



# Viral Clearance Strategies for Biopharmaceutical Safety

## Part II: A Multifaceted Approach to Process Validation

Hazel Aranha\* and Sharlene Forbes

In addition to fulfilling a regulatory requirement, **validation studies maximize productivity and minimize production failures** by setting operational parameters and ensuring product consistency and safety. Viral validation studies should be designed to justify the selected operating conditions and to document their adequacy in achieving expected process performance. (This article was published previously in *BioPharm* 14 [5], 43–54, 90 [2001].)

### Hazel Aranha

is a senior staff scientist at Pall Corporation, 25 Harbor Park Dr., Port Washington, NY 11050, tel. 516.484.3600, fax 516.484.3628, e-mail hazel\_aranha@pall.com, [www.pall.com](http://www.pall.com).

**Sharlene Forbes** is a senior associate at IDEC Pharmaceuticals, [www.idec.com](http://www.idec.com).

\*To whom all correspondence should be addressed.

**P**rocess validation is an integral part of any manufacturing process. It assures a consistent outcome during key manufacturing processes. FDA's Center for Drug Evaluation and Research, Center for Biologics Evaluation and Research, and Center for Devices and Radiological Health define *process validation* as "establishing documented evidence that provides a high degree of assurance that a specific process will consistently produce a product meeting its *predetermined specifications* and quality attributes" (1). Viral clearance validation studies do not meet the requirement for conforming to a "predetermined specification." Nevertheless, the objective of the clearance study is to document product quality and process specificity to assure viral safety. Viral clearance studies also may be called *qualification studies* or *clearance evaluation studies*, terms used synonymously with *virus validation studies* in this article.

Earlier articles in this series addressed general considerations related to viral safety and pragmatic approaches to ensuring virological safety of biologicals (2,3). This article highlights considerations in designing virus safety studies and cautions when developing appropriate virus contamination control programs to be integrated into the manufacturing processes for biological products. Clearance validation case studies help to illustrate these points.

### Study design

Analytical limitations make it impossible to demonstrate absolute viral absence. Viral validation studies are, therefore, conducted both to document clearance of viruses known to be associated with the product (such as HIV, hepatitis, and parvovirus

in plasma products) and to estimate the robustness of the process to clear potential adventitious viral contaminants (that may have gained access to the product) by characterizing the ability of the process to clear nonspecific "model" viruses.

**Process analysis and evaluation.** Ideally, strategic planning for process validation begins early in product development. The first steps in the validation process involve a critical analysis of the bioprocess to determine likely sources of viral contamination, including the pathogenic potential of those contaminants, followed by process characterization to identify which steps in the manufacturing process have the potential for viral clearance.

Each process step to be tested should be evaluated for the viral clearance mechanism; that is, whether clearance will be by inactivation, removal, or a combination of both. A *robust* step is one in which the effectiveness of the viral inactivation or removal is independent of variability in production parameters (4). Both serendipitous methods (steps already in the manufacturing process, such as chromatography and low pH-buffer elution steps, that coincidentally clear viruses) and methods deliberately incorporated for viral clearance (such as filtration and heat inactivation) are usually validated.

**What needs validating?** Guidance in identifying which processes to validate comes from regulatory guidelines, industry trends, and other resources. Excellent resources include vendor information (such as that from manufacturers of chromatographic resins, filtration systems, and membranes) and the experience of others, especially contract testing laboratories, who have extensive experience in study design. Other sources of information include published literature and seminar proceedings. A cautionary note is important here: Data reported in the literature may reflect results of a study rather than actual process validation data and therefore may not address validation-related considerations at the manufacturing scale. Additionally, details related to process specifics (flow rates and product concen-

## Choosing the challenge site

Three options exist for choosing a challenge site when conducting viral clearance studies.

	Advantages	Disadvantages
<b>Option 1:</b> All processes and samples are tested at the vendor site.	<ul style="list-style-type: none"> <li>Immediate testing of samples</li> <li>Reduced data turnaround time</li> <li>Viral containment area on site</li> <li>No titer loss from additional freezing and thawing of samples</li> <li>Access to troubleshooting experts</li> </ul>	<ul style="list-style-type: none"> <li>Costs associated with using the vendor site</li> <li>Scheduling subject to vendor availability</li> <li>Possible need to ship special equipment</li> <li>Possible need to send trained personnel for extended times for larger studies</li> <li>Extension of lab time may be unavailable if studies do not go as planned</li> </ul>
<b>Option 2:</b> Some processes and all samples are tested at the vendor site while others are performed in-house.	<ul style="list-style-type: none"> <li>Control over more complicated processes</li> <li>Inactivation studies completed by vendor (overlapping timeline for study completion)</li> <li>Testing easier to schedule with vendor</li> <li>Potential price break with frozen samples</li> <li>Potentially faster processing with adequate in-house staff</li> <li>Access to troubleshooting experts</li> </ul>	<ul style="list-style-type: none"> <li>Logistics of shipping virus and samples</li> <li>Potential titer loss from additional freezing and thawing cycles</li> <li>Additional cost of shipping, and freezing and thawing controls</li> <li>Viral containment area required in-house</li> </ul>
<b>Option 3:</b> All processes are performed in-house.	<ul style="list-style-type: none"> <li>Control over all processes and testing</li> <li>Testing easier to schedule</li> <li>Potentially faster processing with adequate in-house staff</li> <li>Immediate testing of samples</li> <li>No titer loss from additional freezing and thawing of samples</li> </ul>	<ul style="list-style-type: none"> <li>Viral containment area required in-house</li> <li>Viral vendors usually do not supply viruses without a testing agreement</li> <li>Need viral experts for study design, virus stock supply, and troubleshooting</li> </ul>

tration, for instance), which could potentially affect virus clearance, may be vague. Clearance data reported at these forums should be taken as an indication of possible clearance and not as the expected value across different processes.

