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**Application Note**

# Designing Successful Viral Clearance Studies

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Ensuring that biopharmaceuticals are free from the presence of adventitious viruses is critical to the safety of these products. Viruses can be as simple as a genome surrounded by a protein shell, yet a single infectious virion can replicate in susceptible cells and lead to a widespread contamination. Implementation of a viral safety strategy involves a multi-pronged, complementary approach (**Figure 1**). This includes selecting and testing cell lines and critical raw materials to ensure the absence of adventitious viruses. For products that are directly derived from human or animal plasma or other tissues, testing of those raw materials is especially critical.

During the manufacturing process, key process intermediates are also tested for adventitious viruses. Low levels of virus that may have escaped detection during the screening of cells or other raw materials may be amplified in a bioreactor if the production cells are susceptible to infection. Furthermore, production cells may harbor latent viruses that are expressed during as cells are propagated. Chinese hamster ovary (CHO) and other rodent cell lines contain genetic sequences for endogenous retroviral-like particles (RVLP) that are expressed during production of recombinant proteins. The presence of these adventitious viruses is detected during this in-process testing.

Despite careful testing of starting materials and in-process intermediates, there is still a risk that a virus could enter the manufacturing process undetected. Steps in the manufacturing process that might inactivate or remove virus act as a final safety net to ensure that the final product is safe from a viral contaminant. The capacity of the manufacturing process to remove or inactivate enveloped and non-enveloped viruses is assessed in viral clearance studies. These studies, which we will go into more detail throughout this paper, involve scaling down individual process steps, and for each step, spiking virus into the specific process intermediate, performing the process step and measuring the virus remaining after the process step.

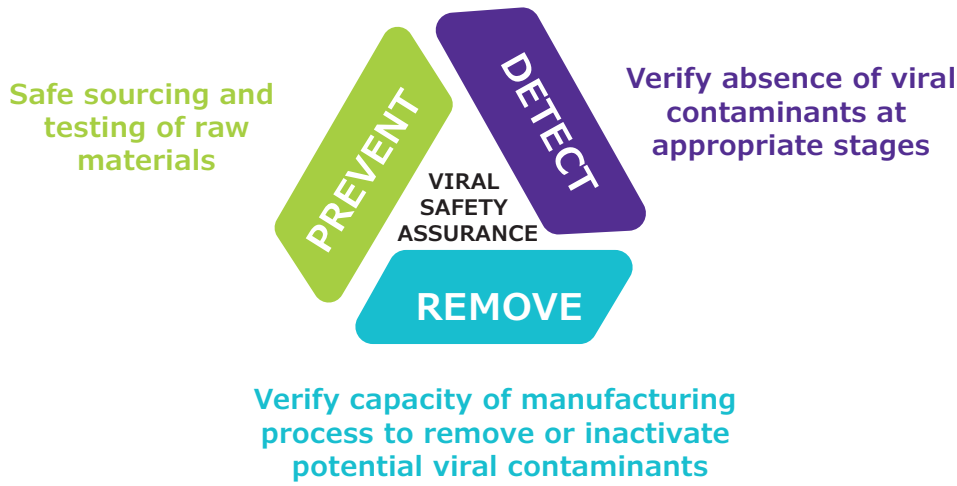


**Figure 1**. A complementary approach ensures viral safety of biological products.



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## Scale Down Model

Before a viral clearance study is undertaken, in support of either an early phase clinical trial or a commercial submission, an appropriate scale down model of the manufacturing steps that will be evaluated should be developed.[1-3] The validity of the scale-down model is key to justifying the validity of the viral clearance data, because unless the scale-down model is a defensible representation of the full-scale manufacturing process step, the clearance data are meaningless. As the product moves toward late stage clinical trials and commercialization, it is anticipated that with ongoing process optimization, changes may be made to the process which in turn may influence the validity of the scale down model and the clearance data generated to support the early clinical trials. Any changes made to the process need to be carefully evaluated using a risk-based approach. Process steps for which any of the changes may impact the validity of the viral clearance data, will require requalification of the scale down model and additional viral spiking studies. It is important to note that a process change not only may impact viral clearance for the step that is changed but also may impact clearance of subsequent steps.

When scaling down the process steps selected for viral clearance evaluation, consideration should be given to the operational parameters specified for the step and how these might influence viral clearance. Some considerations for scaling down key steps are given in **Table 1**. Worst-case operating parameters with respect to viral reduction should be considered where known.[3] It is acknowledged, however, that during the early phase of product development, limits for operational parameters leading to reduced clearance of virus may not be known and in such cases using process set points is acceptable.

Selecting the scale of operation for the scale down model is a balance between ensuring that the process can be operated in a manner that is an accurate representation of the full scale process, the time and resources required to demonstrate the validity of the model and the quantity of the product required to support the spiking studies. To maintain a similar residence time between the scale-down and full-scale chromatography steps, the chromatography is typically scaled down by reducing the column diameter whilst maintaining the same bed height and linear flow rate specified for the full-scale process. While there is no specific recommendation for the scale down ratio, consideration should be given to factors such as wall effects that may be observed when reducing the column diameter below a critical point. This could influence the operation of a chromatography step by improved flow properties in the smaller columns due to the column walls providing a greater support to the chromatography media. It has been demonstrated that wall effects disappear in columns packed at or above 2.6 cm in diameter.[4] However, it is unusual to use columns of this size in a viral clearance study due to the increased product required to support this scale of operation. With higher producing cell lines becoming the norm in recombinant technology, chromatography resin and processes are being developed to maximize loading capacity whilst still achieving the required separation of the product from the process impurities. The use of a 2.6cm diameter column would result in 2.6x more product required per run that the same column step scaled using a 1.6cm diameter. Typically, columns packed using 1.0 and 1.6 cm diameter columns are used for evaluating the capacity of chromatography steps to remove virus. Irrespective of the final scale of operation selected, the critical aspect is to demonstrate that the scale down model is a faithful representation of the full-scale operation with respect to the defined acceptance criteria (e.g. process impurity profile, yield, and elution profile).

It is recommended that when submitting the viral clearance data to the regulatory authorities that a detailed comparison of the operational parameters and specifications for both scales are included with added justification where any parameters differ (e.g., when applying “worst case” parameters). The scaling down of chromatography columns by reducing the column diameters whilst maintaining the same bed height and linear flow rate is a well-established approach to

**Table 1. Considerations for scaling down key steps.**

| Process Step | | |
|---|---|---|
| **Chromatography** | **Inactivation** | **Filtration** |
| Bed height | Temperature | Filter area |
| Column volume | Mixing rates | Capacity (g/m$^2$ and / or L/m$^2$) |
| Residence time | Exposure time | Pressure |
| Operational capacity | Concentration of key components / limits | Flow interruptions routine and or failure mode |
| Pressure | Volume to surface area | Prefiltration |
| Load parameters | Contact surface | Sample handling |
| e.g. pH, conductivity | | |
| Buffer composition | | |
| Raw material sourcing | | |
| Temperature | | |
| Buffer volume: column volume ratio | | |

maintain the same residence time at both scales. In addition, residence time for key parts of the step is maintained by ensuring that the same column capacity is applied, typically the maximum specified capacity is evaluated, and the load sample concentration is within the typical range documented at manufacturing scale. Buffer volume to column volume should be maintained at both scales, specifications applied at manufacturing scale should be applied to the scale down e.g. buffer specification, loading parameters, equilibration criteria and product collection. The product collection is typically triggered when the absorbance at a defined wavelength reaches a specified threshold and collection concluded either based on a specified volume collected or the absorbance falling below a second threshold value. When transferring this to the scale down model, differences in the flow cell pathlength should be considered.

The scale down of viral filtration step is generally a little more straightforward and is defined primarily by the filter modules available from filter manufacturers. The small area filter modules will typically be used to establish the filter capacity and size the filter for the full-scale process. When designing the scale down filtration process, the filtration area will define the scale down factor with the smallest area module typically being selected to minimize sample loss from the full-scale batch.

The key considerations around the scaling down of the viral reduction filtration process include:

- Operational capacity ($L/m^2$ or $g/m^2$)
- Product storage and stability
- Pre-filtration and sample handling
- Virus spike quality and mock spiking studies
- Operational pressure
- Mechanism for generation of driving pressure (compressed gas or pump driven)
- Flow interruptions (planned vs unplanned)

The virus filter is one of the critical steps within the manufacturing process for viral reduction. Due to the nature of the filter, the filter capacity and flow profile are very sensitive to the quality of the feed stream and a small variation in the product quality (e.g., increased product aggregation, addition of virus particles) has the potential to have a major impact on the filter capacity.[5] It is of critical importance that the filter be evaluated to cover the maximum capacity processed at manufacturing scale and desirable to evaluate a higher capacity if possible, to build in contingency for process scale up at a later date. The samples taken for the evaluation of the viral reduction filter therefore need to be handled and stored appropriately to minimize the risk of the filter blocking due to the product quality concerns.

Most viral filters are operated at constant pressure, some driven by compressed air and some by pumps. When designing the scale down model it is important to understand the driving force for the filtration and how the target pressure is achieved. Compressed gas represents a relatively quick transition to the operating pressure whereas the use of pumps may mean that the pressure ramp-up time occurs over 8 to 10 minutes. If scaling down a pump driven process, using compressed air as the driving force, the procedure should mimic the pressure ramp up time in the scale down model at all relevant points in the process (e.g., at the start and following any planned interruptions).

Once the scale down model has been designed, the impact of the viral spike on the filter operation (capacity and flow decay) needs to be evaluated. The quality of the virus spike used in the clearance study is critical to minimize the risk of the filter clogging before achieving the target capacity. Guidance on viral spike quality has been detailed in the PDA technical report TR47[6] with examples on how spike ratio and spike quality can influence the filter performance and the log reduction factor obtained. Unlike chromatography steps, the use of the viral resuspension buffer is not an appropriate mock spike since the buffer will not lead to filter clogging in the same way that the addition of the virus spike will. It is therefore recommended that as part of the viral clearance study, additional runs are scheduled to characterize the filter performance. The first run should be an unspiked baseline run to ensure that the storage and transportation has not influenced the product quality. The second run should be a spiked run for each of the model viruses to be tested or a single run using the “worst case” virus. This run will mimic the proposed spiked runs in terms of the virus spike ratio and viral spike quality and the filter flow decay monitored and confirmed that:

- The target capacity can be achieved using the selected spike ratio
- The flow decay is, where applicable, within the defined specification applied to the full-scale process
- The duration of the filter step, if applicable, meets the target duration

It may not always be possible to achieve the target capacity even with a reduction in the spike ratio using the traditional approach and alternative strategies, such as inline spiking[7], may need to be considered to ensure sufficient capacity has been achieved in the viral clearance study to support normal manufacturing operations. If the filtration problems are not linked to the addition of the virus spike but to the quality of the filter feed this can be addressed by generating the material immediately prior to the spiked runs by processing through the previous process step which is often optimized as an aggregate removal step or by applying an optimized pre filtration strategy to “restore” the product quality.

