Design and Performance of Viral Clearance Studies

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The risk of virus contamination is a feature common to all biotechnology products. Contamination can arise from a source cell line or from adventitious virus introduced during production. Three complementary approaches are used to assure the viral safety of a biotechnology product. First, source cell lines and raw materials are tested for freedom from viruses. Second, in-process testing programs assess the presence of viruses in the crude product and assure freedom from those viruses in the final product.

The third component is the assessment of viral clearance during purification. Viral clearance measures the capacity of a purification process to remove and/or inactivate viruses. Such studies involve deliberately spiking viruses into process intermediates and demonstrating their inactivation or removal during subsequent processing steps. Expectations for design and performance of these studies are detailed in various regulatory documents (listed in the "Regulatory Guidance" box).

PRODUCT FOCUS: ALL PRODUCTS FROM CELL CULTURE PROCESSES

PROCESS FOCUS: DOWNSTREAM PROCESSING

WHO SHOULD READ: QA/QC, PROCESS DEVELOPMENT, MANUFACTURING

KEYWORDS: VIRUS SAFETY, SCALE-DOWN, FILTRATION, CHROMATOGRAPHY, LOG REDUCTION

LEVEL: BASIC

DESIGN OF A VIRAL CLEARANCE STUDY

Decisions made during development of the purification strategy influence the design and conduct of viral clearance studies. And in turn, an efficient process is one that maximizes the potential for viral clearance. Manufacturers should therefore seek expert advice for efficient process design, combining advance planning with early and frequent communication with appropriate regulatory authorities.

Designing a viral clearance study involves the following steps:

- Selection of process steps
- Scale-down of process steps
- Selection of viruses
- Performance of study
- Interpretation of results (see the "Interpretation" box for more detail).

A viral clearance study is performed following a thorough analysis of the potential for virus contamination. Such analysis involves research on the history of a cell substrate, characterization of master and working cell banks and end-of production cells, and raw-material and bulk-harvest testing. Analysis of bulk harvest material by electron microscopy (EM) is performed to obtain a measure of the viral load (generally retrovirus) before

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INTERPRETATION OF VIRUS CLEARANCE STUDIES

- Cytotoxicity and/or interference due to process solutions may cause over- or underestimation of log-reduction values.
- Control assays should be performed to assess the effects on virus titer of each step's duration (and sample manipulations such as freezing or filtration).
- Inaccurate scale-down of a process step may lead to changes in virus clearance.
- Addition of virus to the feedstream may affect the performance of a step.
- Care should be taken to ensure that two steps do not inactivate or remove virus by the same method because virus not cleared by one step may not be cleared by a similar step.
- Inactivation steps should be evaluated several times because inactivation of virus frequently shows nonlinear kinetics.
- Steps with LRV < 1 are not considered to show significant virus clearance and thus should not be included in calculations of total LRV.
- Process parameter changes may significantly change viral clearance.

purification. The detection limit of EM analysis is about 10^6 virus-like particles/mL. Therefore, if no virus is found, the potential virus contamination is set at that level. As a safety factor, a purification process should remove or inactivate at least three to six logs more virus than the potential viral load per dose.

Selection of Process Steps

It is unnecessary to test all steps in a purification process if sufficient clearance can be shown by analysis of fewer steps. Process steps should be selected that present a reasonable expectation of viral clearance. Additional factors for consideration are a procedure's reproducibility under process conditions and its ease of scale-down. Results from such steps provide greater confidence that results of viral clearance studies will translate to process scale.

A typical purification process is diagrammed in Figure 1. Process steps that are likely to inactivate or remove significant amounts of virus include inactivation procedures such as ultraviolet or gamma irradiation, chaotropic salts, pH inactivation, solvent–detergent treatment, and heat treatment. Chromatography and filtration procedures can provide large amounts of virus clearance in some circumstances, but not in others. Other steps such as centrifugation and partitioning are hard to scale and tend to be less effective. Table 1 indicates the performance of typical process steps.

When the starting or source material is less well characterized, the possibility of virus contamination is greater than for products from more defined sources. So for products such as those derived from in vivo sources (e.g., blood, tissue, or ascites), a manufacturing process should incorporate one or more effective virus removal or inactivation steps to increase the level of confidence in clearance during purification. (For products derived from human plasma, two independent robust steps are usually included.)