**Multiple methods.** Regulatory guidelines recommend incorporating multiple orthogonal methods for viral clearance; that is, methods that have independent (unrelated) clearance mechanisms (5,6).

One misconception is that an entire manufacturing process that includes, for example, ion-exchange chromatography, pH inactivation, and detergent inactivation can be tested by challenging with a large spike of virus during the first step and sampling during subsequent steps. Logistically, this is impossible for two reasons: Based on the product and possible contaminants, most processes require a demonstration of greater than 12–15 log of clearance for individual viruses, and it is impossible to grow mammalian viruses to such high concentration. Using a low level of viral challenge will result in an initial low viral load, with each successive step in the bioprocess being challenged by fewer viral particles (assuming the previous steps are effective at inactivation or removal of viruses). That type of study design also restricts the number of viral

clearance steps that can be claimed and reduces the overall claim that can be established for the entire process. The best compromise is to evaluate each of the individual orthogonal steps separately and then sum the amount of clearance obtained for the entire process. Although this method has some limitations and introduces errors by overestimating the clearance, it is the only practical approach to a complex problem.

**Scaling considerations and identifying critical parameters.** Ideally, a process validation is conducted at pilot or full scale, but logistic limitations preclude that approach for virus clearance validations. Evaluation of viral clearance strategies requires demonstration of the equivalence of scalability from bench to manufacturing scale and vice versa. The scale-down must truly represent what occurs in the manufacturing process; that is, the process modeling must be accurate. Depending on the process, critical operating parameters to be conserved in scaled-down studies include volume, flow rates, contact time, and product and contaminant load. The test material composition should be similar in protein concentration, pH, and ionic strength. Product generated by large- and small-scale processes should be similar in purity, potency, and yield. One

approach is to perform a small-scale run and compare the purity and contaminant profiles with the large-scale or development history. Other process parameters should be evaluated for their possible impact on viral clearance to determine whether they should be included in the scaled-down study model.

### Worst-case conditions

Regulatory guidelines recommend using virus validation data to set in-process limits for critical process parameters (7). In general, validations are usually conducted at both process extremes. However, because viral studies are costly and time consuming, testing at both process extremes is seldom done. Instead, testing is performed under worst-case conditions to demonstrate the minimum clearance a step can provide. Worst-case conditions will vary depending on the method used and are determined by those factors that influence the clearance mechanism.

**Virus removal methods.** In chromatographic processes, depending on the resin and binding mode, critical variables might include product and contaminant concentration, buffers, flow rates, wash volumes, and temperature, among others. Because of the competitive binding for interactive sites in product-binding mode

chromatography, depending on the resin being evaluated, the kinetics of virus binding is enhanced by the lowest product concentration, thereby constituting the worst case. Using the minimum wash volume before elution encourages virus to elute with the product. As with all chromatographic processes, flow rate influences kinetics.

For contaminant- or impurity-binding mode chromatography, the worst-case contaminant conditions can be achieved by either increasing the contaminant-to-product ratio or by loading the column with a larger volume of product than is processed during manufacturing. This provides competition between the virus and the expected contaminants and impurities. Using the largest postload wash volume anticipated in the manufacturing process (before the first cleaning step) will remove the maximum amount of virus from the resin along with the product and thus constitutes the worst case.

In filtration studies, depending on the filtration mode (direct or tangential flow), variables include the composition of the solution to be filtered (the nature of the protein, the protein concentration, and other solution characteristics such as pH and ionic strength); the process-associated factors such as the differential pressure and the flux; and the appropriateness of down-scaling, that is, the ratio of filter volume to filter area (3).

**Inactivation methods.** All viral inactivation methods should result in irreversible loss of viral activity. Viral inactivation kinetics are rarely linear, and sometimes a small residual fraction of the viral contaminant, resistant to the inactivation strategy, can persist. The rate of inactivation, and thus the potential margin of safety in the production process, can be assessed by using kinetic inactivation experiments at several points in the process.

Variables in inactivation studies include exposure time, temperature, product concentration, volumes, flow rates, the presence or absence of contaminant proteins, and container equivalence. Samples must be homogenous before the treatment strategy, and equipment (such as timers and chart recorders) must be calibrated and qualified.

In pH inactivation studies, low pH inactivation is generally considered robust at

## Auditing a contract laboratory

A quality system review is needed when choosing a contract testing laboratory. Several aspects of the lab need to be audited.

### Sample-related review

- Confirm log-in and receipt procedures (may be provided in writing if requested in advance).
- Check that the storage offers a controlled environment that meets customer specifications.
- Make sure the identification procedure includes proper segregation between customers' samples to ensure lack of cross-contamination (microbial or otherwise).
- Ensure that samples and test solutions are tracked throughout the testing process.