Chemical based inactivation studies (e.g. low pH, solvent/detergent treatment, formaldehyde treatment, etc.) are generally scaled down by reducing the volume, however, considerations should be given to aspects beyond a simple volume reduction as appropriate:

- Material of contact e.g. plastic tanks, stainless steel vessels, bioprocess containers / bags
- Temperature control
- Mixing strategies, if required
- Online measurement e.g. pH measurement
- Accuracy of chemical addition, if appropriate
- Air liquid interface
- Chemical additional rates (e.g., precipitation with caprylic acid)

When evaluating viral inactivation, it is important to characterize the kinetics of the inactivation process, so it is critical to be able to quench the inactivation effect immediately at each designated time point. For inactivation steps such as formaldehyde or solvent/detergent treatment, quenching is achieved by diluting out the chemical inactivating agent and this dilution strategy is defined as part of the preliminary testing ahead of the spiking study. Where pH extremes (high or low) are being evaluated quenching is achieved by neutralizing each time point. Since neutralization needs to be immediate and controlled, completion of a mock spike run will allow characterization of the pH adjustment required. If we look at a low pH treatment step as an example, we need to consider several elements:

- The adjustment of the bulk sample to the target pH prior to spiking
- The influence of addition of the virus spike on the pH
- The adjustment required, if any, after the addition of the virus
- The neutralization of the individual timepoints.

The mock spike run would be scheduled immediately prior to the spiking runs such that the same pH adjustment buffers are used in both the mock run and spiking run. Since this run would be completed at the testing facility prior to spiked run the “mock spike” would be the same batch of virus to be used in the spiked runs. On completion of the mock spiked run, the mock run would provide detailed information on the target pH to which the sample should be adjusted to accommodate any pH change on addition of the virus spike. This will minimize the need for a post spike pH adjustment and determine a ratio (v/v) of titrant to sample required to neutralize each timepoint. Each timepoint sample can then be dispensed into labelled sample tubes containing the required volume of titration buffer to allow the sample to be immediately neutralized.

The qualification of the scale down process can appear to be a daunting workload however, time spent characterizing the scale down model will mitigate the risk of failure during the viral clearance study. In addition, the workload may be reduced significantly if the scale down model has already been “qualified”. The scale down model used for the viral clearance study may be the same scale as that used for the initial process development in which case there will be comparative data following the scale up from the laboratory through to the pilot scale and finally to production scale. The data obtained through the scale up process can then be applied to justify the use of the same scale down model for the virus study. Furthermore, if there have been other process characterization studies completed (resin reuse or process limits evaluation) where the same scale down model has previously been qualified, then there would be no requirement to requalify the model except for completing the mock spike evaluation. Worst Case

## Worst Case

Where known, the “worst case” parameters within the specifications set for the individual step under assessment, should be evaluated. Definition of “worst case” requires an understanding of how the proposed virus model is removed relative to the product. Understanding the properties of the virus (e.g. structure, isoelectric point (pI), hydrophobicity, charge, and size) will be invaluable in making this assessment. Other useful sources of information that will assist in the selection of “worst case” parameters include in-house data from similar purification platforms that may have included more extensive testing to demonstrate viral partitioning. Published data (publications, presentations, and posters) will also be beneficial in supporting the study design. As the product moves through the different clinical phases, additional work will be completed to optimize and characterize the process. Data from these studies looking at the removal of process related impurities such as host cell DNA may provide supportive data in the assessment of the “worst case”. The proposed “worst case” parameters will be further discussed when looking at the different process steps assessed for their capacity to remove or inactivate viruses.

## Selecting A Virus Panel

The panel of viruses selected for a viral clearance study is tailored to each biopharmaceutical product and is based on the source materials used to manufacture the product. The panel may include both relevant and model viruses. Viruses used in a clearance study should include those that may contaminate the product as well as viruses that represent a range of physical and chemical characteristics of potential contaminants. ICH Q5A(R1)[1], which provides viral safety guidance for products to be submitted for licensure, describes three categories of viruses that are used in clearance studies:

relevant viruses, specific model viruses and nonspecific model viruses (**Table 2**).

**Table 2. Categories of viruses to be included in viral clearance study as described in ICH Q5A(R1).**

| Relevant | Specific Model | Non-Specific Model |
|---|---|---|
| Virus identified as potential contaminant or of the same species | Virus which is closely related to the known or suspected potential contaminant | Virus used to demonstrate that process clearance for viruses is robust (e.g., process can remove general classes of viruses) |

1. **Relevant Viruses** - a relevant virus is a potential contaminant of a given source material that can be readily assayed in the laboratory and should always be included in the panel for clearance studies. For example, human immunodeficiency virus type 1 (HIV-1) is a potential contaminant of products derived from tissues of human origin and should be included in the panel of viruses used for viral clearance studies with these products. Therefore, for products derived from human tissues, HIV-1 is a relevant virus. Similarly, bovine viral diarrhea virus (BVDV) is a potential contaminant where bovine-sourced products are part of the production process and is a relevant virus for these products.
2. **Specific Model Viruses** - some potential contaminants, however, cannot be grown to high titers in the laboratory or cannot be readily cultured in an *in vitro* infectivity assay and therefore are difficult to be used in clearance studies. For these viruses, a virus that is closely related to the potential contaminant and which has similar physical and chemical properties can be used as a specific model virus. Hepatitis C virus (HCV) is a potential contaminant of human blood and tissue-derived products. This virus cannot readily be grown in culture and so a virus from the same family, BVDV, is used as a specific model virus. BVDV and HCV both have similar RNA genomes and have similar virion structure; they also share similar physical-chemical susceptibilities. While BVDV is a relevant virus for bovine-derived products, for a human-derived product it is a specific model virus.

   Rodent-derived cell lines, such as CHO cells, usually contain high levels of endogenous retrovirus-like particles. These particles have not been shown to be associated with human disease, yet regulatory agencies expect clearance of these particles to be demonstrated(8-9). Another retrovirus, often a murine leukemia virus, is used as a specific model virus for these retroviral particles.
3. **Nonspecific Model Virus** - in addition to the known potential contaminants for a biopharmaceutical, the threat of the unknown or emergent contaminant is always a concern to both regulators and manufacturers. Therefore, it is important to include viruses that represent a variety of different properties in clearance studies so that the capacity of the process to clear a wide variety of viruses can be demonstrated. These non-specific model viruses will include both enveloped and non-enveloped viruses, viruses with DNA genomes and viruses with RNA genomes, large viruses, and small viruses, as well as viruses with low and high resistances to physical and chemical methods of inactivation. Relevant and specific model viruses in the panel can also represent nonspecific properties. For example, inclusion of murine leukemia virus (MuLV) in the virus panel for a CHO-derived product will provide a specific model for retroviral particles as well as a non-specific model for medium-sized, lipid enveloped, RNA viruses with low resistance to physical and chemical methods of inactivation.

For products derived from well-characterized cell lines, the number of viruses used to evaluate viral clearance in support of a clinical trial is reduced as compared to the number used to support product licensure.[1-2] To support early phase clinical trials of a biopharmaceutical derived from a well-characterized murine cell line in which the bulk harvest contains retroviral particles, the use of a specific model for the retroviral particles, such as XMuLV, and a parvovirus, such as murine minute virus (MMV) is recommended (**Table 3**). This is the expectation for the European authorities and accepted by the U.S. Food and Drug Administration (FDA) and many other countries. For viral clearance studies to support product licensure, additional nonspecific model viruses are included. Typically, a medium-sized, non-enveloped virus with an RNA genome (e.g., reovirus type 3) and a large, enveloped virus with a DNA genome (e.g., pseudorabies virus) are added to the panel used to support studies for a clinical trial.

**Table 3. Typical virus panel for early- vs. late-phase viral clearance studies for recombinant proteins or monoclonal antibodies derived from CHO cells and other well-characterized rodent cell line.**

| Virus Panel | |
|---|---|
| **Early-Phase Clearance Studies** | **Late-Phase Clearance Studies** |
| Murine leukemia virus (MuLV)<br>Lipid enveloped virus<br>80-110nm in diameter<br>RNA genome<br>Low resistance to inactivation | Murine leukemia virus (MuLV)<br>Lipid enveloped virus<br>80-110nm in diameter<br>RNA genome<br>Low resistance to inactivation |
| Murine minute virus (MMV)<br>Non-enveloped virus<br>18-24nm in diameter<br>DNA genome<br>Very high resistance to inactivation | Pseudorabies virus (PRV)<br>Lipid enveloped virus<br>120-200nm in diameter<br>DNA genome<br>Low resistance to inactivation |
| | Reovirus 3 (Reo 3)<br>Non-enveloped virus<br>60-80nm<br>RNA genome<br>Medium resistance to inactivation |
| | Murine minute virus (MMV)<br>Non-enveloped virus<br>18-24nm in diameter<br>DNA genome<br>Very high resistance to inactivation |

If the manufacturing process utilizes components derived from human or animal sources, it may be

necessary to include additional relevant or specific model viruses as surrogates for potential contaminants from those materials.

## Virus Spike Preparations

In a viral clearance study, virus is spiked into an appropriate process intermediate and the amount of virus recovered following the unit operation determines the level of clearance. The virus used in the study must represent the potential contaminant, both in terms of the relevancy of the virus, as discussed previously, and in terms of the quality of the virus preparation(6). Virus preparations are generated by infection of susceptible cells. The resulting virus is released into the culture medium, which is harvested as a virus stock. This stock contains not only the virus, but also many other components. The salts, carbohydrates, growth factors and possibly serum that make up the culture medium are present with the virus. Proteins, lipids, and nucleic acids from the cells can also be present. Depending on the virus and the cells that are used and the culture medium, the level of impurities in the virus stock can be quite high. Spiking this "crude virus prep" into a process intermediate may introduce impurities that possibly interfere with the performance of the downscaled process step or may not be representative of the purity of the potential contaminant. For example, impurities in the virus spike preparation may plug the small pores of a virus retentive filter, impacting the flux of the filter and potentially limiting the total amount of process intermediate that can be filtered. The impact of the virus spike on the performance of a unit operation can be mitigated by altering the volume and/or the purity of the spike.

As much virus as possible should be added to the process intermediate to assess the capacity of the process step for virus removal or inactivation. While regulatory agencies limit the virus spike to 10% v/v, typically much less volume is used so that the composition of the process intermediate is not altered, or the performance of the process step is not impacted.[3] Use of a high-titer, purified virus stock can reduce the introduction of impurities and limit the volume of the spike that is needed. This is especially useful for a step late in the purification process when impurities introduced by the virus spike could adversely impact the performance of the step.

Various standard virological methodologies are used to purify crude virus. These techniques include ultracentrifugation, either to pellet the virus and re-suspend in a buffer or through a cushion or gradient of a suitable medium. They can also include chromatographic methods. The methods can be used alone or in combination, and they result in preparations with varying degrees of purity. A technical report by the Parenteral Drug Association[6] describes standard purification methods and provides guidance for preparation of virus spikes that are used in viral clearance studies. Each virus is biochemically unique and has evolved its own biologically distinct method for infection of host cells, and therefore it is necessary to optimize propagation and purification procedures specific to each individual virus. Most laboratories that perform viral clearance studies offer purified virus preparations and can provide guidance on the quality of the virus preparation and the optimal spike volume for each process step.

