (for detergent/solvent inactivation). In such cases, the subsequently higher dilution samples were used for determination of the plate count thereby making a conservative estimate of the LRV.

2.5 Quantifying the RT-qPCR-based and infectivity- based phage LRV

Typical viral clearance studies require at least 4 to 6 logs of viral clearance using at least two orthogonal strategies [3]. The extent of VI was determined using infectivity assay for both BSL2 virus and phage and measured in Log Reduction Value (LRV) as follows

$$LRV_{Virus\ Infectivity} = -\log_{10}\frac{[Virus]_{VI}}{[Virus]_{Control}}$$

$$LRV_{Phage,\ Infectivity} = -\log_{10}\frac{[Phage]_{VI}}{[Phage]_{Control}} = \log_{10}\frac{[PFU/mL]_{VI}}{[PFU/mL]_{Control}}$$

For RT-qPCR-based phage inactivation, the LRV's were calculated using the following equation,

$$LRV_{Phage,qPCR} = -\log_{10}\frac{[Phage]_{VI}}{[Phage]_{Control}} ¿ \log_{10} e^{C_{T,VI} - C_{T,Control}}$$

The qPCR-based LRV equation was derived from the threshold cycle. The threshold or C_T cycle in RT-qPCR is the minimum number of amplification cycles needed to generate enough genetic material to detect a response beyond threshold signal. The C_T value is inversely proportional to the original phage population as shown below,

$$C_T = -Slope\log_{10}[Phage] + Intercept \qquad (1)$$

Efficiency, e is the fold increase in population over one amplification cycle; under ideal conditions of amplification, e is 2.

$$e = 10^{-1/Slope} \approx 2 \qquad (2)$$

For control phage sample with no detergent,

$$C_{T,Control} = -Slope\log_{10}[Phage]_{Control} + Intercept \qquad (3)$$

For phage treated for VI,

$$C_{T,VI} = -Slope\log_{10}[Phage]_{VI} + Intercept \qquad (4)$$

Subtracting eq. (3) from eq. (4) and substituting in eq. (2),

$$LRV_{Phage,qPCR} = \log_{10} e^{C_{T,VI} - C_{T,Control}} = -\log_{10} \frac{[Phage]_{VI}}{[Phage]_{Control}} \quad (5)$$

A calibration curve of C_T versus phage concentration was used to determine the linear range of phage detection using **Equation (1)**.

2.6 Statistical analysis

To investigate the relationship between LRV from phage RT-qPCR with that of phage infectivity and BSL2 virus infectivity for the three different virus-protein systems, we fitted statistical models using JMP software version 13.1.0. We evaluated linear correlations based on R^2 and p-values for statistical significance of the linear models. All model assumptions were met, including normality of residuals and constant variance. The studentized residuals indicated no influential outliers for the fitted models, so no additional transformation or removal of observations were made.

3. Results and Discussion

3.1 Optimization of phage RT-qPCR - based assay

The method and probable mechanisms of phage or virus inactivation along with the optimized qPCR-based phage and infectivity-based phage assay are illustrated in **Figure 1**. The optimization for the phage RT-qPCR and infectivity assay are discussed below followed by statistical modeling to compare the inactivation of BSL2 viruses against that of phage infectivity and RT-qPCR assays. In this study, we quantified the extent of VI for different detergent and virus systems in harvested cell culture fluid of monoclonal antibody and fusion proteins. The VI was compared against phage

inactivation evaluated using both qPCR and infectivity assays in the corresponding detergent-therapeutic systems.

Since qPCR typically detects all target RNA or DNA in a given sample, a modified approach was needed in order to use this assay for selective quantification of live phage. In order to distinguish between live and dead phage, we combined the use of proteases with RNase and viability dye to develop an optimized method. [21, 26, 27, 38] The method is summarized in **Figure 1A-B**. This method was implemented to carry out a screening study for low pH and detergent-induced phage inactivation. Following inactivation of phage-spiked sample with VI agent, the phage was recovered by overnight PEG precipitation at 2-8 °C. PEG precipitation had to be further optimized for low pI fusion proteins (pI of 5.0) by lowering the harvest matrix pH from 8.0 ± 0.2 to 6.0, thus reducing the surface charge on the protein itself to promote PEG-induced aggregation. [39] The precipitated phage was recovered by centrifugation and resuspension in water.