### Test procedures and assay validation review

- Review the laboratory's procedures relating to challenge viruses, cell lines, media, and reagents. Confirm that the acquisition of viruses and cell lines are documented; tests are performed to confirm identity and purity; SOPs are available for preparation, storage, and cultivation, and the acceptance criteria are defined for the viruses and cell lines; and shelf life (stability) studies have been performed on the media and reagents.
- Check that equipment is calibrated; that the installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ) have been documented for the equipment; and that SOPs and acceptance criteria have been established for the calibrated equipment.
- Determine whether summaries of protocols can be provided by the vendor (usually on request), and review the protocols (available during the audit).
- Confirm data-recording procedures: whether SOPs are available for general record keeping and data processing, and all project-related items can be traced (lot numbers on cell lines, virus stocks, and reagents and equipment part number, for instance).
- Ensure that computer processes are validated: Software is validated; computer SOPs are available; the IQ, OQ, and PQ for the computer systems are documented, and acceptance criteria have been established; data tables are generated and confirmed appropriately; and procedures are available for control of software changes with revalidations.

### Technician training review

- Ensure that training records are adequate for documenting an extensive training program for the technicians before they perform critical validation studies.
- Monitor vendor safety training program.
- Confirm vendor CGLP training program.

### Investigation program review

- Evaluate vendor SOPs for documenting evidence of investigations, conclusions, and corrective or preventative actions.

### Internal auditing program review

- Determine whether the vendor's quality assurance department reviews process during critical steps.

values of 3.9 or below but can be effective at different ranges for different lengths of time. Choosing a pH value closest to neutral within the range tested provides a worst-case challenge, as does the shortest time. High protein concentrations generally have a protective effect, so during viral clearance studies, product (protein) concentration should be maximized (within process ranges) to ensure worst-case conditions.

**Variables in detergent inactivation studies** include concentration, exposure time, and exposure temperature. Additionally, because detergents are viscous, samples must be homogenous. The lowest deter-

gent concentration combined with the shortest time is the worst-case condition. Temperature can be an important factor and may need to be evaluated at the extremes during development to determine its effect. In general, the lowest temperature provides the slowest kinetics.

**For heat inactivation studies,** temperature distribution must be uniform, and timing must begin only when steady state is reached. "Worst case" in heat inactivation studies would constitute the highest stabilizer concentration used, the highest product concentration, and the lowest temperature. If scaled-down studies are conducted, container equivalence must be

demonstrated. Appropriately calibrated equipment such as timers and chart recorders must be used; equipment qualification is mandatory.

## Study design

**Testing locations.** Once the proposed manufacturing process has been evaluated and the potential viral clearance steps identified, it is necessary to determine whether the viral testing is to be done in-house or at a contract testing lab. We refer to the company scheduling viral validation studies as *the client* and the provider of supplies or services as *the vendor*.

Three options exist for the location of the tests: The viral clearance studies can be conducted at a contract lab; depending on the specific processes and their complexity, some of the testing can be conducted in-house and the rest contracted out; or all test processes can be performed in-house. The “Choosing the challenge site” sidebar lists some of the advantages and disadvantages of each option.

**Virus stock availability** comes into play when scheduling the evaluation of multiple process steps in a short time frame, more so if the viral stocks are purchased from a vendor and the viral clearance studies are done in-house by the client. Before beginning studies that require large volumes of virus, consult the vendor to determine the availability of viral stock and the lag time.

**Contract lab considerations.** Coordinating a multistep validation is logistically challenging and becomes more so when working with outside vendors. When evaluating a potential vendor, consider its technical capabilities, customer service, and cost. Ultimately, the quality of the final product (the viral clearance claim) is influenced by the entire package: its sound study design, the appropriateness of its testing regimens, and correct interpretation of its data. Although contract labs can offer guidance and assistance in study design and data interpretation, it is the purview of the client to agree, acknowledge, and justify the scientific data. Inconsistent or anomalous virus clearance data, which may be a consequence of poor study design or badly conducted tests, cannot just be ignored. The results must be presented to regulatory authorities as part

**Table I: Viruses commonly used in viral clearance evaluation studies.**

Virus	Family	Genome	Envelope	Size (nm)	Shape
Parvovirus (canine, murine, and porcine)	parvoviridae	DNA	no	18–24	icosahedral
Encephalomyocarditis virus	picornaviridae	RNA	no	25–30	icosahedral
Hepatitis A virus	picornaviridae	RNA	no	25–30	icosahedral
Poliovirus	picornaviridae	RNA	no	25–30	icosahedral
SV40 virus	papovaviridae	DNA	no	40–50	icosahedral
Bovine viral diarrhea virus (BVDV)	togaviridae	RNA	yes	50–70	pleomorphic/spherical
Sindbis virus	togaviridae	RNA	yes	60–70	spherical
Reovirus 3	reoviridae	RNA	no	60–80	spherical
Human immunodeficiency virus (HIV)	retroviridae	RNA	yes	80–100	spherical
Murine leukemia virus (MuLV)	retroviridae	RNA	yes	80–110	spherical
Herpes simplex virus	herpesviridae	DNA	yes	120–200	spherical
Pseudorabies virus	herpesviridae	DNA	yes	120–200	spherical
Parainfluenza virus	paramyxoviridae	RNA	yes	150–300	pleomorphic/spherical
Vesicular stomatitis virus	rhabdoviridae	RNA	yes	70 × 175	bullet

of the documentation file, and the justification for negating the unexpected results must be included.