## Virus Detection Assays

In most cases, virus is detected using an infectivity-based assay, since inactivation or removal of infectious virus is most relevant with respect to viral safety. The most widely used assays are tissue culture 50% infectious dose ($TCID_{50}$) and plaque assays. Any assay used for endpoint virus detection in a regulated study must be validated.[2, 6]

In a $TCID_{50}$ assay, a sample that contains virus is serially diluted and each dilution placed on replicate cultures of susceptible, adherent cells in wells of a flat-bottomed plate. In this quantal assay, the infected cultures are incubated and then wells are scored positive or negative, based on the presence or absence of virally induced cytopathology.[10] The proportion of positive replicate wells at each dilution are determined. Using Spearman-Kärber or Reed-Muench formulae, the number of $TCID_{50}$ units per milliliter can be calculated.[11] The $TCID_{50}$ value represents the concentration of virus required to infect 50% of the inoculated cells. If no factors are present that might decrease the infectivity of a virus in either assay, then the 1 $TCID_{50}$/mL can be considered to be equivalent to 0.69 plaque forming units (pfu)/mL.[12]

In the quantitative plaque assay, a sample that contains virus is serially diluted and several dilutions are used to infect susceptible, adherent cells that are usually in a flat dish.[10] After allowing time for the virus to adsorb to the cells and then removal of the inoculum, the cells are overlaid with culture medium containing a semi-solid matrix (e.g., agarose, methyl cellulose). As the cells become infected and release their progeny, the new virions can only infect cells near the site of the original infection because the semi-solid medium prevents virus from moving anywhere except to neighboring cells. Eventually cells surrounding the cell that was originally infected are also infected. If the infection results in cell death, a clear zone or "plaque" is formed. Plaques can be visualized without a microscope, although at times staining the cells makes the plaques easier to see. For most viruses, there is a linear relationship between the number of plaques and the number of infectious particles in the original sample. Results of a plaque assay are presented as plaque forming units (pfu) per milliliter. Not all viruses form plaques, and for those viruses, another assay, such as a $TCID_{50}$ assay, must be used for detection.

While infectivity assays are the "gold standard" for detection of viruses in viral clearance studies, under certain circumstances a quantitative polymerase chain reaction (qPCR) assay can be very useful in assessing viral reduction across removal steps. For example, if a chromatography load contains detergent, which would inactivate enveloped viruses spiked into it, then qPCR could be used to quantify the viral genomes present in the chromatography load and eluate fractions. Protein A affinity chromatography (hereafter referred to as Protein A chromatography) is a common capture step for

purification of monoclonal antibody products. The bound antibody is eluted from the Protein A ligand using a low pH buffer. During the elution phase, it is likely that the enveloped viruses would be inactivated. Typically, the next step in the process is a low pH hold, where the eluted product is adjusted to a low pH and held for a defined time period to allow for inactivation of enveloped viruses. If assessed by an infectivity assay, the mechanism of reduction by the Protein A chromatography step would include both inactivation by the low pH elution buffer and removal by the chromatography process. In this case any inactivation by the low pH hold could not be claimed, because the contribution from low pH mediated viral inactivation would be counted twice and reduction by the same mechanism of action can only be claimed once in a process.[1-3] If enveloped virus removal by the Protein A chromatography step is assessed using qPCR, then any inactivation by the low pH hold, an orthogonal mechanism, can now be claimed since the qPCR assay will detect the viral genome from both infectious and virus inactivated by exposure to the low pH elution buffer.

## Pre-Study Controls

Before a viral clearance study is conducted it is necessary to perform pre-study control experiments that will account for anything in the process intermediate that might lead to inaccurate estimations of viral reduction. The pre-study includes:

- cytotoxicity assay - to determine whether the process intermediates are toxic to the indicator cells used to detect the virus
- viral interference assay - determines whether the process intermediates interfere with the ability of the virus to infect the detector cells or for infected cells to display a characteristic cytopathic effect
- spike recovery assay - is used to determine whether the titer of a virus spike can be recovered following the addition to the load material for a given step
- quench analysis evaluates how the effects of an inactivating substance present in the process intermediate can be mitigated.

These assays are described in more detail below.

## Cytotoxicity Assay

In order to evaluate the cytotoxicity of process intermediates to the virus detector cells, process intermediates, representing every sample that will be generated and assayed in the spiking study, are tested on indicator cells for each virus that will be used. Dilutions of each intermediate are placed on the indicator cells in a manner that mimics the viral infectivity assay;

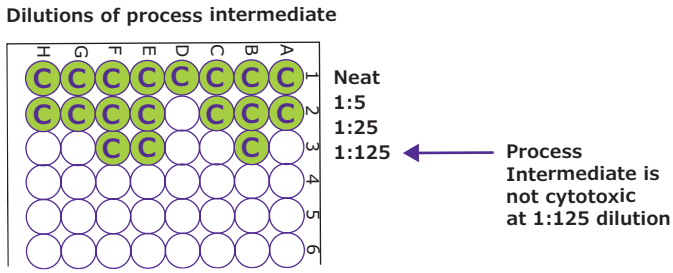


**Figure 2**. Overview of cytotoxicity assay. In this example, the process intermediate is not toxic at a dilution of 1:125. This will be the starting dilution for the viral interference assay.

however, for this assay, no virus is used. Following assay incubation, the indicator cells are microscopically assessed for evidence of toxicity. The lowest dilution that exhibits no cytotoxicity will be the starting dilution for the viral interference assay. **Figure 2** is a schematic overview of a cytotoxicity assay.

## Viral Interference Assay

The viral interference assay addresses whether the product intermediate interferes with the ability of the

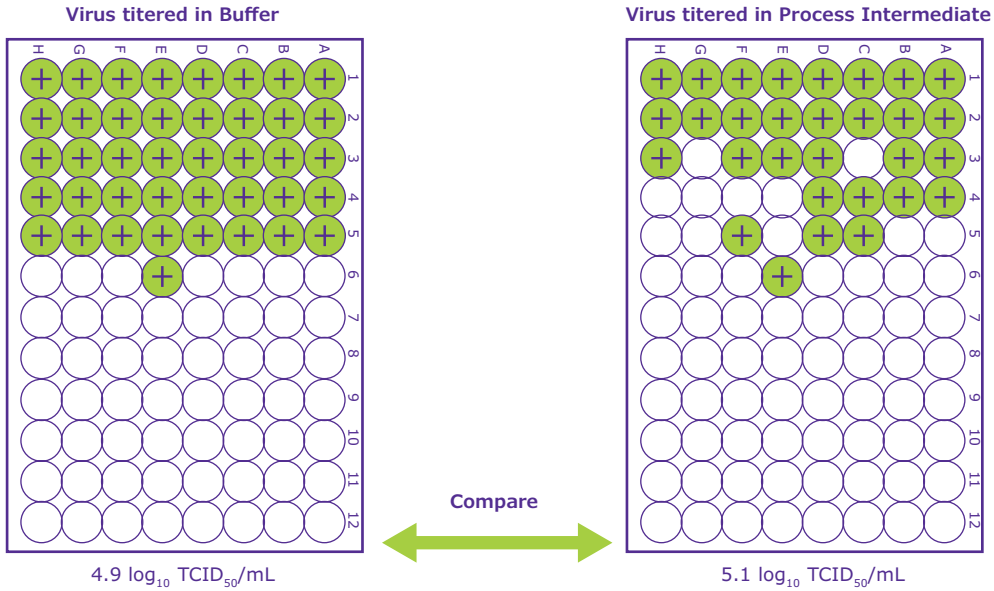


**Figure 3**. An example of a viral interference assay. In parallel, virus is titered in buffer and in product intermediate. In this example, the titers are comparable, and no viral interference is detected.

virus to infect the indicator cells or for infected cells for display a characteristic cytopathic effect. The viral interference assay compares the detection of virus in the process intermediate to the detection of virus in buffer. In the assay shown schematically below in **Figure 3**, two separate samples of virus are titered, one using buffer as a diluent and the second using process intermediate as the diluent. If the process intermediate is cytotoxic, it must be diluted to a non-cytotoxic level before it is used as a diluent in the viral interference assay. The virus titers achieved in buffer and in process intermediate are compared. If the titers are within 0.5 to 1 $\log_{10}$ of each other, then no interference is present. If the difference is greater, then the process intermediate must be further diluted and the assay repeated until no interference is present.

An example of viral interference is the presence of neutralizing antibodies that react with virus in a process intermediate for a human plasma-derived product. Some of the virus present in the process intermediate may not be detected in the infectivity assay if antibodies have neutralized the virus and prevented it from infecting the detector cells. A very simplistic illustration of this is provided in **Figure 4** where the beaker on the left contains ten infectious virus particles. In the beaker on the right, neutralizing antibodies (blue shapes) are present that prevent the detection of five of the infectious virus particles. Consequently, viral interference prevents an accurate estimation of the number of infectious viruses present, and it will be necessary to dilute the interfering process intermediate to accurately quantitate the infectious virus present.

An example of viral interference data for a process intermediate evaluated for the parainfluenza type 3 virus (PI3) and XMuLV infectivity assays is provided in **Table 4**. The titer for XMuLV in undiluted process intermediate was 5.5 $\log_{10}$ $TCID_{50}$/mL and in buffer was 5.7 $\log_{10}$ $TCID_{50}$/mL. The difference in the titers was 0.2 $\log_{10}$; there was no interference. However, the titer of PI3 in undiluted process intermediate was 3.5 $\log_{10}$ $TCID_{50}$/mL, 1.9 $\log_{10}$ lower than the titer of the virus in buffer. This process intermediate required a 1:10 dilution before viral interference was eliminated.

**Table 4**. Example of viral interference data.

| | PI3 | | XMuLV | |
|---|---|---|---|---|
| Diluent Dilution | $Log_{10}$ $TCID_{50}$/ mL | Difference from Buffer Control | $Log_{10}$ $TCID_{50}$/ mL | Difference from Buffer Control |
| No Dilution | 3.5 | 1.9 | 5.5 | 0.2 |
| 1:3.2 | 4.4 | 1.0 | Not Done | Not Done |
| 1:10 | 5.6 | 0.2 | Not Done | Not Done |
| Buffer Control | 5.4 | | 5.7 | |

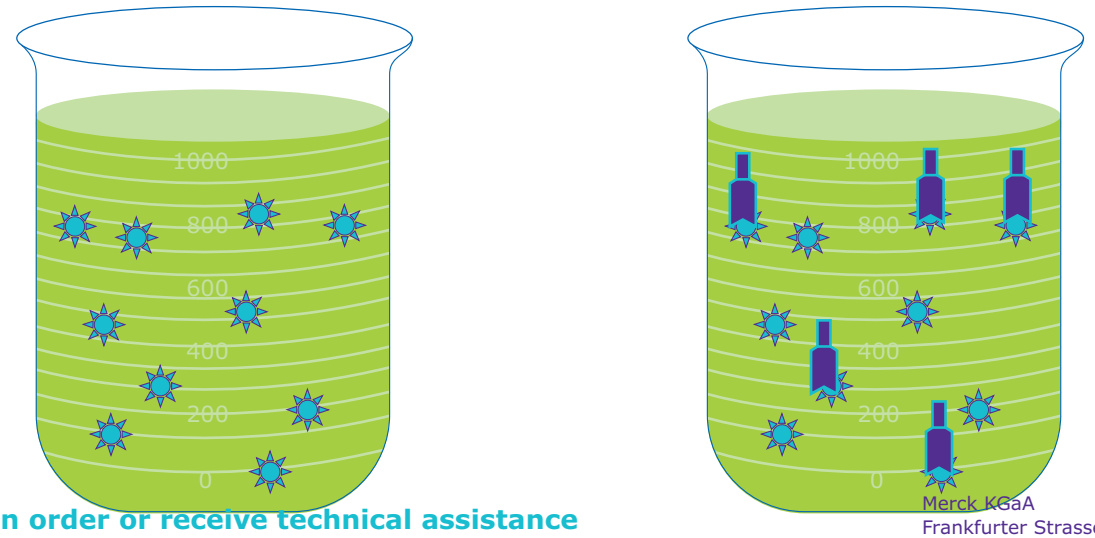


**Figure 4**. An example of viral interference in a product intermediate due to the presence of antibodies. Although ten infectious particles are present in the intermediate, only five are detected, due to neutralization of five virus particles by a neutralizing antibody. It would be necessary to dilute out the neutralizing antibodies in order to accurately quantitate the infectious viruses.