Scale-Down of Process

Viral clearance studies are performed on scaled-down versions of actual manufacturing processes. Column chromatography steps are scaled down by decreasing column diameter while keeping bed height and linear flow rate constant. Filters are scaled by decreasing the surface area but maintaining the linear flow rate and volume-per-surface area.

A scaled-down version should be evaluated in advance of viral clearance studies to assure that it accurately represents the full-scale process. Validity of scale-down should be demonstrated by comparing the process parameters for similarity with the manufacturing scale process. Parameters to consider in scaling down column chromatography steps include ratios of protein load to column volume ratio and flow rate to bed size as well as temperature, buffers, pH, conductivity, yield, and purity. Parameters to consider in scaling down filtration steps include flow rate, differential pressure, and volume-to-area ratio, transmembrane pressure, and yield. When changes must be made, each change should reflect a worst-case scenario.

Each process step is scaled to a convenient bench-scale size, usually 1:50 to 1:1,000 of manufacturing scale. Load volumes should be small to maximize spiking levels. The virus spiking level should be no more than 10% by volume to avoid altering the starting material. Output volume should be as small as practical to increase assay sensitivity. Process parameters should be selected to represent specific operating conditions and acceptable tolerances to the process at the manufacturing scale, including worst-case conditions. Operating conditions would encompass upper and lower processing limits, as well as those within standard operating procedures, but they should not necessarily induce product or process failure.

Virus Selection

Viruses to be used in clearance studies should resemble those that contaminate or may contaminate the product (relevant viruses). Viruses should also represent a wide range of physicochemical properties to evaluate the robustness of the process to remove and/or inactivate a variety of virus types (model viruses).

Table 2 lists viruses that have been used for viral clearance studies. Viruses should be selected that can be grown to a high titer and detected in an efficient and reliable assay system (such as a viral plaque or focus assay). Assays such as PCR that do not measure infectivity can be used for process steps (some chromatography unit operations, for example) in which virus clearance is virus removal.

However, such assays cannot be used to evaluate inactivation because the viral genome may be present even in noninfectious virus. Table 3 lists sample virus panels for a variety of products from different sources. Virus preparations should be controlled for their protein content and degree of aggregation because those factors can affect virus behavior during clearance steps.

Study Performance

Unprocessed bulk material used in viral clearance studies should represent bulk material from manufacturing. Typically, each process step is evaluated independently for viral clearance. An alternative method is to spike unprocessed bulk material and evaluate virus remaining after each process step. However, this method may lead you to underestimate viral clearance because of limitations on the amount of virus that can be spiked. If a clearance study is in support of product licensure, each process step should be performed in duplicate. The statistical variation within and between studies should be evaluated. For chromatography steps, reuse should be evaluated using resins or filters that have been cycled the maximum number of times used in manufacturing. Regeneration solutions should be tested for viral inactivation to evaluate the possibility of virus build-up on columns during multiple uses.

Properties of a product or process step may complicate virus titration. Samples may be cytotoxic to the indicator cells, or they may interfere with the ability of the virus to infect the indicator cells. A single sample

Regulatory Guidance

Center for Biologics Evaluatlon and Research, Food and Drug Administration. *Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals* (1993); www.fda.gov/cber/gdlns/ptccell.pdf.

Center for Biologics Evaluatlon and Research, Food and Drug Administration. *Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use* (1997); www.fda.gov/cber/gdlns/ptc_mab.pdf.

Committee for Proprietary Medicinal Products (CPMP) and the Biotechnology Working Party (BWP). *Note for Guidance on Virus Validation Studies: The Design, Contribution and Interpretation of Studies Validating the Inactivation and Removal of Viruses* (CPMP/BWP/268/95), 1995; www.emea.eu.int/pdfs/human/bwp/026895en.pdf.

Department of Health and Human Services, Food and Drug Administration. ICH Q5A: Note for Guidance on Quality of Biotechnological Products — Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin. *Fed. Regist.* 63(185), 24 September 1998: 51074–51084; www.fda.gov/cber/gdlns/virsafe.pdf.

may be cytotoxic, interfering, or both. Either property can lead to misestimation of viral clearance. Assays for cytotoxicity or interference should be performed in advance of actual viral clearance studies so that the study design can reflect those results as necessary. Regeneration solutions should be tested for viral inactivation to evaluate the possibility of virus build-up on columns during multiple uses.