The next stage involved effectively digesting RNA from inactive or non-infectious phage to ensure that only live phage RNA was detected by RT-qPCR following reverse transcription. Designing this stage required an understanding of the probable mechanisms of inactivation as summarized in **Figure 1A**. The VI agent may solubilize the bilayer thereby solvent exposing the nucleocapsid; the VI agent may intercalate in the lipid bilayer leading to loss of phage or virus infectivity [40]. Alternately, the VI agent may denature a critical surface receptor or binding protein thus rendering the phage inactive [41]. RNA from each possible mechanism of inactivation was digested with a combination of proteases, RNase and RNA-binding viability-selective dye (**Figure 1B**). The resuspended phage was treated with proteases to cleave the nucleocapsid core. RNase was used to

digest solvent-exposed phage RNA. Additionally, viability-selective light-activated dye, propidium monoazide (PMA), was used to penetrate into dead phage particles and intercalate into RNA. The intercalation into RNA is to prevent reverse transcription and subsequent amplification of solvent-exposed RNA from inactivated phage during RT-qPCR. The PMA-modified phage LRV was more closely comparable to the X-MuLV LRV for the same conditions of detergent and therapeutic than the RT-qPCR phage LRV without PMA (**Figure 2A**). The PMA modification led to a two-fold improvement in $LRV_{Phage,qPCR}$ thus leading to a two-fold improvement in the assay sensitivity as seen for three different detergents.

A calibration curve of C_T versus phage concentration without any VI treatment was generated using **Equation (1)** in the Methods section to demonstrate the linear range of detection of the RT-qPCR assay with the upper and lower limit of phage concentration being 10^8 and 10^2 plaque forming unit (PFU) / mL respectively (**Figure 2B**). Thus with RT-qPCR detection sensitivities of 6 orders of magnitude, Ø6 could be a valuable VI or VC surrogate for enveloped BSL2 or higher viruses.

3.2 Optimization of phage infectivity - based assay

Phage infectivity assay was optimized by first ensuring maximum growth of host cell followed by maximizing phage growth. Phage host cell, *P. syringae* was incubated in TSB at 20-25 ℃ and 30-35 ℃ with the former yielding a much higher suspension population of 1.3×10^7 CFU/mL compared to 2.1×10^6 CFU/mL. The growth condition for the phage host cell was further optimized through 72 hour incubation in 3 different culture media, TSB, LBLP20, and FTM (Fluid Thioglycollate Medium) at 20-25 ℃. Population suspension of *P. syringae* was greatest in LBLP20 at 8.6×10^7 CFU/mL followed by FTM at 2.4×10^7 CFU/mL and then TSB at 8.9×10^6 CFU/mL. Phage growth was further

optimized through controlling the sequence of addition of tempered TSA to mixture of phage and host cell (in LBLP20). Adding molten TSA to the phage-host cell mixture led to a phage population of 4.9×10^7 PFU/mL. Reversing the sequence of addition led to a lower phage count of 1.3×10^5 PFU/mL. Thus the greatest PFU/mL was observed when molten TSA was added to the mixture of 1 mL of phage and 1 mL of host cell. Volume of phage added to host cell was also varied from 1 mL to 0.1 mL leading to phage counts of 4.9×10^7 PFU/mL and 1.1×10^7 PFU/mL -respectively, indicating that 1 mL addition had the highest phage count. The final method is summarized in the Methods section and **Figure 1C**.

A direct comparison of the phage RT-qPCR with infectivity assay (**Figure 1B** and **C**) shows the longer turnaround time associated with infectivity assay. Despite optimization of the plating medium and temperature of incubation with host cell, the infectivity assay requires a minimum of 6 days from plating of phage-spiked sample to actual counting of plates. The RT-qPCR assay with its shorter turnaround time of 24 to 48 hours further expedites preliminary screening for different VI conditions.

3.3 Comparing virus LRV with RT-qPCR-based and infectivity- based phage LRV

The extent of inactivation for different protein-virus-detergent systems was initially evaluated at a contract lab by infectivity assay using three commonly used BSL2 enveloped viruses (X-MuLV, A-MuLV and HSV-1) [1, 30] with up to seven different detergent conditions in the harvested cell culture fluid of two different therapeutics, mAb and Fusion protein 1. For each of these protein-detergent combination, LRV values were also calculated using the surrogate bacteriophage system and assayed both by infectivity and RT-qPCR. All of the data is summarized in **Table S2, S3** and **S4**.