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## Spike Recovery Analysis

The spike recovery assay is a variation on the viral interference assay. It is another way to determine the impact of the process intermediate on detection of virus spiked into a process load sample. This assay is used for all load samples instead of viral interference. As shown in **Figure 5**, virus is spiked into a sample of the process step load material, which has been diluted according to cytotoxicity data. Virus is spiked at the same ratio that will be used when the spiking study is performed (often 1% v/v). The sample is held for 60-80 minutes in a biosafety cabinet and then pH adjusted as needed and assayed by $TCID_{50}$ assay. For virus reduction filtration and membrane chromatography loads, the spiked load material is split into two aliquots (**Figure 6**). One aliquot is pH adjusted, as required, and then assayed by $TCID_{50}$. The second aliquot is passed through an aggregate control filter, the pore size of which is selected to remove potential viral aggregates allowing monodispersed virus to pass through (e.g., 0.1µm for MMV , 0.2 µm for MuLV and Reo 3 and 0.45 µm for PRV). The filtrate is then pH-adjusted and assayed by $TCID_{50}$.

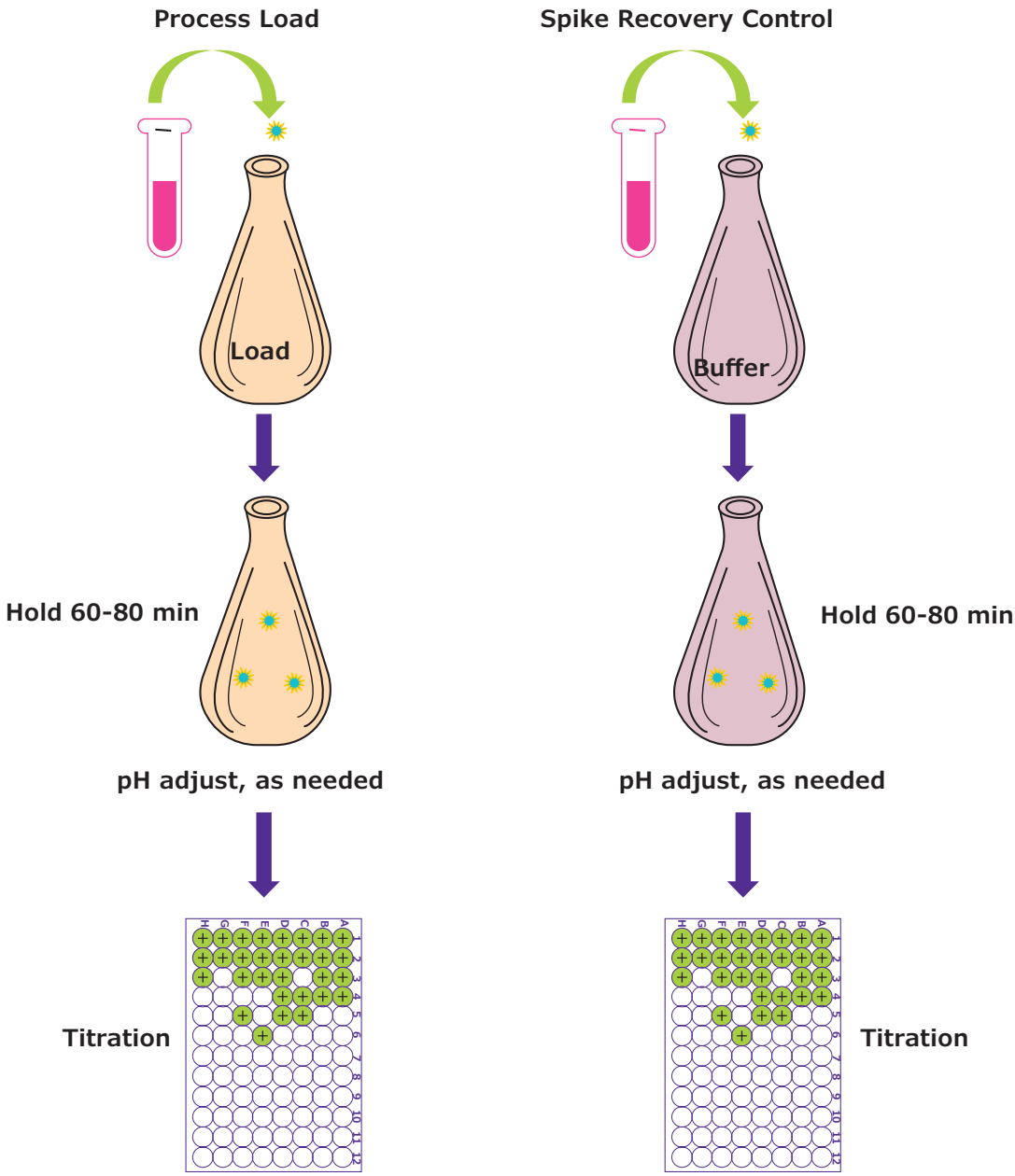



**Figure 5**. An example of a spike recovery assay for process loads (not including virus reduction filtration or a membrane chromatography). Virus is spiked into process load material and buffer at the same ratio used in the clearance study and then held in a biosafety cabinet before being assayed for infectious virus.

In parallel, an aliquot of virus diluent buffer is spiked at the same ratio, held and processed the same way in which the load sample is processed. This spike recovery control is then titrated. The virus titers from the load samples are compared to the respective spiked recovery control samples, and if they are within one $\log_{10}$ of the control titer, the process intermediate has no impact on recovery of the virus spike. Samples that have gone through the aggregate filters, are compared to the sample that has not been filtered and are expected to be within one $\log_{10}$ of each other.

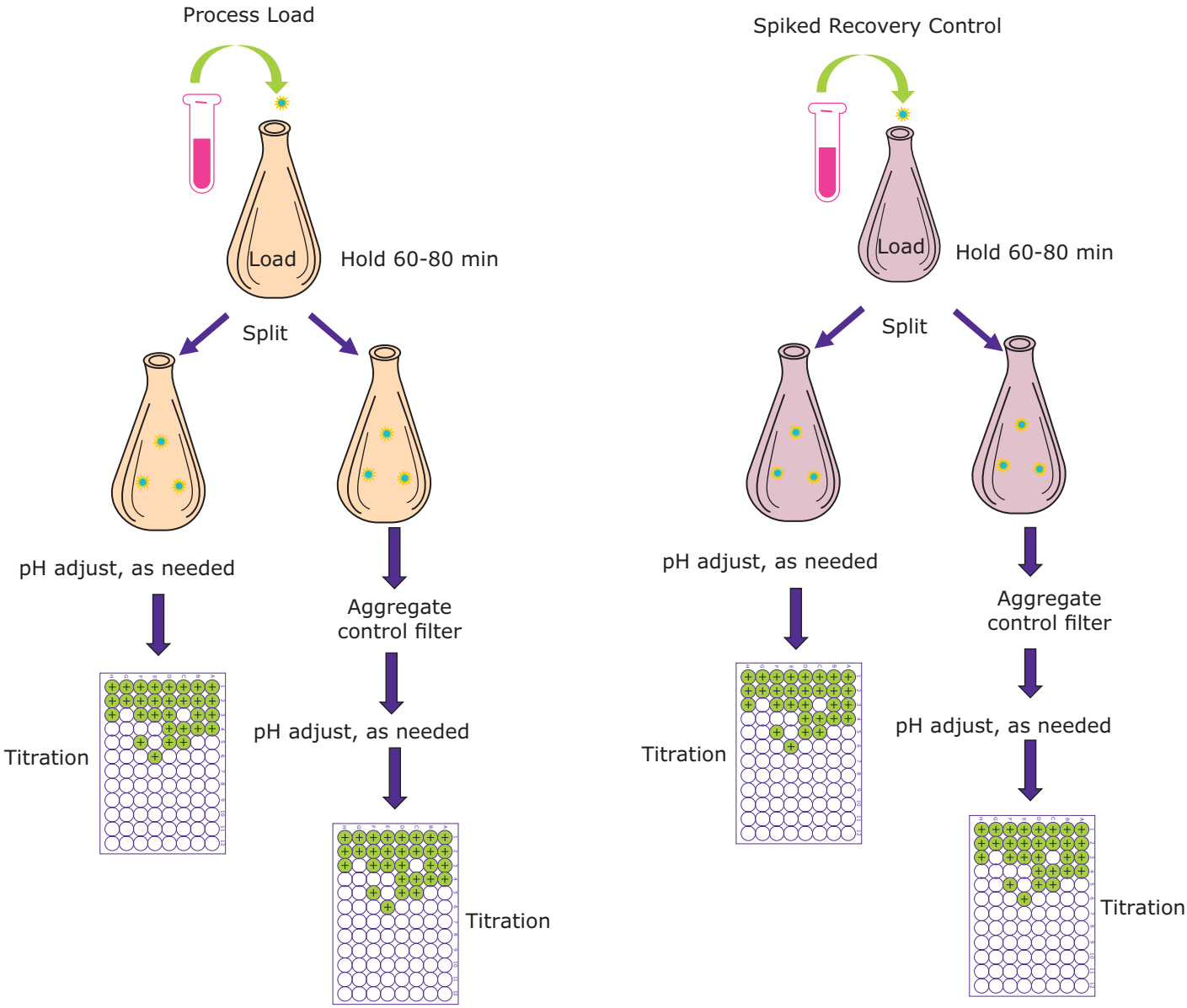



**Figure 6**. An example of a spike recovery assay for a virus reduction filtration or a membrane chromatography load. Virus is spiked into process load material and buffer at the same ratio used in the clearance study and then held in a biosafety cabinet before being assayed for infectious virus.

## Quench Analysis

Quench analysis can be performed on process intermediates that may contain an inactivating agent, such as detergent. This assay will verify that the effect of the inactivating agent present in the sample has been quenched prior to the endpoint $TCID_{50}$ assay. This analysis is important when a potent inactivating agent is used, and it is necessary to determine how to dilute or otherwise mitigate the inactivating agent to "stop" the inactivating agent after a defined hold duration. For example, the process intermediate from a detergent inactivation step may be tested to demonstrate that the detergent can be diluted to a level that no longer inactivates the targeted virus. For this assay, illustrated in **Figure 7**, virus is spiked into non-cytotoxic dilutions of the sample containing the inactivating agent as well as into an aliquot of virus buffer, which will serve as a positive control. Each spiked sample dilution as well as the positive control is assayed, and the lowest dilution with virus titers similar to the buffer control will be used as the quench dilution in the spiking study.

- Selected dilutions of sample containing inactivating agent are prepared
- Virus is spiked into each dilution of sample as well as into buffer (positive control)
- Samples are assayed, and the effective quench dilution is the lowest dilution with a virus titer that is similar to the titer of the buffer control.