It is intuitively obvious that cost should not be the primary consideration in the choice of a vendor. Vendor evaluation often begins with obtaining quotes. Nested in the quote, the vendor usually recommends a study design. Such designs can differ significantly between vendors and often are not directly comparable. Costing can vary and be based either on the number of dilutions to be tested or on the expected clearances, with the cost of additional dilutions built into the quote.

Customer service is an integral but often a neglected aspect of vendor selection. Unforeseen test-related factors can sometimes necessitate changing the strategy or repeating a test. Customer-friendly contacts can smooth the way when midstudy changes are required or questions about billing surface. The time it takes for report turnaround or to receive a “quality reviewed” report also can play a part in last-minute evaluations.

Before commissioning a virus validation study, the vendor should be audited, if possible. The “Auditing a contract laboratory” sidebar lists some of the criteria to use when evaluating a vendor.

## Study design technical features

**Choosing a panel of test viruses.** No single indicator species can be used for virus validations — a panel of viruses must be chosen. Choosing the appropriate panel of viruses depends on the source material (whether derived from plasma or from a cell line) and on the clinical trial phase (1, 2, or 3) when the viral clearance will be tested.

In general, the panel of test viruses should include relevant viruses (known or suspected viral contaminants) and model viruses. Examples of relevant viruses are HIV and hepatitis B and C viruses, which are known blood product contaminants. Some relevant viruses such as hepatitis B and C viruses are difficult to propagate in vitro. In those cases specific model viruses can be used. Specific model viruses are viruses that resemble known viral contaminants. For example, bovine viral diarrhea virus (BVDV) and the Sindbis virus have been used as models for the hepatitis C virus. Similarly, murine leukemia virus (MuLV) often is used as a model for noninfectious endogenous retroviruses associated with rodent cell lines. Additionally, nonspecific model viruses also are included in the test panel to characterize the theoretical clear-



ance capability of the manufacturing process — the robustness of the process. Nonspecific model viruses come in different sizes and varied physicochemical and biophysical characteristics. They are not expected to be associated with the product but are included to address theoretical safety concerns and to increase confidence in the ability of the process to handle unknown or undetected viruses. Table I lists examples of viruses that have been used in virus validation studies.

Because of the high costs associated with an entire virus validation package, preliminary testing with surrogates such as bacteriophages can be undertaken in some cases. Such testing is, of course, relevant only if removal is size-based, as in filtration; if clearance depends on a particular physicochemical or other surface characteristic of the virus, surrogate testing cannot be used. The applicability of bacteriophages as surrogates for mammalian viruses in filter validation studies has been discussed (8).

Different clinical trial phases may require different virus choices. Before phase 1, the clearance of known viral contaminants (HIV in the case of plasma-derived products) or specific model viruses is usually assessed. During phases 2 and 3, viral clearance studies should include both specific model and nonspecific model viruses. The entire virus panel evaluated for phase 2 or 3 products should be evaluated again if final manufacturing conditions change or if significant scale-up occurs during or after phase 3 trials.

**Virus stock considerations.** The quality of the stock preparation and of the virus spike titer will significantly influence the test results and the ability to make a viral clearance claim. Unfortunately, no standardized methodologies are available for preparing and purifying virus stocks. Those methods will vary from vendor to vendor and must therefore be discussed with the vendor when designing the study.

In general, starting with a high viral load to challenge a process step will maximize the potential viral clearance claim. The volume of virus spiked into the challenge material and the virus stock titer combine to determine the total virus titer in the spiked product. The virus density depends primarily on the biology of the virus and can vary from virus to virus. Although it is ad-

**Table II: Examples of reduction in viral clearance claim due to viral spike-related factors during a filter validation study.\***

	Expected**	Actual
Sample titer/mL	$4.9 \times 10^4$	$4.9 \times 10^4$
Volume (mL)	300	82.1
Total challenge (log <sub>10</sub> )	7.17	6.60
Total recovery (log <sub>10</sub> )	≤2.16	≤2.16
Log titer reduction	≥5.01	≥4.44

\* Test virus used was pseudorabies virus (PRV), the product was a ~4.1 mg/mL monoclonal antibody.

\*\*“Expected” refers to the initial spiked volume and anticipated throughput.

visible to work with high titer virus stocks, care must be exercised to ensure that the methods used to concentrate the virus stock and achieve high stock titers are not conducive to aggregation.

The quality of the virus stocks, as measured by the presence of viral aggregates, cell debris, or other particulates, can influence results by falsely enhancing or reducing viral clearance. For example, extra cell debris during a contaminant-binding chromatography process may compete with the virus for binding sites on the resin, causing a decreased clearance value. In a tangential-flow filtration process, a virus stock containing high amounts of cell debris would enhance virus retention by polarizing the membrane. In direct-flow filtration, a membrane prematurely clogged by cell debris cannot filter the entire load volume and, therefore, full log clearance cannot be claimed. Table II shows the clearance expected from a filtration study when using the expected load and filtrate volumes (300 mL) compared with the actual volume (82.1 mL) collected as filtrate. Because less than one-third of the desired volume was filtered, the overall clearance claim made for that filtration step was decreased.