The phage RT-qPCR model was assessed by comparison against BSL2 virus LRV in corresponding protein-detergent systems. Each data point on the plots **3A-C** represents a unique VI agent. The comparison between BSL2 virus LRV and RT-qPCR-based phage LRV showed strong linear correlation for all three protein-virus systems with R^2 ranging from 0.73 to 0.93 (**Figure 3A-C**). The lowest correlation was observed for the A-MuLV- Fusion protein 1 system which can be attributed to a specific detergent, DDM (**Figure 3B** and **Table S3**). Overall, for detergent-mediated inactivation, phage RT-qPCR or phage infectivity LRV≥ 2.5correlated with complete VI of LRV$\geq 4.0 \pm 0.5$ for three different BSL2 enveloped viruses (**Figure 3** and **4**). DDM was the only exception as it showed complete inactivation of LRV≥ 2.5 for phage but only partial inactivation (< 4.0 LRV) of 2.36 for A-MuLV in Fusion protein 1 (**Figure 3B)**. VI is thus not only a function of the detergent type and its concentration but also the particular virus or phage and its interaction with the protein system in question. To this effect the impact of detergent on the purified protein matrices was tested for product quality attributes which include high molecular weight (HMW) formation and potency. However, minimal difference was observed in HMW and potency across different detergent-protein samples (methods in SI, and data summarized in **Figure S1**).

For the detergent-mediated BSL2 VI, HSV-1 shows greater inactivation than either A-MuLV or X-MuLV (**Figure 3**). The higher LRV for HSV-1 in relation to X-MuLV could be attributed to the difference in susceptibility of the viruses to inactivation and / or the difference in the protein matrices tested - Fusion protein 1 (HSV-1) versus mAb (X-MuLV). However, both HSV-1 and A-MuLV inactivation were conducted in Fusion protein 1 (**Figure 3B-C**). The difference in inactivation can be explained by the difference in the viral properties- relative size and fluidity of the viral

envelope. The larger size of HSV-1 (120- 200 nm) compared to MuLV (80-120 nm) could lead to easier translocation of detergent across the lipid bilayer at a lower energy penalty leading to greater VI. [42] The difference in inactivation can also be attributed to greater fluidity and lower resistance to detergent-mediated solubilization or denaturation of HSV-1 relative to A-MuLV. [41, 43, 44] MuLVs have higher sphingomyelin content (22.5 % versus 3.1 %) and a lower PC (19 % versus 51.2 %) compared to HSV-1. [43, 44] The higher sphingomyelin and cholesterol content leads to formation of detergent-resistant microdomains (DRMs) within the bilayer. Membrane proteins critical to viral infection are often associated with DRMs and are thus less amenable to detergent-mediated denaturation. [41, 45] MuLVs thus have a significantly higher resistance to inactivation possibly due to higher DRM content.

Collective BSL2 LRV for different virus-therapeutics systems against RT-qPCR-based phage LRV show strong correlation with model R^2 of 0.86 and ANOVA p-value < 0.0001 (**Figure 3D**). The least squares mean of RT-qPCR-model predicted BSL2 virus LRV is different for different protein-virus systems - the predicted virus LRV of X-MuLV in mAb is 1 unit lower than LRV for A-MuLV or HSV-1 in Fusion protein 1 (**Figure 3E**). Conversely, for the same BSL2 virus LRV, the mAb phage RT-qPCR LRV is lower than that in comparison to Fusion protein 1. Given that phage RT-qPCR LRV is the negative logarithm of the ratio of the phage concentration after VI to that before VI (control) as demonstrated by **Equation (5)**, the lower mAb phage RT-qPCR LRV can be indicative of higher phage concentration in the control. This is observed from the lower CT value of the mAb in comparison to the Fusion proteins as shown in **Figure S2**. The higher phage concentration can be attributed to better phage precipitation and recovery in mAb versus Fusion protein 1 which may

have resulted in experimentally observed larger pellets for mAb. The closer the harvest matrix pH is to the protein and phage pI, the lower the respective surface charge and the greater the tendency to aggregate.[39] The phage pI of 6.94 [46] and the EDTA (Ethylenediaminetetraacetic acid)-treated harvest pH of 8.0 ± 0.2 in all instances indicate that the phage pI is not the likely source of differing phage recovery. We thus investigated the effect of the protein or therapeutic pI on the extent of phage precipitation. The lower surface charge of mAb in pH 8.0 harvest due to its pI of 8.6 may promote greater precipitation. The higher surface charge of Fusion protein 1 with pI of 5.0 in harvest at pH 8.0 may lead to lower extent of its precipitation. The phage precipitation and recovery and thus phage RT-qPCR assay is likely influenced by the specific protein matrix and pI of molecules in the matrix tested such as host cell protein and therapeutic protein.