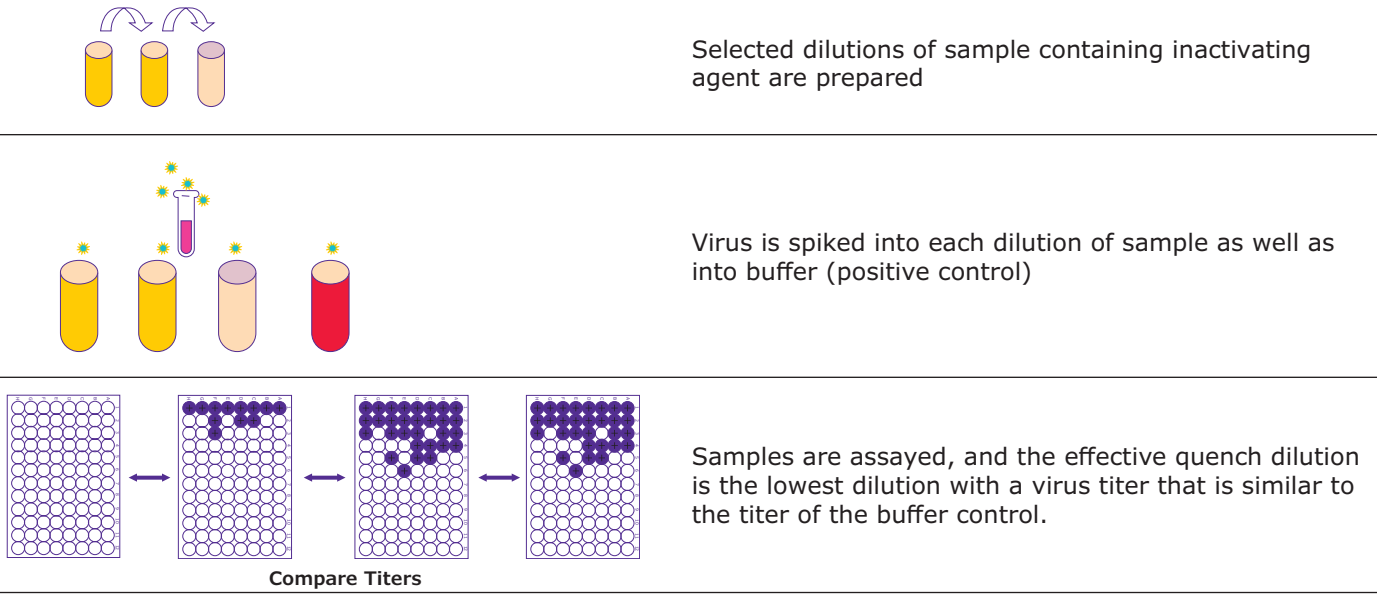



**Figure 7**. Schematic of quench analysis assay.

## Viral Clearance Study Design

### Protein A Chromatography

Protein A affinity chromatography is typically used as a capture step for monoclonal antibodies and Fc fusion proteins whereby the Fc region on the immunoglobin binds to the Protein A ligand that is attached to the chromatography resin.[13-14] Impurities flow through the column, which is often washed to remove additional unbound impurities and other materials that may be loosely associated with the immunoglobulin or the resin before eluting the product at a low pH. When the column load is spiked with virus, much of the virus flows through the column and may be associated with host cell protein.[15-16] Some virus remains non-specifically associated with either the bound immunoglobulin or the resin itself, either through electrostatic or hydrophobic interactions.[16-17] Consequently, manufacturers may include washes before product elution in an effort to disrupt the interaction of the virus with the immunoglobin and potentially increase virus reduction.

Many manufacturing processes for monoclonal antibodies include a low pH inactivation step, where the eluate from the Protein A chromatography step is adjusted to a defined pH, usually pH 3.4-3.6, and then held for a specified period of time to allow for inactivation of any potential enveloped viral contaminants.

When the Protein A chromatography step is evaluated for viral clearance, the column load material is spiked with virus, either a total amount of virus (e.g., 8 total logs) or as a percent volume ratio (e.g., 1%, v/v). Typically, only one virus is spiked into the load at a time. When non-enveloped viruses are spiked into the load, a small aliquot of the spiked load is set aside at the experimental temperature for the course of the chromatography run. This is a "Hold Control" and is

used to account for any virus inactivation in the load that may occur during the course of the chromatography run. A hold control is not used when enveloped viruses are evaluated with a qPCR endpoint. The purpose of the hold control is to determine whether viral inactivation occurs in the load sample over the course of the chromatography run; however, the qPCR does not distinguish between infectious and non-infectious virus.

A schematic overview a viral clearance experiment to evaluate a Protein A chromatography step is shown in **Figure 8**; the relevant samples are in gray and the samples that would be assayed for virus are bolded. In **Figure 8A**, an experiment to support an early phase clinical trial is shown; only the spiked column load and the product-containing eluate are assayed by qPCR for the spiked enveloped virus. For non-enveloped viruses, in addition to the spiked load and eluate, the hold control is assayed for infectious virus. Once the product is ready for late-phase clinical trials, the scope of the study is expanded, and more column fractions are evaluated to better understand the distribution of virus (**Figure 8B**).

**Figure 8**. Schematic of the experimental design for the evaluation of viral clearance by a Protein A chromatography column. A.) Study design for an experiment to support early-phase clinical trial. B.) Study design for an experiment to support a late-phase clinical trial. C.) Study design for an experiment to support a late-phase clinical trial or a commercial licensure, including a carryover run to evaluate the efficacy of the column sanitization step. Samples to be assayed for virus are bolded.

Late-stage studies may also include a limited assessment of the robustness of virus removal. Where a chromatography step provides a critical contribution to the overall viral safety of the process, the step should be shown to provide consistent clearance within the operating ranges of relevant parameters, applying worst-case parameters where known. When worst-case is not known, evaluating viral clearance at the upper and lower limits of the operating range for parameters relevant to viral clearance will provide assurance that viral reduction does not vary within normal operation of the chromatography step.

If chromatography resins are to be re-used, they must be appropriately sanitized/regenerated between runs to ensure reproducibility during subsequent runs as well as inactivation or removal of any process impurities

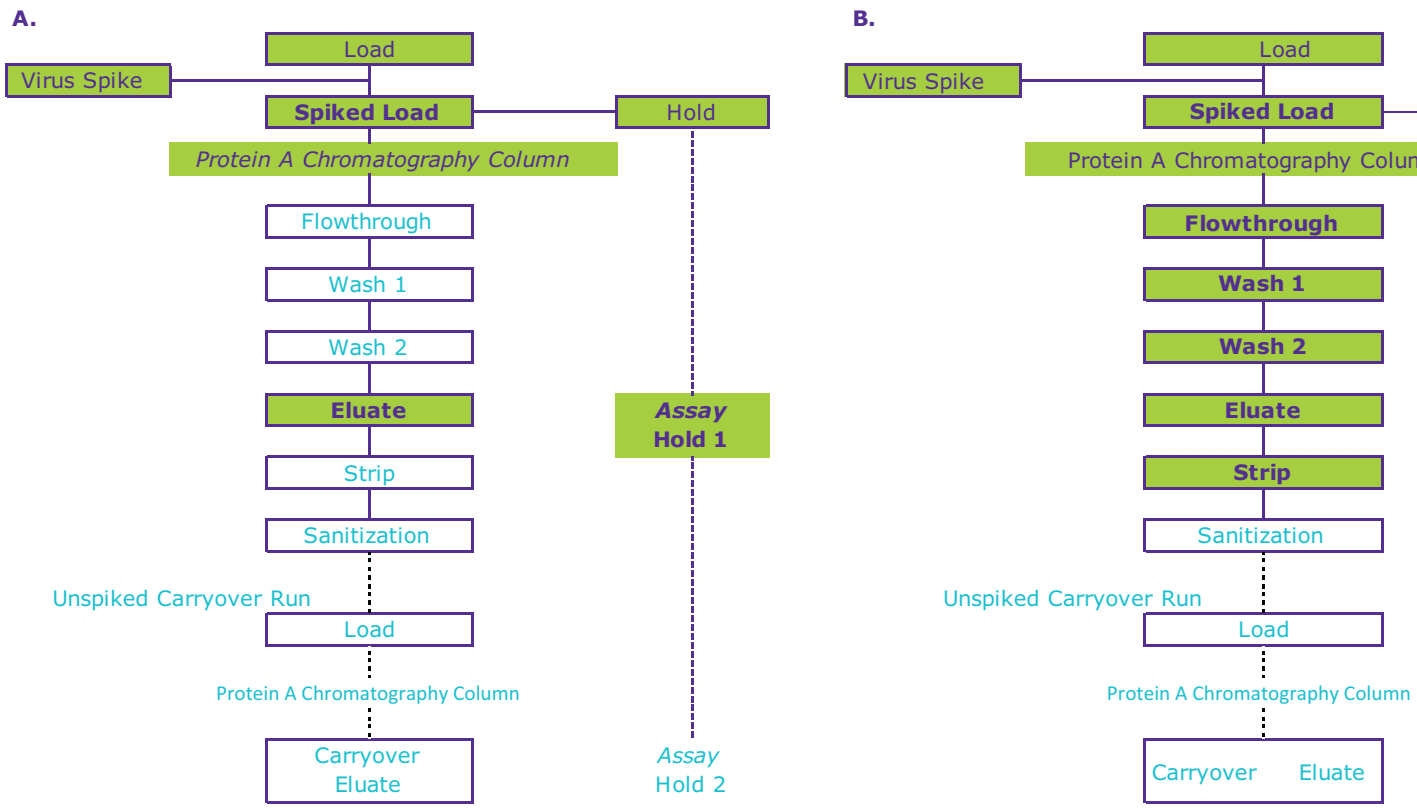
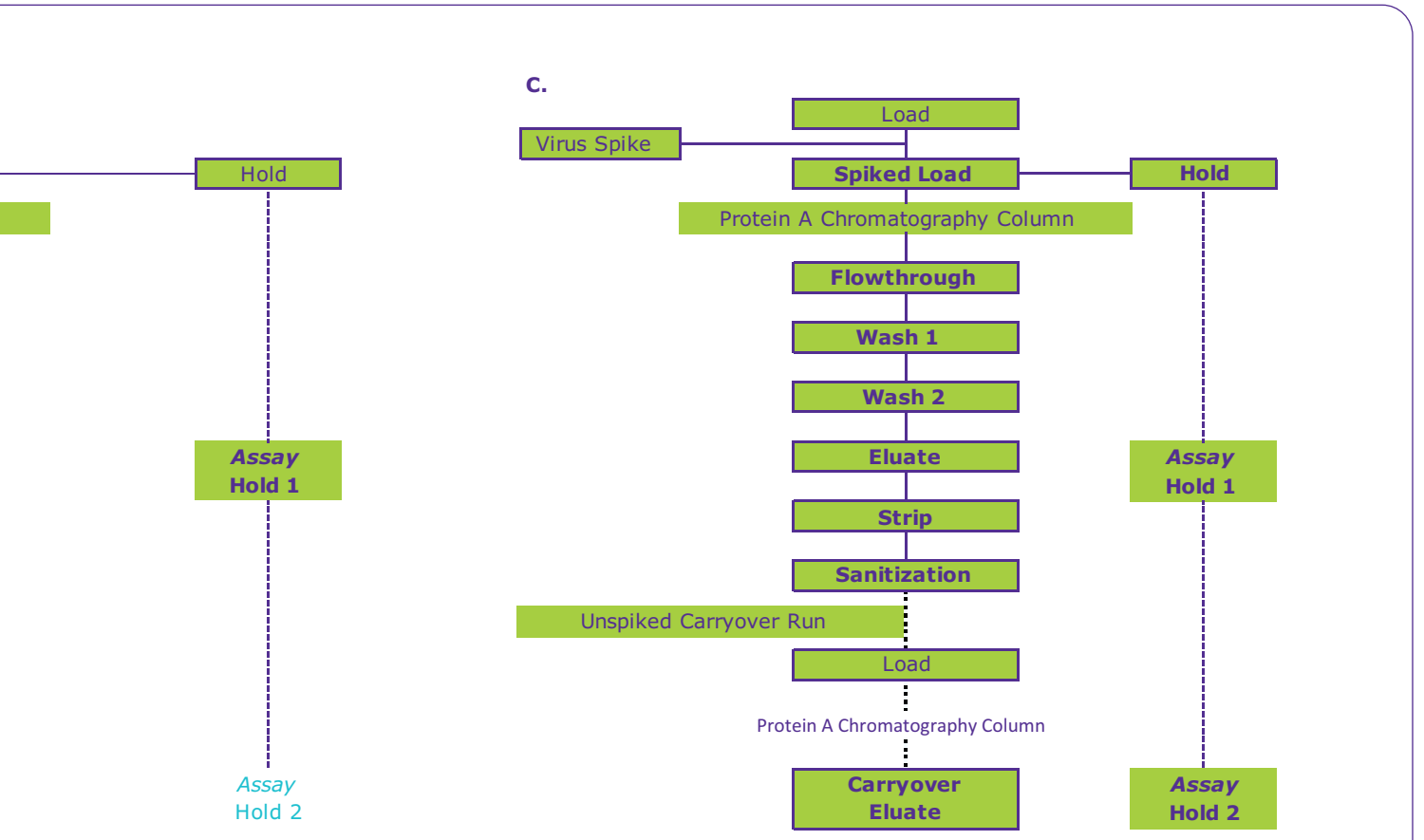