Variables associated with virus preparation (such as stabilizers in stock, aggregation, and debris) can depend on the virus and the vendor. Some vendors take precautions to reduce cell debris, whereas other viral stocks are minimally purified and can contain significant amounts of

**Table III: Loss of virus titer because of prefiltration of spiked test material and its effect on the viral clearance claim.\***

	Sample One**	Sample Two†
Input titer/mL (log <sub>10</sub> )	$6.3 \times 10^4$	$3.85 \times 10^2$
Volume (mL)	127	137‡
Total input concentration (log <sub>10</sub> )	6.90	4.72
Total recovery (log <sub>10</sub> )	4.69	≤1.88
Log titer reduction	2.21	≥2.84§

\* Challenge virus used was xenotropic murine leukemia virus (X-MuLV); the filter was a Ultipor VF grade DV50 (Pall BioPharmaceuticals).

\*\*Sample one is the prefiltered sample and used product filtered through a 0.2-μm filter.

† Sample two was virus-reduction filtered, using 0.2 μm of effluent.

‡ Includes wash volume (to maximize product recovery).

§ Calculated based on input concentration in the 0.2-μm prefiltered load.

membrane particulates. Virus-stock solutions often contain stabilizers (such as bovine serum albumin) and other additives (such as serum), and those can interfere with evaluating the clearance process. Suggested remedies include centrifugation of the virus stock and buffer exchange just before use. Additional manipulation of the stock can lead to increased aggregation. A small-scale run using storage solution for a virus spike (mock spike without the virus) provides useful information and should be done when possible.

**Lot-to-lot differences** can occur even when the lots are produced by the same vendor. For example, in one validation study under identical challenge conditions, we challenged two aliquots from the same batch of test material using a viral spike from two different virus stocks purchased from the same vendor. Each filter membrane was theoretically challenged with approximately the same number of particles as were contained in the certified titer provided by the vendor. Test material spiked with virus lot 1 behaved similarly

Table IV: Virus inactivation by a low-pH product shows the importance of "media control."\*\*

Sample	Input Concentration (TCID <sub>50</sub> /mL)	Volume (mL)	Total Viral Load (Log <sub>10</sub> TCID <sub>50</sub> )	Log <sub>10</sub> Titer Reduction**
T = 0 min	1.12 × 10 <sup>5</sup>	19.5	7.52 ± 0.32	
T = 5 min	≤6.76 × 10 <sup>0</sup>	21.1	≤3.33	≥5.82
T = 30 min	≤6.76 × 10 <sup>0</sup>	21.1	≤3.33	≥5.82
T = 60 min	≤6.76 × 10 <sup>0</sup>	21.1	≤3.33	≥5.82
<b>Media</b>				
T = 0 min	4.79 × 10 <sup>6</sup>	20	7.98 ± 0.24	
<b>Media</b>				
T = 60 min	2.00 × 10 <sup>6</sup>	20	7.60 ± 0.37	0.38

\* Challenge virus used was xenotropic murine leukemia virus (X-MuLV).

\*\*Log<sub>10</sub> titer reduction was calculated based on the starting media control titer (T = 0) because of the virus inactivation by the test product.

Table V: Detection of low levels of virus using large-volume sampling assessment.\*

Sample	Inverse Log <sub>10</sub> TCID <sub>50</sub> /mL	Volume (mL)	Log <sub>10</sub> Adjusted Titer	Log <sub>10</sub> Titer Reduction**
Spiked load	4.79 × 10 <sup>3</sup>	108.4	5.72 ± 0.40	
Adjusted spiked load†	6.31 × 10 <sup>5</sup>	108.4	7.84 ± 0.35	
Flow-through/wash	≤6.76 × 10 <sup>1</sup>	168	≤4.06	≥3.78 ± 0.35
Eluate	≤6.76 × 10 <sup>1</sup>	109	≤3.87	≥3.97 ± 0.35
Eluate	2.57 × 10 <sup>1</sup>	109	3.45 ± 0.15	4.39 ± 0.38
<b>Large-Volume Sampling</b>				
Hold control	1.12 × 10 <sup>4</sup>	108.4	6.08 ± 0.32	1.76 ± 0.47
Media control (start)	6.31 × 10 <sup>5</sup>			
Media control (end)	4.79 × 10 <sup>5</sup>			0.12 ± 0.53

\* Xenotropic murine leukemia virus (X-MuLV) was removed by phenyl-sepharose chromatography.

\*\*Log<sub>10</sub> titer reduction was calculated based on the media control titer (start) because of virus inactivation by the test product.

† Spiked load was adjusted based on the media start control.

to development work done without virus. However, test material spiked with virus lot 2 showed a dramatic decrease in flow rate, and the test had to be aborted prematurely because of flow decay. Differences between those two virus stock lots meant that the challenge level (particles/cm<sup>2</sup>) was different in those tests: Virus lot 1 had a particle load/cm<sup>2</sup> of 9.61 log, whereas the particle load for virus lot 2 was 9.80 log. Considering previous experience with this antibody, we concluded that virus lot 2 probably had a higher level of cell debris. The visible cloudiness of the virus stock lent credence to that conclusion.