For each step, the mechanism of clearance (inactivation, removal, or both) should be identified. Both infectivity assays (plaque or focus) and PCR assays can help elucidate the mechanism of clearance. If clearance is a result of viral inactivation, it is important to determine the kinetics of that process. A typical inactivation study design is shown in Figure 2. Different viruses will most likely have different inactivation kinetics. If clearance is a result of removal, the virus distribution (mass balance) should be evaluated. A typical chromatography study design is shown in Figure 3. Both kinetics and virus distribution can assist in demonstrating the effectiveness of a step and provide additional assurance of safety.

Whenever changes are made in a manufacturing process, the effects of each change on viral clearance (both direct and indirect) should be assessed. Some portions of clearance studies may require repeating because process changes may lead to a change in viral load or viral clearance. When process changes are made after product approval, virus clearance studies must be repeated as validation studies because the acceptance criteria determined in the original study must be met.

Calculation of Clearance

The measure of virus clearance is called the log reduction value (LRV), which is calculated as described in Figure 4. It is calculated for each step, and the values are summed over the steps in the entire process. If the LRV for a step is less than 1, that value is considered nonsignificant and thus not used in calculation of a total value. A statistical estimate describing the accuracy of each result should be calculated. For samples in which no virus is observed, the probability that the fraction being analyzed does not contain infectious virus is estimated using the Poisson distribution.

Two steps that clear virus by an identical mechanism should not be counted as separate steps in calculating total LRV because virus that is not inactivated or removed by a first step may also not be inactivated or removed by the second step. If insufficient viral clearance is obtained by a purification process, it may be necessary to add another inactivation and/or removal step to reach a satisfactory clearance value.

Table 1: Performance of typical process steps

Process Step	Clearance
Low pH	Good
Solvent/detergent	Good*
Irradiation	Good
Ion-exchange chromatography	Good
Hydrophobic-interaction chromatography	Good
Affinity chromatography	Good
Gel-filtration chromatography	Poor
Virus filtration	Good**

*Lipid-enveloped viruses only

**Especially larger viruses

Table 2: Selected viruses used for viral clearance studies

Type	Viruses	Family	Size (nm)	Physicochemical Resistance
RNA lipid-enveloped viruses	Parainfluenza virus	Paramyxovirus	100–200	Low
	Human immunodeficiency virus	Lentivirus	80–110	Low
	Retroviruses*	Retrovirus	80–110	Low
	Vesicular stomatitis virus	Rhabdovirus	70 × 150	Low
	Bovine viral diarrhea virus (BVDV)	Flavivirus	50–70	Low
RNA non–lipid-enveloped viruses	Reovirus 3	Reovirus	60–80	Medium
	Poliovirus	Picornavirus	25–30	Medium
	EMC virus	Picornavirus	25–30	Medium
	Hepatitis A virus (HAV)	Picornavirus	25–30	Medium
DNA lipid-enveloped viruses	Pseudorabies virus	Herpesvirus	120–200	Medium
	Herpes simplex virus	Herpesvirus	120–200	Medium
DNA non–lipid-enveloped viruses	Adenovirus 5	Adenovirus	70–90	Medium
	SV40	Papovavirus	40–50	High
	Porcine parvovirus	Parvovirus	18–24	High
	Minute virus of mice (MVM)	Parvovirus	18–24	High