**Figure 8**. Schematic of the experimental design for the evaluation of viral clearance by a Protein A chromatography column. A.) Study design for an experiment to support early-phase clinical trial. B.) Study design for an experiment to support a late-phase clinical trial. C.) Study design for an experiment to support a late-phase clinical trial or a commercial licensure, including a carryover run to evaluate the efficacy of the column sanitization step. Samples to be assayed for virus are bolded.

(including potential viral contaminants) present on the resin. **Figure 8C** shows how the efficacy of column regeneration can be evaluated. Following a spiked chromatography run, the resin is regenerated. The column is then equilibrated and an unspiked load is applied to the column. The column run is completed, and the product-containing fraction is collected and assayed for infectious virus. An infectivity assay is used to evaluate this "carryover eluate" regardless of whether infectivity or qPCR was used to evaluate samples from the spiked run. This is because residual infectious virus is the relevant determinant of effective column sanitization/regeneration.

Occasionally infectious virus may be detected in a carryover sample. The impact of incomplete virus reduction by the sanitization/regeneration regimens depends on the position of the chromatography step in the purification process and the impact should be assessed using a detailed risk assessment. As part of this risk assessment consideration will be given to the virus detected, and the likelihood of such a virus being present in the process stream. Detection of residual XMuLV is of greater concern than one of the non-specific model viruses such as Reo 3. In addition consideration should be given to the affected step and its position within the downstream process, a capture chromatography step will have viral clearance steps that follow whereas, a late stage polishing step may not have any additional opportunities for viral clearance in the process. In recent years, chromatography resins, and especially resins used for affinity chromatography, have been designed to tolerate sanitization with sodium hydroxide solutions.

If chromatography resins used in the purification of a biopharmaceutical are to be reused, manufacturers must establish a limit to the number of cycles that a given resin can be used. This resin age limit is based on several factors but will also include process data that demonstrate that product purification is equivalent throughout the life of the resin. The viral reduction capacity of resin at the limit of its reuse must also be shown to be equivalent to that with new resin. These data must be generated before a product is licensed. Most clinical trial material is manufactured using relatively few reuse cycles for the resins, and therefore viral clearance studies with aged resin are not required to support clinical trials.[2] However, if resins that have been extensively recycled are used in production of clinical trial material, then viral clearance



data to support the safety of the aged resin must be generated. While evaluation of the viral clearance of aged resin can easily be incorporated into a viral clearance study, generation of the aged resin can require a significant amount of time and must be considered well in advance of the clearance study. Surrogate measurements that would predict a change in viral reduction with resin age have been suggested,[18-19] but regulators are moving away from the need to evaluate aged Protein A resins for viral clearance.

## Viral Inactivation

Viral inactivation steps are included in manufacturing processes for most monoclonal antibodies and recombinant proteins. Since Protein A chromatography is often used as the capture step for monoclonal antibodies and the product is eluted with a low pH buffer, many manufacturers utilize a low pH hold as a dedicated viral inactivation step. Experiments to determine inactivation of enveloped viruses by low pH should include an evaluation of the kinetics of inactivation.[1-2] The minimum exposure time should be included as a timepoint with at least one timepoint that is less than the minimum exposure time.

Using a factorial design, the low pH inactivation step has been extensively studied, evaluating the impact of factors such as protein isoelectric point (pI), buffer type, temperature, sodium chloride concentration and protein concentration on MuLV inactivation at pH 3.8 ± 0.1.[20] The results of this study defined brackets for these parameters under which 4.6 logs of rodent type C retrovirus reduction might be expected. This study formed the basis of an ASTM standard that states that for early phase regulatory filings to the US FDA, 5 $\log_{10}$ of inactivation for non-defective type C retroviruses could be claimed if the process parameters for the step are maintained as described.[21] The maximum pH for the ASTM standard is ≤3.6, and parameter ranges are specified for temperature, time, buffer matrix, sodium chloride concentration and protein concentration. These parameters and their ranges have been further studied to further dissect their impact on rodent type C retrovirus clearance, especially at pH levels between 3.6 and 3.8.[22] At pH 3.6 and lower, significant levels of enveloped virus reduction are often achieved, but as the pH increases above 3.6, the robustness of the inactivation decreases.

Five $\log_{10}$ of MuLV can be claimed for viral clearance studies to support early phase clinical trials if manufacturing conditions meet the ATSM standard; however, for submissions to regulatory agencies other than the US FDA, or for submissions to support late-phase clinical trials, manufacturers must evaluate this step. It is understood that higher pH and lower temperature are worst case conditions for this step, and that the highest pH of the pH range and the lowest temperature of the temperature range should be used when designing a low pH experiment.

A schematic of an experimental design to evaluate enveloped virus inactivation by the low pH step is shown in **Figure 9**. Additional timepoints can be added, to more closely evaluate the kinetics of inactivation, and typically large volume plating is performed on the final timepoint to increase the sensitivity of the infectivity assay and potentially increase the amount of reduction that is claimed. The overall design of a study to evaluate low pH inactivation does not vary between early-phase and late-phase studies; the only difference is that for an early-phase study for a product derived from a CHO or other well-characterized rodent cell line, evaluation of MuLV is the only virus typically required. For a study to support a late-phase clinical trial, PRV is added to the enveloped virus panel.

Other methods of enveloped virus inactivation have been used for manufacturing processes for monoclonal antibodies or recombinant proteins. When solvent/detergent treatment of human plasma was introduced into the manufacturing process for human plasma-derived products, the safety of these products with respect to transmission of enveloped viruses like human immunodeficiency virus, hepatitis C virus and hepatitis B virus increased dramatically.[23-25] Solvent/detergent treatment has been used for monoclonal antibody and recombinant protein manufacturing processes. Polysorbate 80 and Triton X-100 have successfully been used both with and without tri(n-butyl) phosphate, often to treat the bulk harvest before application to the Protein A capture chromatography column.

When octylphenol polyethoxylates, like Triton X-100, are degraded by microorganisms, they can become an alkylphenol, a class of molecules that have estrogenic effects on aquatic life, wildlife and humans.[26] For products that are manufactured in Europe or that may be manufactured in other countries, but exported to Europe, the Registration, Evaluation, Authorization and Restrictions of Chemicals (REACH) required in the REACH Annex XIV of June 14, 2017 that the use of these compounds be stopped. Consequently, manufacturers are replacing Triton X-100 in their manufacturing processes with suitable alternatives.[27]

Evaluation of enveloped virus inactivation by a detergent or solvent/detergent treatment step is done in a similar manner as for a low pH inactivation step. The control for the step is the product intermediate without any detergent or solvent/detergent. The detergent or solvent/detergent can be very cytotoxic to the virus indicator cells used in the endpoint assay, so

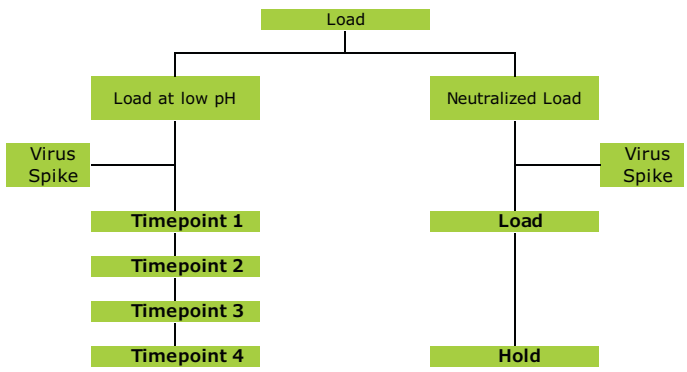



**Figure 9**. Schematic of the experimental design for the evaluation of enveloped viral inactivation by the low pH step. Samples to be assayed for virus are bolded.

it may be necessary to dilute the samples extensively before they can be assayed, resulting in a loss of assay sensitivity. Large volume plating is one approach used to help regain some assay sensitivity.

## Chromatography Steps

Chromatography forms the backbone of the purification process for a monoclonal antibody or a recombinant protein. For a monoclonal antibody process, often a cation exchange column is placed after the Protein A column and the low pH inactivation steps to provide removal of host cell proteins and other impurities, and occasionally it has been explored as an initial capture step. The column is often run in bind and elute mode and the product is usually eluted with a linear or step gradient of increasing salt concentration. While the viral reduction for this step is often lower than for other types of chromatography, when operated at pH 5.0, it has been shown to provide effective removal of MuLV, PRV and Reo 3.[28-29] When the column is operated at pH 5.5 or higher, MuLV reduction decreases significantly. The virus appears to bind to the resin by a tight electrostatic interaction and remains bound during elution of the product.[29] Inactivation of MuLV at pH 5.0 does not appear to be the mechanism of clearance. In contrast to MuLV, cation exchange chromatography does not achieve good parvovirus clearance; at most, MMV clearance levels were similar to those achieved by Protein A chromatography.[29-30]

Anion exchange or mixed mode chromatography steps may be included following the Protein A chromatography and low pH treatment as a polishing step. The protein product flows through the column, but impurities, such as host cell DNA and any potentially contaminating virus, bind to the resin. The isoelectric point of many biotherapeutic proteins is basic and the isoelectric point of most viruses, including the model viruses used in viral clearance studies is on the acidic side of neutral. When the chromatography is run under conditions where the viruses are negatively charged, virus removal has been shown to be due to electrostatic interactions with the positively charged resin.[31] Understanding the mechanism of virus removal by anion exchange chromatography has allowed many manufacturers to adjust buffer pH and conductivity for optimal virus removal.[32] In fact, some manufacturers have developed operating spaces that achieve good virus reduction.[32-33] These operating spaces are often the basis for anion exchange chromatography as a manufacturing platform.