Prefilters are sometimes used before virus removal filtration to remove virus aggregates or debris that can falsely in-

crease clearance. Some titer loss can be expected with a prefilter, which will reduce the amount of virus contacting the test membrane. The amount of loss will depend on the method of virus stock preparation and on the aggregating effect of the test material on the virus. The effect of using a prefilter, both on the product and on the level of viral spike, must be acknowledged. Testing must be performed in advance to determine the amount of product loss and dilution from any filter wash and the volume retained from passage over the prefilter. If the product is lost or diluted, that loss and dilution must be compensated for so that the starting material loaded on the test filter meets the minimum concentration

and mass-to-surface area specification. If the viral spike contains a large number of aggregates (which consequently increase the viral size), the prefilter can effectively clear a significant amount of virus, reducing (by a few log) the viral load to the viral removal filter and decreasing the log-reduction claim that can be made for that step.

In our experiments, prefiltration using a 0.2 μm-rated filter resulted in a 2.2 log reduction (see Table III) and thus resulted in a decreased claim — a log titer reduction (LTR) greater than or equal to 2.84 — because of viral clearance by the prefilter. We are still investigating whether the loss from using the prefilter was from cell debris or aggregation.

Viral spike volumes will affect clearance studies (especially in cases of large amounts of debris) and should generally be maintained at 10% or less of the final volume to keep the feed stream representative of the manufacturing process (7). Using a 5% instead of a 10% virus spike reduces the number of particles only by half, or approximately 0.5 log. For example, in one of our filter validation studies, using a 10% viral spike resulted in clogging of the virus filter (in spite of prefiltering the spiked input volume through a 0.2 μm-rated prefilter). Because filterability tests at the bench and pilot scale had indicated appropriate sizing, filter plugging was probably associated with the virus spike. When the viral spike volume was decreased to 5%, the equivalent volume of batch throughput was readily filtered. (Anecdotally, using a 5% spike allowed even greater volumes to pass through similar virus-removal filters.)

### The importance of controls

The importance of controls in the virus study design cannot be overemphasized. Controls allow clearance effects to be attributed to the treatment procedures rather than to test design artifacts or methodology. Several controls should be included in virus clearance evaluations.

Before viral clearance assays are conducted, the product must be shown to have no inhibitory effect on either the indicator cell line (generalized cytotoxicity control) or the test virus (viral interference studies). Cytotoxicity and viral interference controls are often conducted

considerably in advance of the validation study to ensure that the clearance capacity has not been overestimated because of test-related considerations.

The **cytotoxicity control** is included to ensure that any indicator cell cytopathology observed during the study is due to the virus alone. The cells are exposed to process components (product intermediates and buffers) in the absence of virus for the length of time that the test material will be in contact with the cells. A cytopathic or morphological effect relative to the unexposed control cells is an indication of cytotoxicity.

**Viral interference control** determines whether process components interfere with the capacity of the test virus to infect the indicator cell line. Essentially, following exposure of the indicator cell line to the process component, the cells are exposed to the virus and evaluated to determine any loss of infectivity and thus viral interference by the product. If either the cytotoxicity or the viral interference controls demonstrate positive results, the test material can be diluted (to determine a noninhibitory concentration), or the test solution can be neutralized or otherwise adjusted.

A **media control** consists of virus spiked into the virus cultivation medium at the same ratio as the test material and helps to determine inactivation by the test material. Media “start” and “end” controls demonstrate the stability of the test virus under the test conditions.

Table IV demonstrates the importance of the media control. To validate a pH inactivation step, the test material at neutral pH was spiked with virus (X-MuLV), agitated, and then sampled. The remaining volume was then adjusted to below pH 4. An immediate drop in virus titer was observed: The expected titer at T = 0 min was 6.52 log; the actual titer in the sample was 5.05 log, a viral titer drop of 1.63 log immediately after spiking. No virus was detectable at 5 or 60 min after virus spiking. The media controls demonstrate that the number of virus particles expected was achievable (no reduction in titer for the stock virus). Time and temperature

## Risk calculations

The required level of clearance is assessed in relation to the perceived hazard to the target population and guided by a risk–benefit analysis like the one below, which uses xenotropic murine leukemia virus (X-MuLV) as the challenge virus.

Retrovirus-like particles per mL	$1.62 \times 10^7$ particles/mL
Antibody titer	0.274 mg/mL
Weight of average person	80 kg
Dose per mass	15 mg/kg
One dose	1200 mg
Viral clearance factor	unknown

The total amount of retrovirus-like particles in one dose =

$$\{(1.62 \times 10^7 \text{ particles/mL})(1200 \text{ mg/dose} \div 0.274 \text{ mg/mL})\} \div 10^{-6} \text{ particles/dose} = 7.09 \times 10^{16}$$

or 16.85 log minimum clearance required to achieve a clearance value of one particle per million doses.

effects alone did not affect titers within the assay variability. The probable cause of the decreased input titer was inactivation from the starting material (product) components. Because this is an inactivation step, the media control titer (T = 0) can be substituted as the starting titer, with the final result claimed as inactivation under those process conditions. Media control titers are often substituted as an estimate for the initial titer when the test material is virucidal.

The **hold control** ensures that the test virus is stable in the presence of test materials throughout the duration of the test. The hold control involves virus-spiking the starting material, then holding the starting material at the process temperature for the length of the process time. This control essentially demonstrates inactivation effects that are a consequence of the product (the starting material). Loss demonstrated by a hold control is unrelated to the clearance strategy under study and should be evaluated accordingly.