*Xenotropic, ecotropic, and ecotropic recombinant murine leukemia viruses

Figure 1: Typical manufacturing process

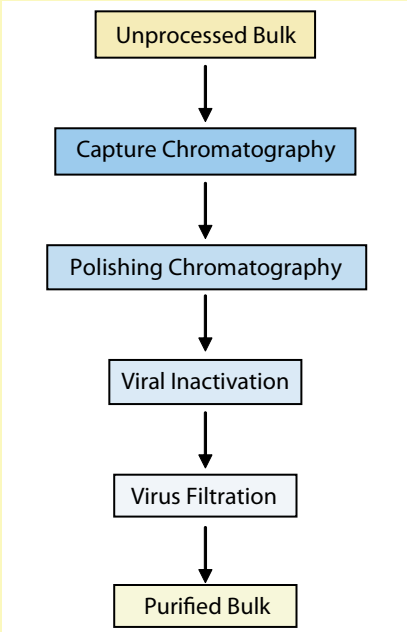

Figure 2: Inactivation study design

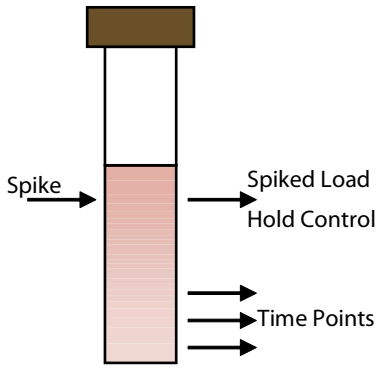

Table 3: Sample virus panels

Species	Source	Viruses
Rodent	Ascites, cell line	Murine leukemia virus, herpesvirus, reovirus, MVM
Human	Cell line	HIV or murine leukemia virus, herpesvirus, poliovirus, parvovirus, or adenovirus
Human	Blood product	HIV, herpesvirus, parvovirus, BVD (model for HCV), HAV

Figure 3: Column chromatography study design

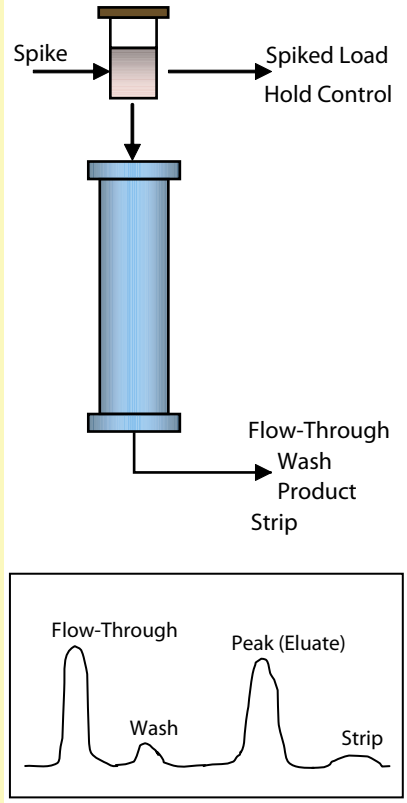

Figure 4: Calculation of virus reduction

Virus reduction for each step:

$$LRV = \text{Log}_{10} \frac{\text{Input titer} \div \text{vol.} \times \text{input vol.}}{\text{Output titer} \div \text{vol.} \times \text{output vol.}}$$

Virus reduction for entire process:

$$LRV_{total} = LRV_{step\ 1} + LRV_{step\ 2} + \ldots + LRV_{step\ n}$$

Ensuring Product Safety

Viral clearance studies are an essential part of a biotech manufacturer's program to ensure product safety. In conjunction with characterization of starting materials and a program of bulk and final product testing, a careful analysis of the capability of a manufacturing process to remove or inactivate a wide range of test viruses plays an important role in establishing the safety of biological products. It provides evidence that the process assures freedom from known viral contaminants as well as from viruses that might be unknown or unsuspected in the starting material.

Although the information provided here is directed toward providing assistance with viral clearance studies for biotechnology products produced in cell culture, it also can be used to guide design of clearance studies for products from other sources. Some details in study design and performance will change; however, the principles are the same for any product. Consultation with the regulatory agencies and expert consultants can assist in the design and performance of such studies.

For Further Reading

Bergmann KF, Schiff LJ. Assuring Viral Safety in Products Produced in Milk from Transgenic Animals: What Can be Learned From Ascites-derived Products. *Bioprocessing J.* January–February 2003: 62–65.

Brorsen K et al. Bracketed Generic Inactivation of Rodent Retroviruses By Low pH Treatment for Monoclonal Antibodies and Recombinant Proteins. *Biotechnol. Bioeng.* 82, 2003: 321–329.

Sofer G. Virus Inactivation in the 1990s — and Into the 21st Century, Part 4: Culture Media, Biotechnology Products, and Vaccines. *BioPharm Intl.* January 2003: 50–57.

Wilkendorf P, Gilbert J. Importance of Adequate Controls in Viral Clearance Studies. *BioProcess International* 2005 3(11, supplement):30–34.

Zhou J, Delghani H. Viral Clearance: Innovative Versus Classical Methods — Theoretical and Practical Concerns. *Am. Pharmaceut. Rev.* 2006.

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