Mixed mode resins have found increased use in recent years. These resins combine multiple mechanisms to provide purification of a protein. Often electrostatic interactions are combined with hydrophobic interactions. Mixed mode anion exchange resins, run in a flowthrough mode, can provide good separation of virus from a protein product. The same considerations of pI, pH and conductivity as done with anion exchange chromatography are necessary to facilitate virus removal.[34] In some cases, mixed mode cation exchange (MMC) resins and cation exchange (CEX) resins show similar levels of viral reduction, while in other cases viral reduction by MMC resins is better than for CEX resins (Merck data).

Hydrophobic interaction chromatography provides purification of a protein of interest by differences in hydrophobicity between the protein and impurities. This type of chromatography has been shown to achieve effective reduction of enveloped viruses, but much less reduction of the non-enveloped MMV.[34]

The design of a study to evaluate viral reduction for any chromatography resin is very similar to the design described in **Figure 8** for a Protein A chromatography step. For studies to support an early phase clinical trial, only the load, controls and product-containing fraction need to be evaluated. As the product progresses through development and approached late phase clinical trials and commercialization, then the scope of the study expands to include evaluation of additional fractions, the efficacy of the column sanitization and aged resin. Data to support the robustness of the chromatography step should be generated during late-stage studies.

## Viral Reduction Filtration

The viral reduction filtration step is typically used as an orthogonal viral reduction step in the downstream process. When ready to evaluate the viral reduction capacity of the filtration step, the design and execution of the study should be carefully considered.

The maximum capacity for the viral reduction filter will typically have been defined through filter sizing trials during the process development, building in a suitable margin of safety that balances process economics and filter fouling during processing. When designing the viral reduction study, two operational targets may be considered when setting the acceptance criteria. The first and lower target will be the minimum volume to be processed to cover all the batches of product manufactured to date. The second will be the maximum target specified for the step based on the filter sizing studies. In some cases, these two targets maybe one and the same and whilst the maximum specified target will always be the aim for the viral clearance study compromise may be required based on the influence the addition of the virus may have on the filter performance.

Storage of the process intermediate is an important consideration for a successful viral filtration study. Anything that promotes aggregation of the process intermediate, even small increases in aggregation, may have a significant impact on the filterability of the intermediate and consequently the capacity of the small-scale filter. Freezing and thawing of intermediates can generate product aggregates, and so storage of the intermediate at 2 to 8°C may reduce aggregation. Shipping of partially filled containers of liquid intermediate will result in sample agitation potentially generating aggregates and impacting filter flux. Complete fill of containers that will be transported may mitigate this issue. Some products are susceptible to precipitation upon standing. This can be problematic during the viral clearance evaluation, because the

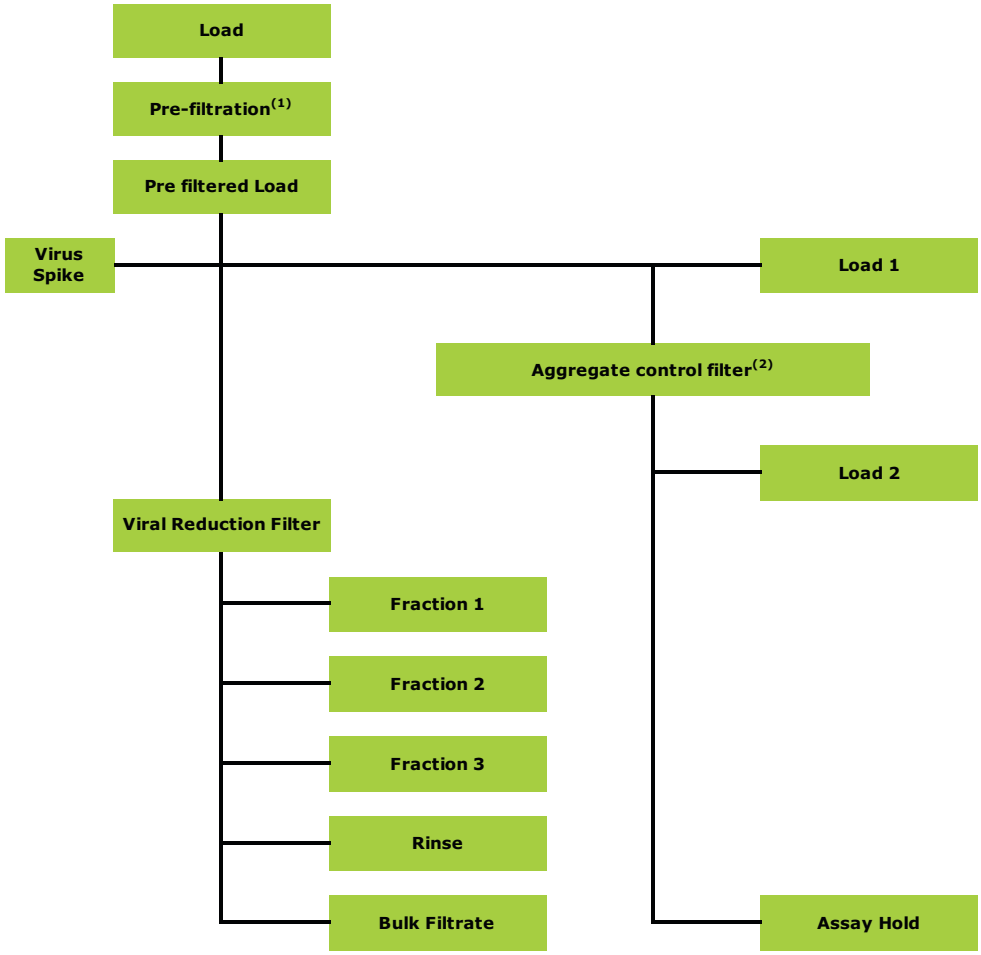



**Figure 10**. Schematic of the Experimental Design for the Evaluation of Viral Clearance by a Viral Reduction Filter. 1) The pre-filtration is the process-specific pre-filter used to "protect" the viral reduction filter. 2) The aggregate control filter is not part of the manufacturing process and is a size exclusion filter carefully selected to remove any viral aggregation that may have formed on addition of the virus to the sample

pre-filtration step is typically decoupled from the virus filtration, and precipitation may occur in the time between completing the pre filtration, addition of the virus spike and initiating the virus removal filtration. This is not a problem observed in manufacturing where the two filters are run in coupled in series. If it is a concern during a viral clearance evaluation, running the pre-filter and viral filter coupled, with inline addition of virus just prior to the virus filter may alleviate the problem.[35-36]

Typically, a maximum pressure is specified for the viral reduction filtration step and it is important that this is not exceeded however, where there is a pressure range specified, viral reduction data supporting the range should be generated. Breakthrough of parvovirus at reduced pressure has been reported[37] with some filters having a greater susceptibility to pressure related breakthrough. Regulators therefore expect to see viral reduction data that covers the minimum pressure specified for manufacturing operations. In addition to pressure considerations there maybe routine flow interruptions within the viral reduction filtration process at manufacturing scale. For example, these may be due to connection of a new feed tank to the system or transition from the product filtration to the rinse. In addition, unplanned pauses may occur as a result of power interruptions or equipment failure. The impact of flow interruptions on viral retention properties, particularly, parvovirus or those viruses that are similar in size to the filter pore size, has been well documented.[38] It is important to include routine flow interruptions in the design of the viral clearance study and consider failure mode robustness testing for MMV prior to license.

For the larger viruses (those in excess of 30 nm) fractionation is not required since it is anticipated that these viruses will be retained by the filter. For small viruses, like MMV (18 to 24 nm in size), detection of virus in the filtrate sample is not entirely unexpected and increased breakthrough has been correlated with flow interruptions[38] and capacity or viral load.[39] It is recommended that the filtrate for parvovirus-spiked runs is collected in fractions (**Figure 10**). By collecting filtrate fractions, it is possible to characterize any capacity related viral breakthrough. These data can be used to adjust the specified filter capacity for future batches to target a specific reduction capacity for example a minimum of 4.0 $\log_{10}$.

## Optimizing Viral Reduction

When evaluating viral reduction for a given process step, the reduction is the difference between the total virus in the input sample and the total virus in the post-purification, product-containing fraction. The results of viral assays are typically provided as a titer; that is viral units (e.g., $TCID_{50}$, PFU, genome equivalents, etc.) per unit volume. For calculation of total virus in each fraction, the volume of each fraction be must be considered, and this is done by multiplying the volume of the input and output fractions by the virus titers. Virus reduction, then, can be calculated as (1):

$$10\text{^reduction} = \frac{10\text{^log titer}_{input} \times \text{volume}_{input}}{10\text{^log titer}_{output} \times \text{volume}_{output}}$$

For example, if a $TCID_{50}$ assay was used to determine viral clearance across a bind and elute chromatography step, and the virus titers and volumes of the input and output fractions were:

| Column Fraction | Volume | Virus Titer |
|---|---|---|
| Column Load Volume (Input) | 200 mL | 6.5 $\log_{10}$ $TCID_{50}$/mL |
| Eluate Volume (Output) | 50 mL | 3.2 $\log_{10}$ $TCID_{50}$/mL |

then reduction would be calculated to be:

$$10\text{^3.9} = \frac{10\text{^6.5} \times 200}{10\text{^3.2} \times 50}$$

If the volume of each fraction has been considered, then virus reduction can be calculated simply as the difference between the log total virus of the input fraction and the log total virus of the output sample. In the example above, 3.9 log reduction is the difference between 8.8 log total virus in the input sample (10^8.8 = 10^6.5 x 200) and 4.9 log total virus in the output sample (10^4.9 = 10^3.2 x 50). The reduction for each purification step in the manufacturing process that is evaluated for viral clearance can be summed to determine the overall process reduction. Due to the inherent variability in the biological assays used for virus detection, however, reductions of one log or less cannot be included in the overall process reduction.

Viral reduction is expressed on a logarithmic scale, and while virus can be reduced to very low levels, it can never be reduced to zero. For a given sample (e.g., virus reduction filter filtrate), only a small portion of the sample can be tested in the virus infectivity assay. If no virus is detected in that small volume, the amount of virus that would need to be present in the larger sample to achieve a positive result can be calculated using the Poisson distribution at a 95% confidence limit (1; **Figure 11**).

This endpoint can be calculated using the following equation:

$$c = \ln p / -v,$$

where:

c = concentration of infectious virus particles in the process intermediate,

*p* = probability (typically at 95%)

v = the volume of the sample that is actually tested in the assay.