In one of our chromatography validation studies, a monoclonal antibody solution in 150 mM NaCl, 50 mM Tris pH  $7.7 \pm 0.1$  buffer was spiked with pseudorabies virus (PRV). This buffer was not expected to affect the stability of PRV. However, the test material spiked with the virus and held for the process time (that is, the hold control) did reduce the titer by 1.21 log, suggesting that the decrease in the titer could be attributed to inactivation. The media controls confirmed that conclusion. Data interpretation for chromatography steps can sometimes be complicated by the combination of inactivation and removal occurring during the

same clearance step. Hold controls are therefore essential when evaluating viral clearance.

A **freeze–thaw control** is defined differently by different vendors. One definition is an aliquot of virus stock thawed and held unopened at process temperature and time. That test provides information about the thawing of the virus in concentrated form compared with the effects of thawing on a diluted form of the virus from the media controls. Another approach is using the media control to verify that the freeze–thaw cycle does not affect the stock titer. Enveloped viruses especially can be affected by a slow transition between frozen and thawed states.

**Stability and storage controls** are primarily a concern if process challenges are performed somewhere other than at the virus vendor’s site. If virus stocks are to be shipped to another location, the stocks are thawed, processed for the manufacturing step to be challenged, and frozen for later shipping. These controls may differ from challenges performed at the vendor site because many vendors assay the test material immediately. A reduction in this test can affect the final clearance claim, so the freeze–thaw stability should be reviewed.

**Shipping controls** determine whether temperature changes that may occur when the virus is shipped to a different site would affect the titers.

## Pitfalls and cautions

As mentioned, a “good” viral clearance validation study is detailed and well designed. Scaled-down studies are, at best, approximations of the conditions that will be achieved during manufacturing, and

the validity of the clearance data reflects the accuracy of the process modeling and study design. Several pitfalls can be associated with small-scale validation studies.

**Virus-related considerations.** Viral spike-related perturbations can make a process nonrepresentative of the actual manufacturing conditions. Also, model viruses used in process validation studies are at best just that — models — and a wild-type strain may not behave the same way as a laboratory strain.

**Inaccurate process modeling.** Conditions in small-scale validations may be incongruent with the process-scale conditions. For instance, columns used only once for a validation study may not reflect the ability of columns used repeatedly (during manufacture) to remove virus consistently: Certain sites on resins can become blocked with repeated use, reducing the effectiveness of virus removal over the resin lifetime.

**Sample-related considerations** include the use of nonrepresentative samples in viral validations. For example, the proper intermediate or the actual product sample may not have been used; the sample may not be representative of the protein concentration, the pH, or other solution characteristics such as ionic strength; and samples may be nonhomogenous because of inadequate mixing.

**Assay-related considerations** include failure to evaluate buffer toxicity, poor model virus selection, lack of appropriate controls, and poor standardization of viral assays. Critical criteria for assay performance are accuracy, reproducibility, repeatability, linearity of range, limit of detection (LOD), and limit of quantification (LOQ), and all must be validated (9).

**Product dilution steps** (because of viral interference or other toxicity-related considerations) will affect assay results and the ability to make a high viral clearance claim. For example, high salt concentrations, pH extremes, or other sample conditions can interfere with virus titration. Decreasing the volume assayed (because the sample is diluted) will result in decreased sensitivity and is especially important when no virus is detected and a theoretical limit titer for the sample is calculated.

In general, greater viral reduction can be claimed with more observations from using larger volumes and from testing the lowest

available limits of detection. Virus quantitation methods can be modified to enhance sensitivity by using additional replicates and increasing inoculation volumes.

It is important to avoid overestimating the effectiveness of a viral clearance process that fails to detect low levels of residual virus. Large-volume assessments can be used as supplements to conventional titration methods to increase the probability of detection for extremely low virus concentrations. Table V demonstrates that when a routine sampling protocol (volume assayed 0.4 mL) was used, no virus was detected in the eluate from a Phenyl Sepharose chromatography column. However, large-volume sampling (total sample volume of 3.2 mL) allowed detection of low levels of the challenge virus ( $2.5 \times 10^1/\text{mL}$ ).

## Estimating viral clearance

Establishing clearance for an entire process (the overall clearance value) requires at least two orthogonal, robust methods of viral clearance. The individual steps must possess fundamentally different mechanisms of virus removal or inactivation for the values to be considered cumulative. Only data for the same model virus can be cumulative because viruses vary greatly in their inactivation or removal profiles. Clearance estimates and their variances are calculated for each orthogonal unit operation; total virus reduction is the sum of individual log reduction factors. In cases of complete clearance, a theoretical titer value is based on a statistical distribution (the Poisson distribution). Table VI provides cumulative virus clearance values for MuLV.

Few guidelines describe how to perform final data interpretation. All existing guidelines state that clearance steps yielding one log or less of clearance are considered negligible and are not to be included in the overall clearance for a process. The FDA-ICH guidelines recommend running replicate challenges presumably to increase confidence in the data (9). However, no specific guidance is provided on how to report replicate data, nor on how data interpretation can be made consistent for each process step and across all processes. One suggestion is to average the replicates if each value is within the assay variability. If replicates differ by more than the

## Example of the calculation of viral clearance (log titer reduction) for the overall process for a monoclonal antibody product.\*

	Log Reduction Factor
Ion-exchange chromatography	>5.39
Nanofiltration	>5.14
Low-pH inactivation	>5.85
Detergent inactivation	>6.06
Total clearance	>22.44

\*Challenge virus used was xenotropic murine leukemia virus (X-MuLV).

assay variability, report the lowest clearance value as the conservative approach. Other approaches, if justified, may be acceptable. The procedure chosen should be logical, defensible, and defined.