We complemented findings for phage RT-qPCR analysis with infectivity-based LRV for phage inactivation (**Figure 4**). Collective BSL2 LRV for different virus-therapeutics against infectivity-based phage LRV show strong correlation with model R^2 of 0.93 and ANOVA p-value < 0.0001. Only the finite LRV values of phage infectivity were included in the model. Any detergent-protein system where no phage particle was detected has ≤1 PFU/mL yielding ≥ LRVs which were thus excluded. Overall, infectivity-based phage inactivation greater than or equal to 2.5 LRV was correlated to BSL2 VI greater than or equal to 4.0 LRV. Unlike the phage RT-qPCR model which showed dependence on the protein system, we did not see an impact of system tested on the phage infectivity model-predicted BSL2 virus LRV.

Further confirmation of the utility of phage RT-qPCR technique for assessing VI was obtained through comparing the overall results of phage RT-qPCR with phage infectivity (**Figure 5**). Only the

finite values of phage infectivity were included in the model. The phage RT-qPCR and infectivity LRV shows a statistically significant linear trend (R^2 = 0.55, ANOVA p-value: 0.0022). For a 1 unit increase in phage RT-qPCR, we would expect a 1.00 increase in phage infectivity. Phage RT-qPCR explains about 55% of the variation in phage infectivity (R^2 = 0.55). This relatively low R^2 could be attributed to the variability in phage RT-qPCR LRV (**Figure 3**) due to difference in extent of phage recovery in different protein systems**.** All model assumptions were met, including normality of residuals and constant variance. The studentized residuals indicated no influential outliers, so no transformation or removal of observations were made.

In addition to phage RT-qPCR and phage infectivity LRV cut-off of 2.5, the phage LRV for the commonly employed VI agent, Triton X-100 can also serve as a valuable reference to assess the efficacy of a VI agent. Any VI condition which yields phage LRV greater than or equal to that of Triton X-100 would be a promising candidate for successful inactivation of the BSL2 model enveloped viruses.

The phage RT-qPCR technique can be valuable as a screening tool for VI in different therapeutic modalities. Its shorter turnaround time of 48 hours compared to 6 days for phage infectivity and 4 to 7 months for off-site third-party testing of BSL2 virus makes it an efficient alternative for VI screening. The phage RT-qPCR technique circumvents the need for host cell toxicity and interference studies as needed for infectivity assays. Additionally, phage RT-qPCR assay can provide comparable data in-house for a fraction of the cost. To test up to nine independent VI conditions, phage RT-qPCR costs 5 % and phage infectivity costs 10.5 % of the cost for standard BSL2 virus infectivity.

4. Concluding remarks

The long turnaround time of 4 to 7 months and cost associated with viral validation delays assessment of viral clearance to later stages in process development. In the event of VC failure, this delay in VC validation hinders commercialization and patient access to therapeutics. We developed an in-house assay which can screen VI conditions at ~5% of the cost and turnaround time of third party testing of BSL2 viruses. The RT-qPCR assay, which distinguishes between active and inactive phage, was used to determine phage inactivation in different therapeutic -detergent systems with Triton X-100 as positive control for complete inactivation. We also developed a complementary phage infectivity assay which can be used when there is minimal risk from cross-contamination of cell lines with phage host cell. Statistical models were built to compare RT-qPCR-based and infectivity-based phage inactivation with three BSL2 virus inactivation in different therapeutic modalities. Despite the influence of protein matrices on the phage precipitation and recovery in RT-qPCR-based phage inactivation, RT-qPCR-predicted BSL2 virus LRV explained 86% of the variation in BSL2 VI. Our data suggests that any VI condition where phage RT-qPCR and / or infectivity demonstrated ≥ 2.5 LRV or ≥ $LRV_{\text{Triton X-100}}$ would lead to complete inactivation of ≥ 4.0 LRV for the tested BSL2 enveloped viruses. This in-house low-cost faster turnaround RT-qPCR-based bacteriophage surrogate can prove valuable for a-priori prediction of BSL2 enveloped VI in downstream purification of biotherapeutics.