From this it is readily apparent that as the volume of sample tested increases, the concentration of virus (or what we colloquially refer to as our assay limit) decreases. Consequently, when the presence of cytotoxicity or viral interference necessitates dilution of the process intermediate, the volume of the actual intermediate in the sample tested decreases, and the assay limit will increase (**Figure 12**). Testing less volume increases the assay limit but testing more volume will decrease the assay limit. This is the basis of a large volume assay, in which much more sample is assayed. For every ten-fold increase in the volume assayed, the assay limit decreases by approximately one log. There are practical limitations, however, to the volume that can be assayed; while a 10-fold increase in sample volume may be manageable for the laboratory, larger increases in volume may prove too unwieldy to be used. Large volume testing is only useful when no virus is detected in the sample. It must be remembered, too, that the more volume that is assayed, the greater the probability that an infectious virus will be detected.

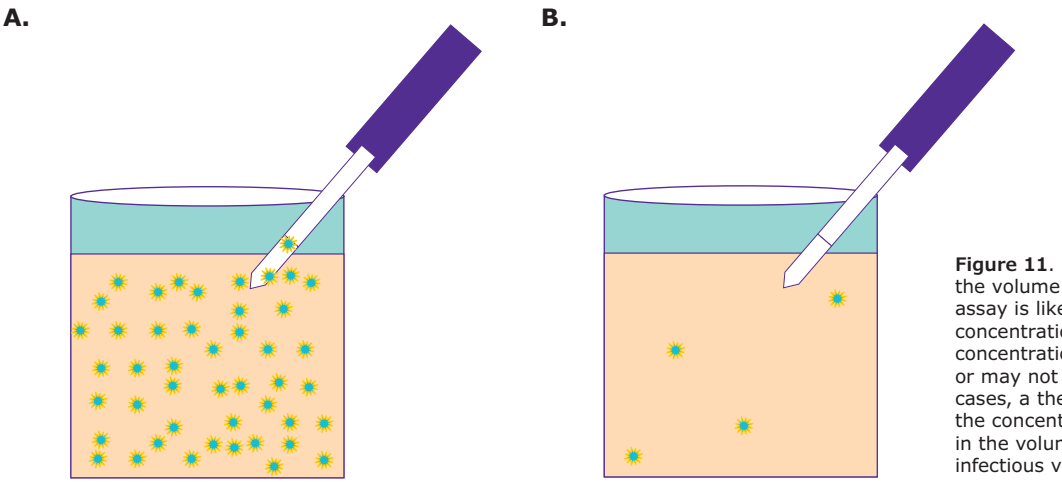



**Figure 11**. At high virus concentrations (A.), the volume sampled for the virus infectivity assay is likely to contain a representative concentration of virus. At very low virus concentrations (B.), the volume sampled may or may not contain infectious virus. In these cases, a theoretical endpoint is calculated as the concentration of virus that would result in the volume sampled not containing an infectious virus 95% of the time.

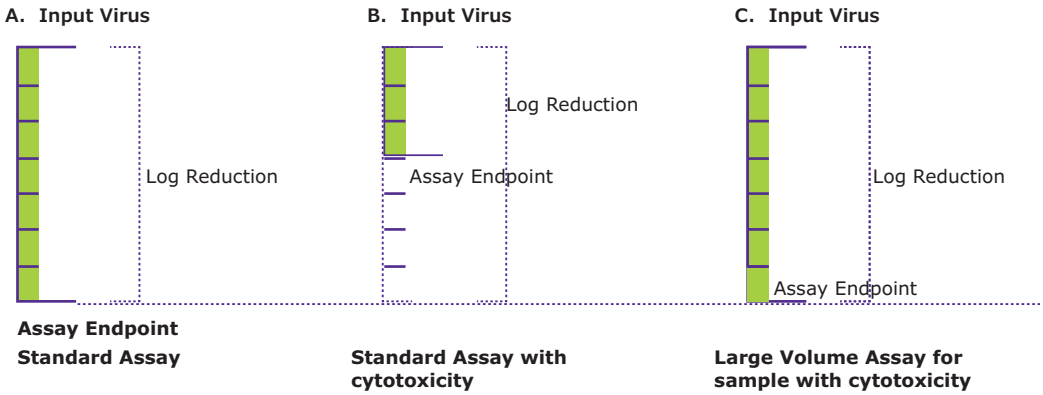



**Figure 12**. Schematic representation of log virus reduction (blue bar) in (A.) a standard assay, (B.) in a standard assay with a highly cytotoxic process intermediate and (C.) using a large volume assay for the same highly cytotoxic process intermediate.

Large volume testing can be used to increase the log reduction factor for a process step. In the situation where a process intermediate must be diluted due to cytotoxicity or viral interference, the log reduction is reduced because the assay limit is increased (**Figure 12B**). Large volume testing can potentially increase the level of clearance that can be claimed by decreasing the assay limit (**Figure 12C**). Knowing the extent that a process intermediate is cytotoxic or interferes with the virus detection assay prior to initiation of the spiking study will allow large volume assays to be utilized to maximize potential reduction.

## Reduction Expectations

A common concern for manufacturers of biopharmaceutical products is whether their process achieves sufficient viral clearance. There is no fixed level of viral reduction that all manufacturing processes for biopharmaceutical products must achieve. Each biopharmaceutical product uses unique source materials and a unique manufacturing process, and so each product has its own unique risk for potential viral transmission. ICH Q5A tells us that "the entire purification process should be able to reduce "substantially more" virus than is estimated to be present in a single dose equivalent of bulk harvest".[1] Because the RVLP load and the purification process are unique to each process, the level of viral reduction required to achieve an acceptable viral safety risk is specific to each product. Although the calculated number of viral particles per dose of final product is an important factor in a risk assessment, clinical parameters (e.g., indication, dose, frequency of administration, immunological status of patients, etc.) may also play a role.

For products derived from rodent cell lines that produce endogenous RVLPs, often to high concentrations, these particles should be quantified in the bulk harvest. Using the concentration of this viral contaminant present in the bulk harvest (or limit of detection of screening assay), the volume of starting material used to manufacture a dose of the final product and the viral clearance achieved across the manufacturing process, an estimated number of viral particles per dose can be calculated. In the example below, the following assumptions are made:

- Concentration of endogenous RVLPs in cell culture harvest is $10^6$/mL
- Volume of cell culture harvest required for a dose of final product is 1 liter ($10^3$ mL)
- Viral clearance for XMuLV (specific model for RVLP) across manufacturing process is $\geq 10^{15}$

The number of viral particles per dose is then calculated:

$$\frac{10^6 \text{ viral particles/mL} \times 10^3 \text{ mL/dose}}{\geq 10^{15} \text{ clearance factor}} =$$

$$\frac{10^9 \text{ particles/dose}}{\geq 10^{15} \text{ clearance factor}} = \leq 10^{-6} \text{ particles/dose}$$

In this example, less than one viral particle per million doses would be expected. In general, for recombinant products, safety factors of $10^{-4}$ to $10^{-6}$ are expected.

### Early vs. Late Stage Studies – A Summary

The differences between viral clearance studies to support early stage or late stage products are summarized below; some of the points were discussed in greater detail in the Viral Clearance Study Design section. Before a recombinant protein or monoclonal antibody that is derived from a well-characterized cell line can be evaluated in clinical trials, the viral clearance capacity of the manufacturing process must be evaluated. The scope of the study to support these products in early stages of clinical development is reduced compared to studies that support products that are in late stage development or ready for commercial licensure. A viral clearance study is typically required before a phase 1 clinical trial to support the viral safety of that clinical trial material. Another study may not be performed until the process is set and data is needed to support a phase 3 clinical trial or commercial product licensure. However, any time there is a change in the manufacturing process that directly or indirectly impacts a step for which viral clearance is claimed, the viral clearance data must be updated.

For products derived from a well-characterized rodent cell line, such as a Chinese hamster ovary (CHO) line, a minimum evaluation of two orthogonal steps using a retrovirus and a parvovirus is recommended.[1] The retrovirus is a model for retroviral-like particles (RVLPs) expressed by CHO and other rodent cell lines and found in bulk harvests and the parvovirus, usually murine minute virus (MMV), is a small, worst-case virus. The purification process should be able to clear 4-6 logs or more of the model retrovirus for RVLPs than

is estimated to be present in a single dose equivalent of unprocessed bulk.[2] In most cases, this means that more than two process steps need to be evaluated for their viral clearance capacity. For submissions to regulatory agencies in most countries, duplicate experiments are required to provide an estimate of the reproducibility of the viral clearance capacity of the manufacturing process.

Early phase viral clearance studies are designed to evaluate viral reduction; only the pre- and post-processing intermediates for a given step need to be evaluated, along with appropriate controls. Evaluation of additional samples to understand the distribution of virus across fractions is not required at this stage. Chromatography resins used to manufacture early-stage clinical trial materials are generally quite new i.e. are used for a limited number of reuse cycles; therefore, chromatography column sanitization/regeneration studies are not required, nor are studies with aged resins. If the columns used to produce clinical trial material have been extensively re-used, then column sanitization and aged resin studies will be required.[1]

For viral clearance studies to support early stage submissions, operational parameter limits may not have been established and clinical trial material is generated at the parameter set point. In these instances, the viral clearance study is also performed at the parameter set point, unless worst-case conditions for viral inactivation or removal for a given step are understood. The manufacturer must verify that the parameters used for the viral clearance study reflect those used to manufacture the clinical trial material. When worst case operating conditions for viral clearance are known, however, they should always be used in a clearance study.

Once the manufacturing process has been established, often before phase 3 clinical trials and always in advance of commercial licensure, another viral clearance is required. The scope of late-stage clearance studies is expanded, compared to studies that support early phase clinical trials. For products derived from CHO cells or other well characterized rodent cell lines, the virus panel is increased from two model viruses, XMuLV and MMV to four model viruses by adding pseudorabies virus (PRV), another enveloped virus, this one with a DNA genome, and reovirus type 3 (Reo 3), a non-enveloped virus with an RNA genome (**Table 3**).

For late-phase clearance studies, the number of fractions from a given virus removal step is expanded from only the load and product-containing fractions so that it can be understood how the virus partitions throughout the step. As an example, for a chromatography step, in addition to the load and eluate, the flow through, wash(es) and strip fractions would also be assayed for virus (**Figure 8**).

As development of the product proceeds toward licensure, process parameter ranges will be established; for those parameters that may impact viral clearance, data should be generated to verify that viral reduction will not adversely be affected during manufacture within those process limits. These studies are especially important when variations in processing parameters may impact viral reduction and a worst-case parameter point is not known.

During commercial manufacturing, chromatography columns will be re-used many times. Therefore, for late stage clearance studies, the efficacy of chromatography sanitization procedures should be evaluated to demonstrate that if an adventitious virus were introduced into a chromatography step, the column sanitization and regeneration procedures would prevent carryover to subsequent runs or batches. To evaluate this, following a virus-spiked run, the column is sanitized, regenerated, and then equilibrated for a subsequent run. The column is then loaded with unspiked load material and the usual chromatography procedure followed. The product-containing fraction of this subsequent run is assayed for infectious virus. If the column sanitization procedure is effective, no infectious virus should be detected.

In addition, clearance studies with aged resins must be performed to confirm the viral clearance performance of each chromatography step does not deteriorate with extended use of the resin.

Irrespective of the stage of product development, viral clearance studies should be carefully designed to ensure that sufficient clearance can be demonstrated. The scaled down process used for the spiking studies should accurately reflect the commercial process such that the data generated is considered representative. Process changes should be assessed through a detailed risk assessment to ensure that the change does not directly or indirectly influence a validated step.

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