**Achieving the goal.** A key factor affecting viral clearance for the overall process is the amount of product required to produce a single dose. The required level of clearance is assessed in relation to the perceived hazard to the target population and is guided by risk-benefit analysis. For example, murine cell lines are frequently contaminated with endogenous retrovirus-like particles that pose, primarily, a theoretical safety concern (10–12). The putative risk stems from the cell lines' morphological and biochemical resemblance to tumorigenic retroviruses. Chinese hamster ovary (CHO) cell lines containing endogenous retroviruses at levels of  $10^6$ – $10^9$  particles/mL (as seen by electron microscopy) are deemed acceptable if adequate retrovirus clearance is demonstrated in the manufacturing process.

Risk calculations to determine retroviral load per dose are shown in the "Risk calculations" sidebar. This example assumes a one-time dose of 1200 mg to the patient. Using the conservative goal of assuming a probability of viral contamination of one particle per million doses of product, and assuming a retroviral load of  $1.62 \times 10^7$  particles/mL in the starting material, the purification process for this product would have to demonstrate a minimum log clearance of 16.85 log to achieve the stated goal of one viral particle per  $10^6$  doses. The clearance goal is usually chosen based on product use and the risk to the patient population. The extent



of necessary product testing depends on the source and nature of the product, the stage of product development, and the clinical indication. Serious or immediately life-threatening conditions for which no effective alternative treatment exists can justify abbreviated testing.

### Offering assurance

Virus clearance studies are an integral component of the multifaceted approach recommended to ensure the safety of biologicals and biopharmaceuticals. Ultimately, the quality of the final product (the viral clearance claim) is influenced by the entire package, by sound study design, appropriate testing regimens, and correct data interpretation.

The study design is critical to viral validations. Process modeling must be accurate. Careful and comprehensive studies should be conducted to ensure scientifically and statistically sound results. Virus quantitation methods can be adapted to suit specific study needs by including additional replicates, increasing inoculation volumes, and testing at multiple sampling periods. Factors such as test volumes, batch volumes, and test sensitivity determine the probability of detecting low-level virus concentrations.

Process validation coexists with and supplements in-process testing. In addition to fulfilling a regulatory requirement, it maximizes productivity by setting operational limit parameters, minimizing production failures, and providing a high assurance of product consistency and safety. Viral validation studies should be designed to justify the selected operating conditions and to document their adequacy in achieving expected process performances. Although any validation study only approximates the real situation, it identifies critical parameters affecting viral clearance and provides a framework for setting operational limits and worst-case conditions.

This article has highlighted the various cautions and considerations to be borne in mind when designing and developing appropriate virus contamination control strategies into manufacturing processes for biological products and is designed to provide suggestions and highlight pitfalls based on personal experience and industry trends.

### References

1. Center for Biologics Evaluation and Research, *Guideline on General Principles of Process Validation* (FDA, Rockville, MD), May 1987, pp. 5–9.
2. H. Aranha, "Viral Clearance Strategies for Biopharmaceutical Safety, Part 1: General Considerations," *BioPharm* **14** (1), 28–35 (2001).
3. H. Aranha, "Viral Clearance Strategies for Biopharmaceutical Safety, Part 2: Filtration For Viral Clearance," *BioPharm* **14** (2), 32–43 (2001).
4. H. Willkommen, I. Schmidt, and J. Lower, "Safety Issues for Plasma Derivatives and Benefit from NAT Testing," *Biologicals* **27**, 325–331 (1999).
5. Committee for Proprietary Medicinal Products (CPMP), *Rev. 2: Note for Guidance on Plasma-Derived Medicinal Products* (CPMP/BWP/269/95), July 1998.
6. "Guidance on Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin," *Federal Register* **63** (185), 51074–51084 (1998).
7. Committee for Proprietary Medicinal Products (CPMP), *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), February 1996.
8. H. Aranha-Creado and H. Brandwein, "Application of Bacteriophages as Surrogates for Mammalian Viruses: A Case for Use in Filter Validation Based on Precedents and Current Practices in Medical and Environmental Virology," *PDA J. Pharm. Sci. Technol.* **53**, 75–82 (1999).
9. A.J. Darling, J.A. Boose, and J. Spaltro, "Virus Assay Methods: Accuracy and Validation," *Biologicals* **26**, 105–110 (1998).
10. K.P. Anderson et al., "Defective Endogenous Retrovirus-Like Sequences and Particles of Chinese Hamster Ovary Cells," *Dev. Biol. Stand.* **75**, 123–132 (1991).
11. C. Liptrot and K. Gull, "Detection of Viruses in Recombinant Cells by Electron Microscopy," *Animal Cell Technology: Developments, Processes, and Products*, R.E. Spier, J.B. Griffiths, and C. MacDonald, Eds. (Butterworth-Heinemann Ltd., Oxford, UK, 1991), pp. 653–656.
12. K.K. Lueders, "Genomic Organization and Expression of Endogenous Retrovirus-Like Elements in Cultured Rodent Cells," *Biologicals* **19**, 1–7 (1991). **PT**