Supplementary Materials: Supplementary information (SI): Detergent spiking, Phage and Virus LRV, Product quality attributes. Phage qPCR and infectivity data and statistical analysis files are also provided.

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Figure Legends

Figure 1. Illustration of (A) Mechanisms of detergent-mediated phage inactivation and subsequent evaluation of log reduction of phage using (B) RT-qPCR (reverse transcriptase quantitative polymerase chain reaction) assay developed in-house with RNase, proteases and viability selective dye, PMA (propidium monoazide) to selectively quantify live phage from a population of live and dead phage and (C) Infectivity assay optimized for host cell, *Pseudomonas syringae* growth and plating conditions. The turnaround time for RT-qPCR is 24 to 48 hours whereas infectivity assay takes up to 6 days.

Figure 2. (A) The RT-qPCR (reverse transcriptase quantitative polymerase chain reaction) optimization with viability dye, PMA (propidium monoazide). PMA shows ~2 fold increase in phage LRV (Log reduction value) in comparison to RT-qPCR without PMA. The results are consistent across different detergents and comparable to the LRV for virus, X-MuLV in mAb (monoclonal antibody) harvested cell culture fluid. All RT-qPCR systems included RNase and proteases for digestion of RNA and nucleocapsid respectively. (B) Calibration curve for RT-qPCR based assay. Threshold cycle, C_T shows linear correlation with phage concentration spanning ~6 orders of magnitude; the lower limit of concentration being 10^2 PFU/mL.

Figure 3. Detergent-mediated inactivation of different enveloped viruses in clarified cell culture fluid of therapeutics, i.e., Fusion protein 1 and mAb (monoclonal antibody). Virus inactivation data for virus / protein systems: (A) X-MuLV / mAb (B) A-MuLV / Fusion protein 1 and (C) HSV-1 / Fusion protein 1 was compared against RT-qPCR(reverse transcriptase quantitative polymerase chain reaction)-based phage inactivation in the corresponding detergent-therapeutic systems. Each data point on each plot corresponds to a different detergent

condition. Overall, RT-qPCR-based phage inactivation greater than or equal to 2.5 LRV (Log reduction value) was correlated to virus inactivation greater than or equal to 4.0 LRV. Detergent, DDM was an outlier as it showed complete inactivation for phage but no or incomplete inactivation (< 4.0 LRV) for A-MuLV / Fusion protein 1 respectively. (D) Combined model for virus LRV from data in (A), (B) and (C) indicate strong linear correlation of overall virus inactivation against phage inactivation (R^2= 0.86, *ANOVA p value* <0.0001 (E) Least squares mean of model-predicted $LRV_{Virus\ infectivity}$: the predicted virus LRVs for X-MuLV / mAb are 1 unit lower than A-MuLV / Fusion protein 1 or HSV-1 / Fusion protein 1. The model-predicted virus LRV for the different detergents thus shows dependence on the virus / protein system.

Figure 4. Detergent-mediated inactivation of different enveloped viruses in clarified cell culture fluid of Fusion protein 1 and mAb (monoclonal antibody) compared against corresponding detergent-mediated phage inactivation using infectivity-based assay. For model development (R^2 = 0.93, *ANOVA* p *value < 0.0001*) all instances that showed complete clearance of phage, i.e.," ≥" $LRV_{Phage\ infectivity}$, i.e., no detectable plaque, were excluded. Overall, infectivity-based phage inactivation greater than or equal to 2.5 LRV correlated with virus inactivation greater than or equal to 4.0 LRV.

Figure 5. LRV (log reduction value) for phage infectivity versus LRV for phage RT-qPCR (reverse transcriptase quantitative polymerase chain reaction) is a straight line through origin with a slope of 1.003 (R^2 = 0.55, *ANOVA* p *value =0.0022*). For model development, all instances that showed complete clearance of phage, i.e.," ≥" $LRV_{Phage\ infectivity}$ or no detectable plaque, were excluded.